ARSENIC HYPERACCUMULATION BY *Pteris vittata* L. AND ITS POTENTIAL FOR PHYTOREMEDIATION OF ARSENIC-CONTAMINATED SOILS

By

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This work is dedicated to my wonderful husband, Kenneth Tartar, for his unending love, patience and encouragement.

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Pteris vittata L, an arsenic-hyperaccumulating fern, was examined to understand its hyperaccumulating ability and for its use in remediating arsenic-contaminated soils. Transport of arsenic in xylem sap of *P. vittata* was investigated. Ferns were subjected to arsenate, arsenite, dimethylarsinic acid (DMA) or monomethylarsonic acid (MMA). Xylem sap was collected and analyzed for arsenic concentration, speciation and phosphorus concentration. When inorganic arsenic was supplied, arsenate appeared to be the preferred species transported in the xylem sap. When arsenic was supplied in methylated form, it was transported mainly in that form. Results from glutathione reductase (GR) and catalase (CAT) enzymatic studies in *P. vittata* revealed that, upon arsenic exposure, CAT activity was induced but GR activity was not. Further, GR was not inhibited or activated by arsenic. However, CAT activity appeared to be activated by arsenate. This activation may allow *P. vittata* to more efficiently mediate stress caused

by arsenic. A field study was conducted to determine the efficiency of *P. vittata* in phytoextraction of arsenic contaminated soil. The study suggested *P. vittata* is capable of accumulating arsenic from contaminated sites, and a single harvest per year yields the greatest arsenic removal. Further, results from sequential arsenic fractionation analyses suggested that *P. vittata* is able to access arsenic from more unavailable soil fractions. Phytoextraction of arsenic-contaminated soils using *P. vittata* may be competitive with conventional remediation systems, but its application may be more practical for low-level contamination. The phytoextraction study revealed a discrepancy in mass balance. One hypothesis was that combination of over watering and solubilization of arsenic by root exudates caused leaching. Therefore, it was important to identify if leaching was occurring. It was also hypothesized that leaching may be harnessed for development of an innovative *ex-situ* soil remediation method, *phytoleaching.* Water and chemical solutions were added to promote arsenic leaching, while ferns removed arsenic via uptake. More arsenic was leached from soil when ammonium phosphate solution was applied. When ferns were present in contaminated soil, less arsenic was leached, indicating that *P. vittata* does not promote arsenic leaching. Phytoleaching may be a feasible remediation option with additional studies and refinement.

CHAPTER 1
INTRODUCTION

Arsenic (As) contamination of soil is a growing concern worldwide because it is toxic and is a suspected a carcinogen. When arsenic is in soil and water, it can be taken up by plants and indirectly ingested by animals and humans. Arsenic occurs naturally in the environment, but significant arsenic levels result from anthropogenic sources, such as mining and smelting operations, fuel combustion, biosolids, tanning, wood preservatives and pesticides (O'Neill 1990).

Recent attention has been focused on chromated copper arsenic (CCA) treated lumber, which has been widely used as a preservative. The treated lumber can serve many purposes: telephone poles, decks, pilings, home construction and playground equipment. However, there is concern regarding the leaching of arsenic from CCA treated lumber, prompting numerous studies that have addressed this issue (Cooper, 1991; Stilwell and Gorny 1997; Lebow et al., 2003). Arsenic contamination occurs through other sources, such as those previously mentioned. It is important to address contamination of soil by arsenic and target it for appropriate remediation to prevent possible impacts on the ecosystems.

Hyperaccumulators are plants that can take up and concentrate greater than 0.1% of a given element in their tissue. Recently, an arsenic hyperaccumulator, *Pteris vittata* L. (Chinese brake fern), was discovered (Ma et al., 2001). This arsenic hyperaccumulator may offer an alternative to more traditional remediation technologies for arsenic contaminated soils.

Phytoremediation is the use of plants to remove or render contaminants harmless in the ecosystem. Phytoremediation actually includes several methods, such as phytovolatilization, phytostabilization and phytoextraction. Phytovolatilization refers to the uptake, translocation and volatilization of contaminants from plants. The contaminants may or may not be transformed during this process. Phytostabilization employs plants in order to contain contaminants in the soil, preventing migration of the contaminant off site. Phytoextraction is the use of plants, preferably hyperaccumulators, to take up contaminants. Subsequently, the plants are harvested, transported and disposed off site (Schnoor, 2002).

Phytoextraction has become increasingly popular because of its low cost compared to more traditional remediation technologies. The costs involved in phytoremediation may include planting, maintenance, harvesting and disposal of plant biomass. The volume and mass of the plant disposal are significantly less than the disposal of soil when excavation is required. However, because phytoextraction is dependent on the plant, conditions at the site must be able to maintain plant production, and the contaminant must be accessible to the roots for uptake. In addition, soils with very high contaminant concentrations may inhibit plant growth and/or significantly prolong the amount of time required for remediation (Schnoor, 2002). Much research is still required to ensure proper employment and utilization of phytoextraction.

In general, arsenic is toxic to plants, especially in high concentrations. Arsenate can disrupt oxidative phosphorylation, and the production of ATP (Meharg and MacNair 1994; Oremland and Stolz 2003), while arsenite affects the function of enzymes and proteins by binding to sulfhydryl groups (Leonard and Lauwerys 1980; Oremland and

Stolz 2003). Also, the conversion of arsenate to arsenite, the more toxic form of arsenic, in the plant may create reactive oxygen species (ROS) that can damage plant cells. The toxicity of arsenite may be ameliorated through the production and use of glutathione (GSH) and/or phytochelatins (PC). Antioxidants and antioxidant enzymes may also assist in stress management by responding to the increased levels of ROS in the plant.

It is important to understand the ability of *P. vittata* to hyperaccumulate arsenic and its usefulness in the phytoremediation of arsenic-contaminated soils. The experiments included in this project were conducted to better understand *P. vittata*. The two main objectives were: 1). to increase understanding of the ability of *P. vittata* to hyperaccumulate arsenic; and 2). to determine the practicality, efficiency and ability of *P. vittata* to phytoremediate arsenic-contaminated soil.

The first objective was addressed by examining the speciation and transport of arsenic in *P. vittata* and the effects of arsenic on the antioxidant enzymes glutathione reductase (GR) and catalase (CAT) in *P. vittata*. The second objective was achieved through phytoextraction field studies at a CCA-contaminated site, which specifically investigated into the effects of *P. vittata* on the leaching of arsenic and its as a new phytoremediation technique, and examined the availability of arsenic in CCA-contaminated soils.

CHAPTER 2
LITERATURE REVIEW

Arsenic

Arsenic has a long history being employed for medicinal uses, pigments and poisons. Around 1775, Carl Scheele developed the compound Paris Green, which was used as a pigment in wallpaper, paints and fabrics. However, persons living in the homes containing Paris Green often became ill from direct contact with arsenic or from arsenic volatilization from the pigment. There are also numerous documented accounts of individuals using arsenic to intentionally poison others (Buck, 1978). There is even a theory suggesting that the distressing fate of the 90% of the Jamestown colonists who perished during the winter of 1609-1610 may have been a result of arsenic poisoning and not of starvation (Marengo, 2001; Gundersen, 2002). It is apparent that much of the history of arsenic is blemished with its poisonous properties.

Chemistry of Arsenic

Arsenic, element number 33 in the periodic table; atomic weight 74.9216, is a crystalline metalloid or transition element (Group 5a). Its outer electronic configuration is $4s^2 4p^3$. Arsenic can exist in an allotropic form of alpha (yellow), beta (black) or gamma (gray). It can be present in several oxidations states such as -3, 0, +3 and +5. However, the most common forms of arsenic found in the environment include are arsenate [As(V)] and arsenite [As(III)] (Fig. 2-1) (Adriano, 1986; Matera and Le Hecho, 2001).

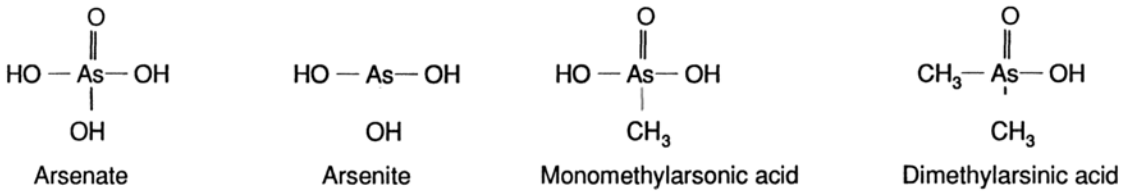


Figure 2-1. Chemical structures of arsenate, arsenite, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA).

Toxicity of Arsenic

A human may ingest as much as 900 μg of arsenic per day, depending on the environment (Fowler, 1977). Generally speaking, inorganic forms of arsenic, arsenite and arsenate, are more toxic than the organic forms of arsenic. This is unlike most other metals (O'Neill, 1990). Overall, the arsenic toxicity pattern is as follows: $AsH_3 > As^{3+} > As^{5+}$ > organic arsenic.

Arsine gas (AsH_3) is considered to be an extremely toxic form of arsenic. As little as 4 μg l^{-1} inhaled into a human body can interfere with many metabolic processes. Arsine gas inhalation can result in decreased erythrocyte osmotic resistance, reduced hemoglobin and erythrocytes and increased reticulocytes. Ultimately, the number of red blood cells may decrease by 50% in as little as one hour after exposure to arsine gas (Fowler, 1977).

Of the inorganic forms of arsenic, the trivalent form, or arsenite, is considered more toxic than the pentavalent form, or arsenate. This is because arsenite can readily combine with thiol (SH) groups. In addition, many enzymes and enzymatic processes may be inhibited by arsenite (Fowler, 1977; Oremland and Stolz 2003). In general, arsenite compounds are considered to be carcinogenic to humans (Hathaway et al., 1991;

Gochfeld, 1995). However, it is interesting to note that arsenite is a component of the drug, Trisenox®, which is used in the United States to treat people with afflicted acute myeloid leukemia (Hall, 2002).

Because of the chemical similarities between arsenate and phosphate, arsenate has the ability to replace phosphate in many biochemical processes. For example, arsenate can disrupt mitochondrial oxidative phosphorylation and thus the production of the nucleotide, adenosine triphosphate (ATP), which is a main energy source for cells. This process is known as arsenolysis, or the hydrolytic process whose first step is the replacement of arsenate for phosphate (Meharg and MacNair, 1994; Hall, 2002; Oremland and Stolz, 2003). Arsenate also has the ability to replace phosphate in DNA, ultimately compromising DNA processes (Fowler, 1977).

Ironically, arsenate is often converted to the more toxic form, arsenite, via enzymatic or non-enzymatic processes in the environment. The arsenate reductase enzyme has been identified in animals, bacteria and yeast. However, this enzyme has not yet been identified in plants (Mukhopadhyay et al., 2002; Rosen, 2002). Arsenite may be detoxified through methylation to monomethylarsenate (MMA) or dimethylarsenate (DMA) (Fig. 2-1). The methylated forms of arsenic, which are generally excreted from the body, are considered to be less toxic compared to inorganic forms of arsenic (Johnson and Farmer, 1991).

Arsenic in the Atmosphere

Arsenic is cycled between the lithosphere, pedosphere, biosphere, hydrosphere and atmosphere (Fig. 2-2). The atmosphere contains 0.8×10^6 kg (Walsh et al., 1979) to 1.74×10^6 kg of arsenic (Chilvers and Peterson, 1987). Approximately 85% of this arsenic is

located in the northern hemisphere, due to a higher number of industrialized countries and a larger land mass (Matschullat, 2000).

Arsenic may be emitted into the atmosphere through natural sources (i.e., volcanoes) or anthropogenic sources. Approximately 60% of the anthropogenic arsenic emissions results from coal combustion and copper smelting. Wood preservation, herbicides, steel production, lead and zinc smelting, and incineration account for the remaining 40%. Most of metallic arsenic emitted into the atmosphere is present as particulate matter, and it may be retained in the atmosphere for seven to 10 days (Matschullat, 2000).

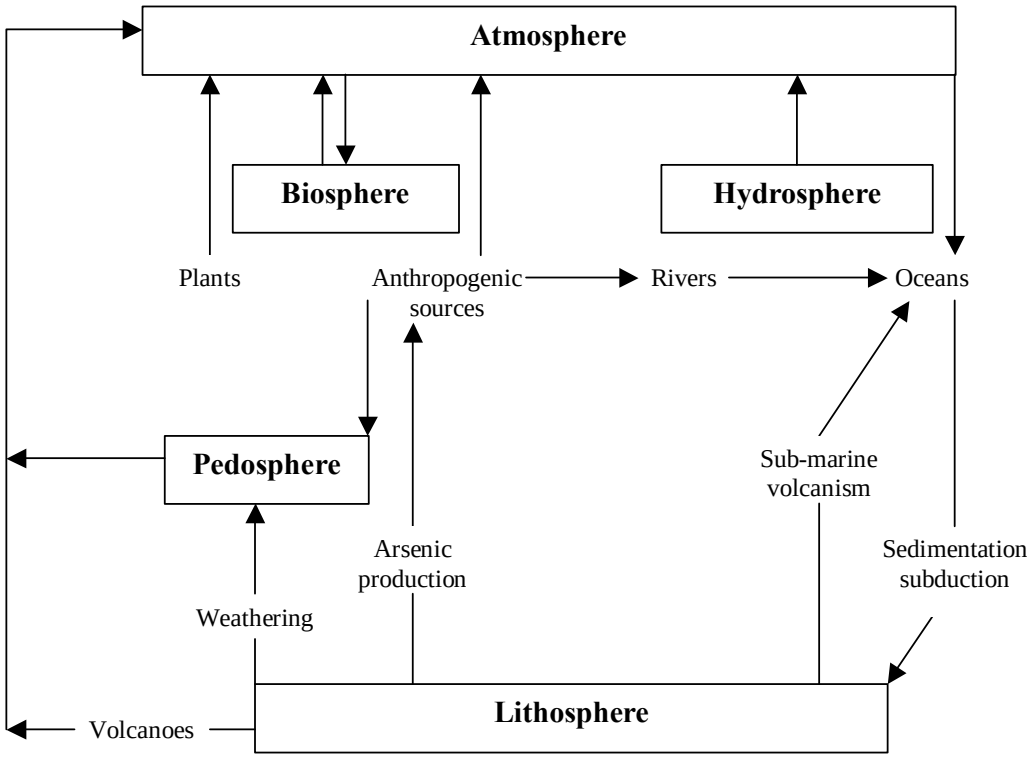


Figure 2-2. Global arsenic cycle (adapted from Matschullat, 2000).

Arsenic in Minerals

Arsenic, which is the 52nd most abundant element in the earth's crust, has an average crustal concentration of 1.5 to 2.0 mg kg^{-1} (Adriano, 1986). Approximately 4.01 x 10^6 kg of arsenic is present in the earth's crust (Matschullat, 2000). On average, shales, granites and sandstones have 13.0, 3.0 and 1.0 mg As kg^{-1}, respectively (Onishi, 1969). In general, arsenic concentrations in igneous rocks range from <1 to 15 mg As kg^{-1}; argillaceous sedimentary rocks (such as shale, sandstone and slate) from <1 to 900 mg As kg^{-1}; limestones from <1 to 20 mg As kg^{-1}; and phosphate rocks from <1 to 200 mg As kg^{-1} (O'Neill, 1990). However, rocks associated with uranium may contain much higher concentrations of arsenic [Committee on Medical and Biological Effects on Environmental Pollution (CMBEEP), 1977].

More than 200 arsenic-containing minerals exist. Of these minerals, most are arsenates (60%), with the rest being sulphides and sulphosalts (20%) and arsenides and arsenites oxides (20%). Arsenopyrite ($FeAsS_2$) is the most common arsenic mineral (O'Neill, 1990). Arsenic-containing sulfides, such as arsenopyrite, tend to be important arsenic-containing minerals. Examples of these are realgar (AsS), niccolite (NiAsS) and cobaltite (CoAsS) (Allard, 1995; Reimann and deCaritat, 1998).

Arsenic in Water

Arsenic is relatively soluble in salt and fresh waters, and it can be present as arsenite, arsenate or methylated arsenic. Mobilization of arsenic from soils or inputs from anthropogenic sources can cause increases in stream concentrations and eventually ocean concentrations (Matschullat, 2000). Groundwater contamination can be a result of the dissolution of minerals from rocks and soil or anthropogenic sources.

Contamination of drinking water by arsenic is a serious threat to millions of people worldwide. Of most prominence are the severe health problems of thousands of people in Bangladesh and west Bengal, India. Health concerns arise due to arsenic-contaminated groundwater (Chatterjee et al., 1995; Das et al., 1995; Abernathy et al., 1997). The regulated upper limit of arsenic in drinking water in the United States is 10 $\mu g\ l^{-1}$. All public drinking water systems must meet this standard by 2006 [United States Environmental Protection Agency (USEPA), 2001]. Figure 2-3 shows estimated concentrations of arsenic in groundwater in the United States. Concentrations tend to be highest in parts of the West, Midwest and upper Northeast.

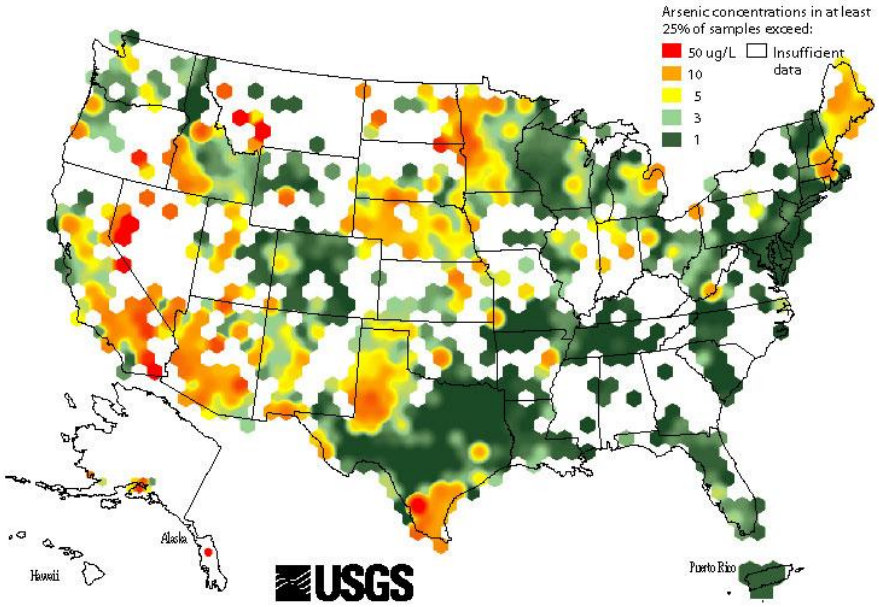


Figure 2-3. Arsenic concentrations in groundwater sampled in the United States (Ryker, 2001).

Arsenic in Soils

As previously mentioned, arsenic is found in many different minerals and rocks. As a result, arsenic is also found naturally in soils due to the weathering of these rocks and minerals (Adriano, 1986). Arsenic concentration generally ranges from 0.2 to 41 mg kg^{-1} in soils worldwide (Kabata-Pendias and Pendias, 2001). Arsenic concentration averaged 7.2 mg kg^{-1} for surface soils in the United States (Shacklette and Boerngen, 1984). However, agricultural surface soil exposed to repeated arsenic pesticides can have an arsenic concentration as high as 600 mg kg^{-1} (Adriano, 1986), and soil arsenic may range from 400 to 900 mg kg^{-1} in areas of arsenic mineral deposits (National Research Council Canada [NRCC], 1978). Also, soils in areas near coal mining and those overlying sulfide ore deposits may have even higher arsenic concentrations.

Behavior of Arsenic

Arsenic and phosphorus (P) have similar chemical properties; therefore, they act similarly in the soil. Phosphorus and arsenic may compete with each other for soil fixation sites and for plant uptake (Adriano, 1986). The phytotoxicity of arsenic may increase with decreasing soil phosphorus levels (Rumburg et al., 1960; Juska and Hanson, 1967). Still, other experiments have indicated that additional phosphorus may increase arsenic phytotoxicity by releasing more arsenic into solution (Schweizer, 1967; Jacobs and Keeney, 1970).

Arsenic Availability

The total arsenic concentration in soils does not necessarily determine the arsenic phytoavaliability (Adriano, 1986). Although a finite amount of the total arsenic in the soil is readily mobile, the rest is not available to plants because it is associated mostly

with iron (Fe) and aluminum (Al). The arsenite form (reduced form) is generally more soluble in soil than the arsenate form (oxidized form). The concentration of soluble arsenic is directly proportional to plant arsenic toxicity, although soil properties are also important in the determination of arsenic availability (Kabata-Pendias and Pendias, 2001).

The availability of arsenic in soils may be affected by many soil factors, such as soil pH (Adriano, 2001). In general, soil pH is important because it affects arsenic speciation and leachability. The adsorption optimum for arsenite is approximately at pH 7.0; however, arsenate adsorbs optimally at pH 4.0 (Pierce and Moore, 1982). Overall, at a low soil pH the hydroxyl groups on the outside of clays, amorphous silicates and metal oxides become protonated. These sites are then able to adsorb arsenic anions present in the soil. Therefore, arsenic is less mobile at lower pH because most of the arsenic is present as arsenate in (aerobic) soils, and there are high concentrations of arsenic-binding species, such as iron and aluminum at low pH (Sposito, 1989). As the pH increases there are fewer protonated sites, allowing the arsenic to become more mobile.

However, arsenic does have the ability to form a strong association with calcium (i.e., calcite) allowing it to possibly be retained at a higher pH. This association may be found under high arsenic concentrations, where arsenic has a secondary preference to calcium over aluminum (Woolson, 1983). At lower pHs, calcite is dissolved by the acidic conditions, and the arsenic is released.

Soil texture is another important factor affecting arsenic availability (Adriano, 2001). For example, soil texture affects the soil surface area. Finer textured soils (silts and clays) have much more surface area than coarse (sandy) soils; therefore, they are

more reactive. Finer-textured soils are more likely to retain higher amounts of trace elements compared to sandy soils (Chen et al., 1999; Berti and Jacobs, 1996). Apart from increased surface area, fine textured soils also have higher cation exchange capacity (CEC). A greater CEC leads to higher retention for cationic species like copper (Chen et al., 1999).

It is also possible to find more organic matter (OM) in finer textured soils with a high CEC, compared to sandy soils with low CEC. Often, high OM leads to high CEC, mostly from the pH-dependent charge. Conditions in fine textured soils are also more conducive to OM accumulation and retention. Organic matter increases retention of both cationic and anionic species. This is achieved through cationic bridging by iron and aluminum, resulting in anion retention, and the dissociation of edges of organic complexes in response to changes in pH. This allows for the retention of both cations and anions, depending on pH.

Soils with sandy textures may increase the toxicity of arsenic to plants and arsenic mobility; compared to soils with clayey textures (Jacobs and Keeney, 1970; Woolson, 1973; Akins and Lewis, 1976; Adriano, 1986). The presence of iron and aluminum oxides also plays an important role in the ability of a soil to retain arsenic (Adriano, 2001; Jacobs et al., 1970; Lumsdon et al., 1984). Further, soil iron and phosphorus concentrations are important factors influencing arsenic concentrations in Florida soils (Chen et al., 2002).

Research on phosphorus indicated that sand grains with clay coatings have a higher ability to retain elements compared to bare quartz grains (Harris et al., 1987a, b). The common coating components, for example, metal oxides and aluminosilicates, have a

high affinity for trace elements, such as arsenic. Some soil horizons (i.e., albic horizons in Spodosols) have been exposed to extreme weathering and leaching. This weathering results in the sand grains being stripped of their clay coatings (Harris et al., 1987a, b). However, Rhue et al. (1994) found that some of these horizons are able to retain their clay coatings. As such they exhibit greater retention ability compared to those that did not retain their coatings.

Arsenic Speciation

As previously mentioned, arsenic can be present in four oxidation states: (-3), (0), (+3) and (+5). However, arsenate (+5) and arsenite (+3) are the more prevalent forms in the soil environment. The form of arsenic in soil is very important, as it can dictate the behavior. Arsenite is considered to be more water soluble, or mobile, in soils compared to arsenate (Pierce and Moore, 1982). This is important because arsenite is considered to be more toxic form of arsenic. The occurrence of arsenite or arsenate in soil is a function of both the pH and redox potential (Eh) (Masscheleyn et al., 1991)

In aerobic soils, arsenate constitutes up to 90% of the total arsenic. However, under anaerobic conditions, only 15 to 40% of the arsenic is present as arsenate (O'Neill, 1990). Arsenate can form insoluble compounds with aluminum, iron and calcium in the soil. As previously mentioned, arsenate and phosphate are chemical analogues. Therefore, arsenate behaves similarly to phosphate, and it often competes with phosphate in the soil.

When soil conditions are moderately reducing (Eh 0 to -100 mV), the solubility of arsenic is dictated by iron oxyhydroxides, as the arsenate precipitates with iron compounds. In soils that are flooded, conditions are extremely reducing (Eh -200 mV),

and arsenic is much more mobile (Matera and Le Hecho, 2001). However, the transformation of arsenate to arsenite is very slow. Therefore, arsenate can often be detected in highly reduced soils (Onken and Hossner, 1996).

Microorganisms also play a role in the speciation of arsenic in soils. There are arsenite-oxidizing bacteria that can transform arsenite to arsenate. Similarly, arsenate-reducing bacteria can convert arsenate to arsenite (Cullen and Reimer, 1989). Also present in soils are microorganisms that can convert arsenite to methylated forms of arsenic (Pongratz, 1998).

Arsenic Contamination

Arsenic contamination of soil and water can result from several anthropogenic activities, such as: pesticide use/production, mining, smelting, combustion and sewage/solid waste (O'Neill 1990; Davis et al., 2001; Oremland and Stoltz, 2003).

Pesticides

Arsenical compounds have been used in pesticides for over one hundred years. However, since the 1970's their total use has declined (O'Neill, 1990). Arsenic is effective as an herbicide, in wood treatment and as a desiccant of cotton. Worldwide average uses have been estimated at 8,000 t yr^{-1} for herbicides, 12,000 t As yr^{-1} for cotton desiccants and 16,000 t yr^{-1} for wood preservatives (Chilvers and Peterson, 1987).

Of recent concern has been the use of chromated copper arsenate (CCA). Chromated copper arsenate is a pesticide that helps to reduce microbial and fungal decay of wood products. Arsenic and copper (Cu) act as the insecticide and fungicide, respectively. Chromium (Cr) fixes the arsenic and copper to the wood's cellulose and other components (Dawson et al., 1991). In January 2004, domestic use of CCA-treated

wood was voluntarily discontinued (USEPA, 2002b). Prior to that, it constituted approximately 75% of the treated wood market by volume (Solo-Gabriele et al., 1999), which is a strong statement to its effectiveness (Warner and Solomon, 1990).

In the Southeastern United States CCA-treated wood use was particularly high. This high use is a result of the hot, humid summers and mild winters of the region. Such conditions increase both the rate of weathering and biological (i.e., microbial and fungal) activity and subsequent decay (Chirenje et al., 2003). However, the massive manufacture, use and disposal of CCA-treated wood has led to increased loadings of these elements into the environment (Carey et al., 1996; Lebow, 1996; Cooper and Ung, 1997; Stilwell and Gorny, 1997; Solo-Gabriele et al., 2000; Townsend et al., 2000; Rahman et al., 2004). For example, when treating wood with CCA, up to 250 l of CCA solution are applied under pressure for every 1 m^3 of wood. This results in treatment solutions containing arsenic, chromium and copper concentrations in the range of 1000–5000 mg kg^{-1} (Aceto and Fedele, 1994). A single 12 ft x 2 inch x 6 inch piece of lumber treated by type C CCA contains approximately 27 grams of arsenic. This is enough arsenic to poison more than 200 adults. On average, one tablespoon, or approximately 20 grams, of CCA wood ash can contain enough arsenic to kill an adult human.

In CCA solution, arsenic is generally used in its anionic form, arsenate. Solo-Gabriele et al. (2000) have shown that although new CCA wood contains predominantly arsenate, which is moderately toxic and carcinogenic, the concentrations of arsenite, which is highly toxic and carcinogenic, increased as the wood aged. These results show that the form of arsenic changes in both soil and in CCA treated wood. Also, the increase in arsenite concentrations is of concern for both human and ecosystem health.

The concentrations of the three elements in CCA-treated wood, copper, chromium and arsenic, are not very different from each other. However, compared to copper and chromium, arsenic can leach out as much as an order of magnitude more from the treated wood products. The type of CCA, wood type, orientation of the wood and surface area may affect the degree of arsenic leaching from the treated wood (Hingston et al., 2001). Climate and moisture conditions also affect arsenic leaching from treated lumber (Kaldas and Cooper, 1996; Lebow et al., 2004). Arsenic concentrations of approximately 550 mg kg^{-1} have been reported in the vicinity of CCA-treated utility poles (Cooper and Ung, 1997).

Mining and Smelting

Arsenic is often a by-product of smelting lead, zinc, copper, iron, gold and manganese (Benson et al., 1981). A CMBEEP report (1977) indicated that copper, zinc and lead smelting and refining releases 955, 591 and 364 metric tons of arsenic for every million metric tons produced, respectively. During mining and smelting processes, arsenic may be released as a gas or as fly ash. Soils in the vicinity of smelters can become contaminated through deposition by rain or the settling of fly ash. An examination of a smelter in Tacoma, Washington found 7 to 152 t As yr^{-1} were deposited, while a smelter in Canada deposited 19 to 2600 t As yr^{-1} (Woolson, 1983).

Mine spoils/dumps can also cause arsenic contamination. Arsenic may leach from these spoils and/or finer material may be dispersed by wind. Arsenic concentrations were found to be over 4 g kg^{-1} in the vicinity of old mine spoils in Virginia. The condition may be exasperated by the difficulty or inability of plants to grow and thrive on these

soils/spoils. A lack of productive vegetation may decrease the stability of the soil, thus increasing water and wind erosion (O'Neill, 1990).

Combustion of Fossil Fuels

Fossil fuels naturally contain arsenic. On average fuel oils contain 0.015 mg As kg^{-1} (O'Neill, 1990). However, arsenic concentration in coal can range from 15 to 150 mg kg^{-1} (Cullen and Reimer, 1989). The increase in the burning of fossil fuels has also increased the opportunity for arsenic contamination in soil.

During the combustion of fossil fuels, such as coal and oil, arsenic may be volatilized. The amount of arsenic volatilized is dependent on the form of the arsenic in the coal. For example, arsenical sulfides are more volatile than organically-complexed arsenic. Approximately 600 million tons of coal were burned in 1983 in the United States; this resulted in the emission of an estimated 800 tons of arsenic (CMBEEP, 1977; Woolson, 1983). Coal combustion also produces ash, which contains approximately 7 to 60 mg As kg^{-1} (O'Neill, 1990).

Biosolids

An increase in industrialization has also lead to an increase in the amount of arsenic present in biosolids. Deposits from the atmosphere, runoff and from effluents of industries often increases the concentration of arsenic in biosolids. Woolson (1983) reported a range of 0 to 188 mg As kg^{-1} dry weight of biosolids. Biosolids are often disposed on land and may subsequently increase arsenic concentrations in the top 20 cm of soil by up to 0.15% (O'Neill, 1990).

Remediation of Arsenic Contaminated Soils

Currently there are several options that exist for the remediation of arsenic contaminated soils. Remediation methods vary greatly in cost, intensity and necessary treatment length. No single soil remediation technique is suitable for all situations. Therefore, careful investigation of the contaminated site characteristics, contaminant problem, treatment options and treatment timeframe must be considered in order to achieve a successful clean up of a site. Several of the current arsenic remediation methods are summarized in Table 2-1, and several will be discussed in the following sections.

Physical Remediation

Excavation, capping and solidification are three examples of physical remediation methods. Excavation is a commonly used remediation method. It is simply the physical removal and disposal of contaminated soil. This method produces rapid remediation results. However, it is often expensive because of the operation, transport and special landfill requirements (Sparks, 1995; USEPA, 2002a).

Capping is also a rather simple method. It requires covering contaminated soil with a hard cover (i.e., concrete or asphalt) to reduce exposure. However, this method does not remove contaminants from the soil, as the contaminants are still present in the soil (Sparks, 1995; USEPA, 2002a).

Stabilization and solidification are *in situ* physical treatments where soil is mixed with cement or stabilizers to create a hardened mixture. Solidification reduces the mobility of arsenic in the soil. Vitrification is a type of solidification. Soil is chemically bonded inside a glass matrix, where the arsenates become silicoarsenates. The drawbacks

to solidification and stabilization remediation techniques are that they can be relatively costly. Also, soil conditions often dictate the feasibility of implementing these methods (Tadesse et al., 1994; USEPA, 2002a).

Table 2-1. Summary of select current remediation technologies for arsenic-contaminated soil (adapted from USEPA, 2002a).

Arsenic Remediation Technology	Description
Excavation	• *Ex-situ* method that removes soil from site • Contaminated soil stored in designated landfill
Capping	• *In-situ* method • Hard cover placed on soil
Solidification and stabilization	• Reduces the mobility of arsenic in soil • Contaminated soil is mixed with stabilizers *in situ*
Vitrification	• Arsenic is chemically bonded inside a glass matrix • Arsenates become silicoarsenates • *In situ* treatment
Soil washing/Acid extraction	• Arsenic is suspended or dissolved in a wash solution • Concentrates contaminants • Water-based and *ex-situ* treatment
Soil flushing	• *In situ* method uses water, chemicals or organics to flush soil • Arsenic is mobilized and is collected for removal or treatment
Pyrometallurgical treatment	• Uses heat to concentrate arsenic • Arsenic is volatilized and collected
Electrokinetic treatment	• Arsenic is mobilized as charged particles by using a low-density current • Arsenic removed through several means, such as electroplating and precipitation • *In situ* treatment
Phytoremediation/phytoextraction	• *In situ* method using plants to take up arsenic from soil • Biomass is harvested and disposed

Chemical Remediation

Chemical remediation can include soil washing/acid extraction and soil flushing. Each of these methods utilizes chemicals to aid in the removal of arsenic from the soil. Soil washing/acid extraction is an *ex situ* remediation method used to dissolve and concentrate arsenic. The concentrated arsenic can then be disposed. Similarly, soil flushing uses chemicals and/or water to remove arsenic from the soil. However, this method is performed *in situ*, which can create concern regarding groundwater contamination (USEPA, 2002a).

Bioremediation

Bioremediation includes any method that uses microbes or plants for remediation, such as: bioleaching, bioaccumulation and phytoremediation. Methods of bioremediation often require inputs into the soil or system to enable the microorganisms and/or plants to produce or grow properly.

Bioleaching involves the use of microbes to alter soil factors, such as pH and redox potential, to increase the solubility of arsenic. This can be accomplished through organic acid production. Once the arsenic becomes more mobile, it can be leached and collected from the soil. On the other hand, bioaccumulation utilizes microbes to absorb contaminants from the soil (Zwieten and Grieve, 1995; USEPA, 2002a).

Phytoremediation is an all-encompassing term to include any remediation method that utilizes plants. Phytoremediation involves plants to either remove pollutants or render them harmless in soil and water systems. This practice has been growing in popularity because of its overall cost-effectiveness (Salt et al., 1995; Watanabe, 1997;

Kabata-Pendias and Pendias, 2001). The term phytoremediation includes several methods, and a few will be discussed in greater details in the following section.

Phytoremediation

Plants can phytoremediate soil and/or water by degrading, removing or containing contaminant(s). The degradation of chemicals can take place in the rhizosphere or possibly the bulk soil through plant root exudation of compounds to convert the contaminant into non-harmful chemical forms. This is known as phytodegredation. Sometimes the plants can take up the contaminant, at which point several things can happen. The chemical can be transported through the plant and to the leaves, and then volatilized via the plant's transpiration. This is termed phytovolatilization. Another fate of a plant-absorbed chemical is storage and sequestration somewhere in the plant (i.e., roots or leaves). These are termed phytoextraction (leaves) or rhizofiltration (roots) (Raskin and Ensley, 2000; Lasat, 2002; McGrath et al., 2002). Ideally in phytoextraction, the contaminant will be translocated to the aboveground biomass where it can be harvested and transported off-site. Lastly, phytostabilization is the use of plants in order to contain the contaminant by reducing its leaching potential.

There are several things that must be considered prior to the initiation of any phytoremediation project. The most important is the ability of a given plant to actually remediate the contaminant in question. Some plants will simply tolerate a contaminant, some will die and some will thrive. The site factors, such as soil properties, source of contamination, extent of contamination, etc., must also be considered.

Phytoremediation is generally thought to be an inexpensive alternative to traditional remediation technologies. This is due to the fact that often less labor and

heavy equipment is needed. For example, in phytoextraction the harvested plants are much lighter to transport than soil (i.e., excavation). Phytoextraction is also considered to be more aesthetically appealing than some traditional remediation technologies. The plants may be relatively easier for the public to accept, as they are more attractive compared to bare soils or caps. Also, in some cases, the plants can actually breakdown the contaminant(s). This is unlike many other remediation technologies where the contaminants are simply contained and/or transported off-site (Schoor, 2002; USEPA, 2002a; Wolfe and Bjornstad, 2002).

However, phytoremediation is a fairly new technology and is very dependent on the plant in the system. This impacts the efficiency and dependability of phytoremediation; therefore, there are many questions or areas of concern that need to be addressed. First, if the plant has a shallow root system it may not be able to fully remediate the soil or water because contaminants may be out of uptake range of the roots. Second, the plants may be limited to low or moderately contaminated sites. If the contaminant levels are too high there is the risk of killing the plant or compromising its growth, which would impede the remediation. Third, clean-up rate is generally much slower than traditional remediation methods. This may pose a problem when the requirements for remediation of the soil are more immediate. Fourth, there is not always a full understanding of the physiology, biochemistry, uptake, etc. of the plants employed (Schoor, 2002). Therefore, it is not always clear what is occurring between the plant and soil (i.e. volatilization or leaching).

Phytoextraction

Phytoextraction is an *in situ* remediation method that employs the use of plants to remove contaminants from soil or water. The plants are able to take up the contaminant

and store it in its roots or shoots. Some plants can efficiently translocate the contaminant to its aboveground biomass (Cunningham et al, 1997; USEPA, 2002a). In this case, the aboveground biomass can be removed and disposed of in a properly constructed landfill or incinerated. If the contaminant is of value, such as nickel or copper, it can be removed, or phytomined, from the plant. Ideally, the plants used for phytoextraction are hyperaccumulators of the contaminant in question.

Hyperaccumulators

Plants often contain trace concentrations of many contaminants of concern. At low levels, plants can usually metabolize or dispose of these compounds without any significant injury. Generally, at high contaminant concentrations in soil or water, plants often suffer and/or die because of their inability to metabolize these harmful elements. However, some plants can survive and/or thrive when they accumulate high concentrations of toxic elements.

Hyperaccumulators are plants that contain more than 1000 mg kg^{-1}, or 0.1%, of an element or compound. Ideally, hyperaccumulators should have a high rate of accumulation, be fast growing, and have a high production of biomass (Wantanbe, 1997; Brooks, 1998). The concentration of the contaminant is generally very high in these plants when grown in contaminated media. They must have both a bioconcentration factor (BF) and transfer factor (TF) greater than one. The BF is the plant to soil ratio for a particular contaminant, while the TF is the ratio of contaminant concentration in the plant to the contaminant concentration in the growing media.

The fern, *P. vittata* L., (Fig. 2-4) is an example of a plant that removes arsenic from soil and/or water, and it can be defined as an arsenic hyperaccumulator (Ma et al., 2001).

Ferns are lower plants, unlike many of the other identified hyperaccumulating plants, which are dicots or monocots. For example, several of these other hyperaccumulating plants are in the mustard family (dicots), such as *Thalaspi* spp. and *Brassica* spp. Also, many of these plants are able to hyperaccumulate a given metal, but are not very efficient at transporting that metal from the roots to shoots, unlike the identified arsenic hyperaccumulators (Schoor, 2002).

When a plant can hyperaccumulate a metal, such as *Thlaspi* spp. with zinc or *Alyssum* spp. with nickel, these metals can be mined from the plant, purified and reused, thus, increasing the value of these plants. However, arsenic is generally not an element of great value for mining.

Figure 2-4. *Pteris vittata* L. growing at an arsenic-contaminated site.

***Pteris vittata* L.**

Pteris vittata was recently discovered as the first known arsenic hyperaccumulator. *Pteris vittata* is very efficient at removing arsenic from soil. It cannot only take up high amounts of arsenic from soil and water, but it can transport arsenic very efficiently from its roots to its fronds (Ma et al., 2001).

This fern also produces a relatively high biomass, it and is a fast-growing plant. *Pteris vittata* is a perennial, and it survives winter fairly well in Florida and warmer climates, thus increasing its value as a hyperaccumulator. It is also tolerant of full sun, unlike many other ferns, but it also grows well under shady conditions.

Pteris vittata prefers an alkaline soil environment. This can contribute to its ability of arsenic-hyperaccumulation because, in general, arsenic is more available at a higher pH. *Pteris vittata* is also able to take up many forms of arsenic (Tu and Ma, 2002). Because of its fast-growth and arsenic hyperaccumulation, this fern exhibits potential for use in the phytoremediation of arsenic-contaminated soils.

After 20 weeks of growth, *P. vittata* accumulated arsenic of 11.8 to 64.0 mg kg^{-1} when grown in uncontaminated soil; however, it accumulated 1,442 to 7,526 mg kg^{-1} arsenic when grown at an arsenic-contaminated site. The arsenic concentration was much higher in the fronds than roots. Therefore, it has both a high TF and a high BF. Although *P. vittata* is capable of taking up many different arsenic species but did not readily take up $FeAsO_4$ and $AlAsO_4$. These arsenic species are generally insoluble in the soil (Tu and Ma, 2002).

After 12 weeks of growth *P. vittata* produced more aboveground biomass in soils containing 50 and 100 mg kg^{-1} arsenic compared to ferns grown in the soil not contaminated with arsenic. These results indicate that low levels of soil arsenic may actually be beneficial to the growth of this fern. However, when the soil arsenic concentration was 200 mg kg^{-1}, there was a slight decrease in fern biomass. No significant differences in root biomass were found at any arsenic concentration. Overall, the mature fronds and the old fronds had the highest arsenic concentrations, while roots

had the lowest after 23 weeks of growth (Tu and Ma, 2002). Phosphorus levels in fronds were higher in ferns grown in soil containing 50 and 100 mg kg^{-1} arsenic versus 0 or 200 mg kg^{-1} arsenic. *Pteris vittata* roots, however, had higher phosphorus concentrations in the control soil (Tu and Ma, 2003).

Arsenic has been shown to leach from *P. vittata* fronds as they senesce. This may pose a potential drawback to the use of *P. vittata* in phytoremediation of arsenic contaminated soils, as the arsenic may be returned to the soil (Tu et al., 2003).

Other Arsenic Hyperaccumulating Ferns

Since the initial identification of *P. vittata* as an arsenic-hyperaccumulator, other ferns have been identified to hyperaccumulate arsenic. However, not all ferns are able to hyperaccumulate arsenic (Kuehnelt et al., 2000; Meharg, 2002; Visoottiviseth et al., 2002; Zhao et al., 2002; Meharg, 2003). To date, the majority of ferns that do hyperaccumulate arsenic belong to the *Pteris* genus. *Pteris cretica*, *P. longifolia* and *P. umbrosa* have been shown to hyperaccumulate arsenic to the same extent as *P. vittata* (Zhao et al., 2002). However, not all members of the *Pteris* genus are able to hyperaccumulate arsenic. For example, Meharg (2003) found that *Pteris tremula* and *Pteris stramina* do not hyperaccumulate arsenic. In this same study, the author found that ferns that are able to hyperaccumulate arsenic developed comparatively late, evolutionarily speaking, for ferns.

In a study performed by Zhao et al. (2002), four other non-*Pteris* ferns were examined. However, they did not exhibit any ability to hyperaccumulate arsenic. Numerous fern species were also examined by Meharg (2003), and most of these fern species also did not hyperaccumulate arsenic. To date the only non-*Pteris* fern to exhibit

this ability is *Pityrogramma calomelanos* (Francesconi et al., 2002). Its fronds were able to accumulate 2760 to 8350 mg As kg^{-1} when grown in soil containing 135 to 510 mg As kg^{-1}. However, the authors were not able to establish a direct correlation between the arsenic concentrations in the fronds to those in the soil. Such a correlation was seen with *P. vittata* (Ma et al., 2001). Interestingly, the fronds with the greatest arsenic concentration were collected from ferns growing in the lowest soil arsenic concentration (135 mg As kg^{-1}). The authors stated that *Pityrogramma calomelanos* may be readily able to remove arsenic from soils that are less contaminated. Therefore, these ferns have the ability to effectively reduce soil arsenic concentrations. However, the hyperaccumulating ability under higher arsenic concentrations was not addressed. It was also suggested that *P. calomelanos* is a better phytoextraction candidate than *P. vittata* because it appeared able to grow better in the arsenic-contaminated soils from which both species were collected. However, there was no formal experimental comparison performed to evaluate this theory.

Arsenic in Plants

Arsenic is not an essential element for plants, and it is generally considered poisonous. However, plants have varying sensitivities to arsenic. Legumes are known to be highly sensitive to arsenic (Adriano, 1986), while *P. vittata* may grow better in the presence of arsenic (Ma et al., 2001).

Arsenic distribution within plants also varies. At high soil arsenic levels, old leaves and roots tend to have higher arsenic concentrations. At lower soil arsenic concentrations, plant arsenic levels are greater in leaves than in roots (Kabata-Pendias

and Pendias, 2001). However, this is not the case with *P. vittata*, where arsenic concentrations are generally greater in the aboveground biomass than the roots.

Arsenic toxicity may be evident in plants in several ways. Characteristic symptoms of arsenic toxicity in plants are: wilting of leaves, slow root growth and shoot growth, leaf necrosis, violet leaf color and ultimately plant death (Liebig, 1965; Woolson et al., 1971; Adriano, 1986). In general, arsenic inhibits metabolism in most plants (Kabata-Pendias and Pendias, 2001). More specifically, arsenate can disrupt oxidative phosphorylation and the production of ATP (Meharg and McNair, 1994, Oremland and Stolz, 2003). Arsenite affects the function of enzymes and proteins by binding to sulfhydryl groups (Leonard and Lauwerys ,1980, Oremland, and Stoltz 2003).

Arsenic Uptake by Plants

Plant arsenic uptake is influenced by arsenic source and solubility (Marcus-Wyner and Raines, 1982). It is hypothesized that arsenite is taken up passively via aquaglyceroporins, or channels allowing movement of water and neutral solutes, in the roots. Arsenate is a chemical analogue of phosphate, and is taken up via the phosphate transport system (Asher and Reay, 1979). However, in *Holcus lanatus* L., *Deschampsia cespitosa* L. and *Agrostis capillaries* L. an altered phosphorus transport system has been found. This transport system enables these plants to be arsenic tolerant by lowering the V_{max} and affinity for arsenate uptake (Meharg and MacNair, 1990; 1991a; 1991b; 1992).

Antioxidants and Antioxidant Enzymes

Exposure of plants to arsenic and heavy metals may result in the production of reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicals, resulting in damage to cell components (Weckx and Clijsters,

1996, Conklin, 2001; Hartley-Whitaker et al., 2001a). It is thought that the production of ROS when plants are exposed to arsenic is the result of the conversion of arsenate to arsenite (Hartley-Whitaker et al., 2001a; Meharg and Hartley-Whitaker, 2002). The metabolism of arsenite within the plant, for example through methylation, can result in the production of additional ROS (Zaman and Pardini, 1996).

In response to the creation of ROS, plants synthesize enzymatic and non-enzymatic antioxidants (Meharg and Hartley-Whitaker, 2002). Through the use of antioxidant molecules, such as L-ascorbic acid, reduced glutathione (GSH), α-tocopherols and carotenoids, plants can manage the detrimental effects of ROS. Specifically, ascorbic acid, which makes up approximately 10% (wt/wt) of the plant soluble carbohydrate, is an important and major plant antioxidant due to its high abundance (Noctor and Foyer, 1998).

As an antioxidant, ascorbic acid can manage ROS through the direct elimination of superoxide anions, hydrogen peroxide and hydroxyl radicals (Padh, 1990). It can also act as a secondary antioxidant through the maintenance of reduced α-tocopherol, another plant antioxidant (Liebler et al., 1986; Noctor and Foyer, 1998; Conklin, 2001), or obliquely through the action of ascorbate peroxidase (Foyer and Halliwell, 1976; Asada, 1992).

Under no arsenic stress, *P. vittata* was found to have intrinsically higher concentrations of non-enzymatic antioxidants, ascorbate (Asc) and glutathione (GSH), in its fronds compared to *Pteris ensiformis* (a non arsenic hyperaccumulator). This suggests that the ascorbate-glutathione pool may play a significant role in the ability of *P. vittata* to tolerate and hyperaccumulate arsenic (Singh et al., unpublished).

It is also possible for plants to bind oxygen free radicals and to detoxify organic contaminants using GSH. Through a reaction catalyzed by glutathione *S*-tranferases (GSTs), GSH can respond to oxidative stress by binding the organic contaminants or their metabolites, storage or incorporation into plant cellular components (Lamoureux et al., 1994; Xiang and Oliver, 1998). Glutathione is composed of the amino acids, glutamate, cysteine and glycine. Its significance lies mostly in its role as a reductant, as well as in its ability to detoxify harmful components within a cell. Glutathione is also a precursor for phytochelatins (PC) (Kneer and Zenk, 1992; Zenk, 1996; Pawlik-Showronska, 2001). Therefore, GSH has been implicated in aiding plants to cope with various environmental stresses, either directly by binding and detoxifying, or indirectly through conversion into phytochelatins.

Glutathione forms glutathione disulfide (GSSG) after oxidation as a result of its antioxidant activity. The GSSG can be reduced or recycled back to GSH, the reduced form, by the antioxidant enzyme glutathione reductase (GR). There are also several antioxidant enzymes in plants, including catalase (CAT) and superoxide dismutase (SOD) (Xiang and Oliver, 1998).

Glutathione reductase is the enzyme that, in conjunction with NADPH, catalyzes the reduction of GSSG to GSH (Eq. 2-1) (Carlberg and Mannervik, 1985).

$$GSSG + NADPH \xrightarrow{GR} GSH + NADP^{+} \qquad \text{(Eq. 2-1)}$$

Glutathione reductase has been detected in bacteria, yeast, plants and animals. It is essentially responsible for maintaining the GSH levels in the cell. Glutathione reductase activity has been shown to increase in plants exposed to environmental stress. For

example, GR in *Triticum durum* increased due to temperature stresses (Keles and Oncel, 2002). Exposure to copper also induced GR in the roots of *Phaseolus vulgaris* (Gupta et al., 1999).

Superoxide dismutase eliminates superoxide anions ($O_2^{\cdot -}$), yielding oxygen and hydrogen peroxide (H_2O_2) (Eq. 2-2).

$$2O_2^- + 2\,H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2 \qquad \text{(Eq. 2-2)}$$

Superoxide dismutase is associated with various metal cofactors: CuSOD and ZnSOD are located in cytosol, peroxisome, plastid and root nodules; MnSOD is located in the mitochondria; and, FeSOD is located in the plastids. Because SOD can degrade superoxide anions, it can play a very important role in the defense of cells upon stress (Fridovich, 1978; Fridovich, 1986; Fridovich, 1995).

Catalase, which can be found in primarily in the peroxisomes and root nodules, converts hydrogen peroxide (H_2O_2) to water and oxygen (Eq. 2-3).

$$O_2 + H_2O_2 \xrightarrow{\text{CAT}} H_2O + \tfrac{1}{2}\,O_2 \qquad \text{(Eq. 2-3)}$$

There are several forms, or isozymes, of CAT. These isozymes may respond differently under the same conditions. For example, in *Zea mays* L., the activities of two CAT isozymes (CAT-1 and CAT-2) were examined for their responses to salicylic acid inhibition. The CAT-1 isozyme was inhibited upon exposure to salicylic acid; however, the CAT-2 isozyme was not (Horvath et al., 2002).

A study on enzymatic antioxidants found that SOD and CAT are induced in the fronds of *P. vittata* upon arsenic exposure arsenic. However, under the same conditions

they are not induced in the fronds of *P. ensiformis* (Srivastava et al., 2005). This further suggests a role for antioxidant enzymes in arsenic tolerance and/or hyperaccumulation by *P. vittata*. Similarly, SOD and CAT activities were found to increase in *Zea mays* L. embryos upon arsenic exposure (Mylona et al., 1998).

Phytochelatins

Plants that take up heavy metals from soil or water often use phytochelatins to help limit the toxic effects of the metals. Phytochelatins, which contain thiol (SH) groups, are peptides in the plant with the ability to chelate heavy metals. Their general make-up is two or more γ-glutamylcysteine units that repeat and have glycine as the terminal residue.

Glutathione is a source of non-protein thiols, and is the precursor for phytochelatins. Using GSH, the phytochelatins are synthesized by the transpeptidase phytochelatin synthase enzyme (Kneer and Zenk, 1992; Zenk, 1996; Chen et al., 1997; Xiang and Oliver, 1998; Rhodes et al., 1999; Pawlik-Showronska, 2001). The synthesized phytochelatins are able to bind some metals in the cytosol, and the phytochelatin-metal complex is transported to the plant vacuole (Rauser, 1990).

Plants have several metal-sensitive enzymes, such as alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and ribulose-1,5-diphosphate carboxylase. Kneer and Zenk (1992) found that these enzymes were able to tolerate cadmium (Cd) at 10 to 1000 times greater concentration when it was complexed with phytochelatins. They also concluded that when heavy metals are at concentrations below the lethal level phytochelatins completely complex them.

However, Leopold et al. (1999) found that when *Silene vulgaris*, a heavy metal tolerant plant, was exposed to copper and cadmium there was no detectable heavy metal-

phytochelatin complexation. They concluded that not all plants are able to form stable heavy-metal-PC complexes. Another study involving *Silene vulgaris* exposed to arsenic-contaminated soil showed that there was a continuous accumulation of phytochelatins as exposure time increased (Sneller et al., 1999). Higher phytochelatin levels were also found in freshwater algae (*Stigeoclonium tenue*) when the metal solution pH was 8.2 versus 6.8 (Pawlik-Skowronska, 2001).

Pteris vittata was shown to have only 4.5% of its arsenic complexed with phytochelatins, as a glutathione-arsenite- phytochelatin complex (Zhao et al., 2003). In a study by Raab et al. (2004), it was determined that the arsenic hyperaccumulator, *P. cretica,* had only 1% of its arsenic complexed with phytochelatins. The conclusion reached in both studies was that the phytochelatins may act as shuttles for the arsenic for transport in a non-toxic form through the cytoplasm and into the vacuoles. They theorized that the vacuolar membrane may contain an arsenic-phytochelatin shuttle to aid in this process. Therefore, phytochelatins may not be the main source of detoxification of arsenite in arsenic hyperaccumulators.

CHAPTER 3
ARSENIC SPECIATION AND TRANSPORT IN *Pteris vittata* L.

Introduction

Plant arsenic uptake generally depends on arsenic source and solubility (Marcus-Wyner and Raines, 1982). It has been suggested that arsenic uptake by plants is passive and directly related to water flow (Kabata-Pendias and Pendias, 2001). Arsenic and phosphorus are chemical analogues, thus they often compete with each other for soil fixation sites (Adriano, 1986). It has also been hypothesized that arsenic may be taken up as arsenate and transported by the plant via the phosphate transport system (Asher and Reay, 1979). However, in grasses *Holcus lanatus* L., *Deschampsia cespitosa* L. and *Agrostis capillaris* L., an altered phosphorus transport system has been identified, aiding these plants in arsenic tolerance (Meharg and MacNair, 1990; 1991a; 1991b; 1992).

Pteris vittata is able to remove large amounts of arsenic from soil (Komar et al., 1998; Komar, 1999; Ma et al., 2001). Typical of hyperaccumulators, arsenic concentrations in *P. vittata* are mostly concentrated in the fronds (Ma et al., 2001; Tu et al., 2002; Zhang et al., 2002). This suggests efficient transport of arsenic from roots to fronds in *P. vittata*.

Arsenic speciation analysis of *P. vittata* grown in an arsenic-contaminated soil showed that >67% of the total arsenic in the aboveground biomass is present as the reduced form of arsenic, arsenite, which is considered to be the more toxic form. However, in roots only 8.3% of the arsenic is present as arsenite. The remaining arsenic was present in the oxidized form, arsenate (Zhang et al., 2002). Tu et al. (2003) found

similar results when arsenic was supplied to the ferns in several different forms. Regardless of the arsenic species supplied to the fern, >90% of the total arsenic in the roots is present as arsenate, versus approximately 94% arsenite in the fronds. In both studies, very low concentrations of organic arsenic were found in the fern, indicating that the arsenic is being reduced in the fern. A study conducted by Wang et al. (2002) examined the uptake kinetics of arsenate and arsenite, and the effects of phosphate on arsenic uptake by *P. vittata*. However, the study did not address methylated forms of arsenic or the form of arsenic that was transported within the plant. Therefore, no data exist regarding the forms of arsenic that are transported in *P. vittata*. Water and solutes are mostly transported via xylem in plants (Marschner, 1995), making xylem sap an important constituent for understanding arsenic transport in *P. vittata*.

In addition, transport of other constituents, such as phosphorus in the xylem sap may be impacted by, or may impact, arsenic transport. The chemical similarity between phosphate and arsenate raises the possibility for competition. Studies have shown that the presence of phosphate in the growing media affects the uptake and concentration of arsenic in the fern roots and fronds (Wang et al, 2002; Tu and Ma, 2003). Therefore, the presence of arsenic in the xylem sap may cause phosphorus deficiency in the fern.

The main objective of this study was to determine the forms of arsenic that are transported in the fern. More specifically, this study examined how the forms of arsenic supplied to the roots of *P. vittata* affect the forms of arsenic being transported to its fronds. In addition, the effects of arsenic concentrations and species on concentrations of inorganic phosphorus (P_i) in xylem sap were examined. The information obtained from

this study should be useful for a better understanding of the mechanisms of arsenic translocation in *P. vittata*.

Materials and Methods

Experimental Setup

Pteris vittata used for the experiments were of similar age and size. Plants were germinated from spores and grown in a mixture of commercial potting soil, sand and peat moss until they were approximately 8 months old. Roots of each fern were washed free of soil using deionized water before being transferred to a hydroponics system. Each fern was placed into individual 500 ml brown plastic bottle, which contained 0.2-strength Hoagland-Arnon nutrient solution (Hoagland and Arnon, 1938). The volume was maintained, and the plants were allowed 7 d to acclimate to the hydroponics conditions prior to treatment. All ferns were kept in a controlled environment with 65% humidity and day and night temperatures of 25^{o}C and 20^{o}C, respectively. The ferns were exposed to an 8 h light period with a light intensity of 350 μmoles m^{-2} s^{-1}.

Arsenic was added at concentrations of 0, 10 or 50 mg l^{-1}. This study was divided into two parts. In experiment A, *P. vittata* were treated with arsenic in the form of either As (III), as sodium arsenite ($NaAsO_2$), or As (V), as sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$). In experiment B, organic arsenic as monomethlyarsonic acid (MMA) or dimethylarsinic acid (DMA), was used. Arsenic treatments were added to each bottle using stock solutions diluted with 0.2-strength Hoagland-Arnon nutrient solution. Plants were harvested three days after treatment and separated into fronds and roots. Roots were rinsed with deionized water before analysis.

Xylem Sap Extraction

Xylem sap samples were extracted from 1 to 2 fronds of similar age and appearance from each fern. The xylem sap was collected using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA) (Schurr, 1998). A constant and high pressure, up to 40 bars, was applied to all fronds, and a micropipette was used to collect the extruded xylem sap. Xylem sap samples were preserved at -80^{o} C immediately following extraction.

Chemical Analysis of Arsenic and Phosphorus

Fronds and roots of *P. vittata* were dried for 24 h at 65^{o} C, and were ground in a Wiley Mill to pass through a 1 mm-mesh screen. The ground tissue samples (0.25 g) were subjected to hot block (Environmental Express, Ventura, CA) digestion using USEPA Method 3051 for arsenic analysis. The digested plant samples were analyzed for total arsenic using graphite furnace atomic absorption spectroscopy (GFAAS) (Perkin Elmer SIMMA 6000, Perkin-Elmer Corp., Norwalk, CT).

Due to arsenate interference with inorganic phosphate (P_i), the determination, P_i concentration in the xylem sap was performed using a modified molybdenum blue method (Carvalho et al., 1998). This method employs L-cysteine to prevent arsenate interference. Samples were analyzed at 880 nm using VIS-spectrophotometer detection (Shimadzu UV160U, Shimadzu Corp., Columbia, MD).

Arsenic Speciation in Plant and Xylem Sap Samples

Arsenic speciation was performed on *P. vittata* samples from experiment A using frond and root samples that were stored at -80^{o}C. Arsenic was extracted ultrasonically using a 1:1 methanol:water solution and was repeated two times for 4 h at 60^{o}C. This

extraction method results in 85 to 100 % recovery of arsenic from the fronds. However, arsenic extraction efficiency in the roots is approximately 60% (Zhang et al., 2002).

The combined extracts were diluted in 100 ml with deionized water; the pH of extract was ensured to range from 5 to 9. A solid phase extraction using an arsenic speciation cartridge (Metal Soft Center, Highland Park, NJ) was performed. The arsenate, which was retained in the disposable cartridge, and arsenite, which is passed through the cartridge, were separated (Meng et al., 2001). The total arsenic and arsenite fractions were determined using GFAAS. The arsenate fraction was calculated by the difference between the total arsenic and the arsenite fractions.

Arsenic speciation of the xylem sap for samples from experiment A was determined by high-performance liquid chromatography coupled with hydride generation atomic fluorescence spectrometry (HPLC-HG-AFS). The HPLC system consisted of a P4000 pump and an AS3000 autosampler with a 100 μl injection loop (Spectra-Physics Analytical, Inc. Fremont, CA). Arsenic species were separated using a Hamilton PRP-X100 anion exchange HPLC column (250 x 4.6 mm, 10 μm particle size) with a 0.015 mol l^{-1} potassium phosphate mobile phase (pH 5.8) at a flow rate of 1 ml min^{-1}. A hyphenated HG-AFS was a P. S. Analytical Millenium Excalibur system (PS analytical, Kent, UK) with hydride generation sample introduction. The outlet of the column was connected to a Teflon reactor and mixed with 12.5% HCl, then with the reductant solution containing 14 g $NaBH_4$ and 4 g NaOH in 1000 ml DDI water. The arsine gas produced was separated through a gas-liquid separator and sent to an integrated atomic fluorescence system for arsenic concentration detection. Data were acquired by a real-time chromatographic control and data acquisition system. Arsenic was quantified

through external calibration with standard solutions containing arsenite, arsenate, MMA and DMA. The lower detection limits for the HPLC-HG-AFS were approximately 1.0 μg l^{-1} for arsenite, 3.0 μg l^{-1} for MMA and DMA and 2.5 μg l^{-1} for arsenate. Quality assurance was obtained through the use of blanks, standard curves, standard check solutions and spiked samples, which were run during sample analysis.

Arsenic speciation of xylem sap for samples from experiment B was determined by coupling HPLC to inductively coupled plasma mass spectrometry (ICP-MS) (Chen et al., 2004). A VG Plasma Quadrupole II (VG Elemental, Winsford, Cheshire, UK) ICP-MS was used. The sample was injected via a peristaltic pump (Rainin, Woburn, MA) to a Meinhard TR-30-A concentric nebulizer (Precision Glassblowing, Englewood, CO). The HPLC system was composed of a SpectraSYSTEM P2000 Binary gradient pump (Thermo Separation Production Inc., Fremont, CA), an Auzx 210 injector valve with a 20 μl loop and a Haisil 100 (Higgins Analytical Inc., Mountain View, CA) C18 column (150×4.6 mm, 5 μm particle size). The mobile phase contained 10 mM hexadecyltrimethyl ammonium bromide (CTAB) as the ion-pairing reagent, 20 mM ammonium phosphate buffer, and 2% methanol at pH 6.0. Arsenic was quantified through external calibration with standard solutions containing arsenite, arsenate, MMA and DMA, which were prepared daily. Lower detection limits for the HPLC-ICP-MS were 0.5, 0.4, 0.3 and 1.8 μg l^{-1} for arsenite, DMA, MMA and arsenate, respectively. Quality assurance measures were the same as those used for HPLC-HG-AFS detection method.

Experimental Design and Statistical Analysis

Experiments A and B employed a randomized complete block design with 4 replications. All data were analyzed using the General Linear Model (GLM) with the Statistical Analysis System (SAS Institute, 2001).

Results

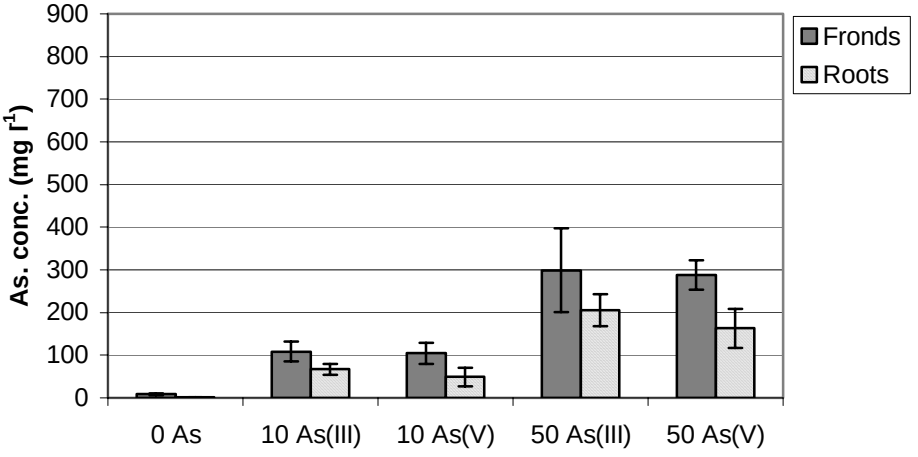
This experiment was designed to determine the effects of arsenic concentrations and species on the arsenic concentrations and species, and concentration of inorganic phosphate in the xylem sap of *P. vittata*. Three arsenic concentrations, 0, 10 and 50 mg l^{-1}, and four arsenic species, arsenite, arsenate, MMA and DMA, were used during the 3-d hydroponics experiments.

Arsenic Concentration and Speciation in Roots and Fronds

In this experiment, the arsenic concentration in the fronds and roots was directly proportional to the arsenic concentration supplied. Plants treated with 50 mg As l^{-1} had the highest frond and root arsenic concentrations compared to the control and 10 mg l^{-1} treatment (Fig. 3-1 A and B). No significant differences were found in plant arsenic concentrations (fronds or roots) between the arsenite and arsenate treatments. However, ferns treated with 50 mg As l^{-1} as MMA had the highest frond arsenic concentrations compared to the DMA treatments.

Compared to the roots, there was a higher, but not significant, level of arsenic concentrated in the fronds (Fig. 3-1 A and B). However, in ferns treated with 10 mg As l^{-1} as DMA or MMA arsenic was distributed evenly between the fronds and the roots. Compared to ferns treated with 10 mg As l^{-1} as As(III) or As(V), arsenic concentrations in the fronds treated with 10 mg l^{-1} DMA or MMA were approximately the same (92.5 to

109 mg kg^{-1}). However, arsenic concentrations in the roots treated with 10 mg As l^{-1} as DMA or MMA were higher than those treated with 10 mg As l^{-1} as As(III) or As(V). In other words, more arsenic remained in the roots when supplied with organic arsenic than inorganic arsenic. Such a trend was not observed when the arsenic treatment was increased to 50 mg l^{-1}, i.e., more arsenic was concentrated in the fronds.



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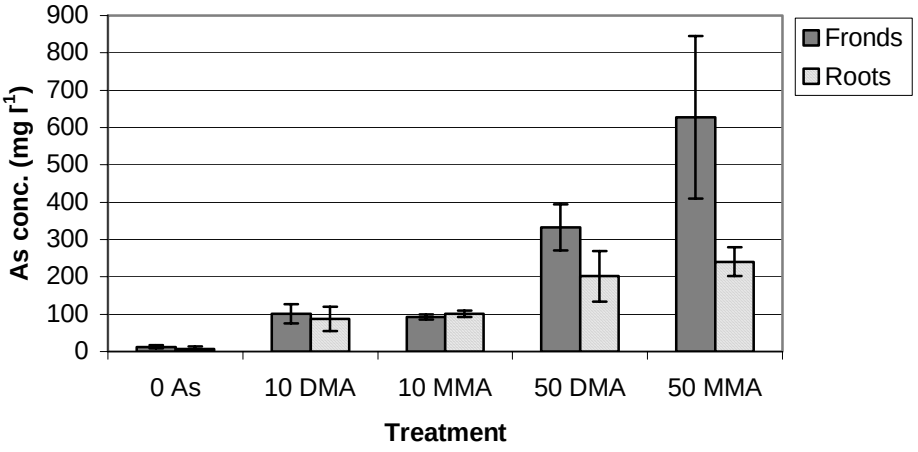


B

Figure 3-1. Total arsenic concentrations in the fronds and roots of *P. vittata* exposed to 0, 10 or 50 mg l^{-1} arsenic as As(III), As(V), MMA or DMA. (A) arsenic supplied as As(III) or As(V). (B) arsenic supplied as DMA or MMA. Values represent means ± std. dev.

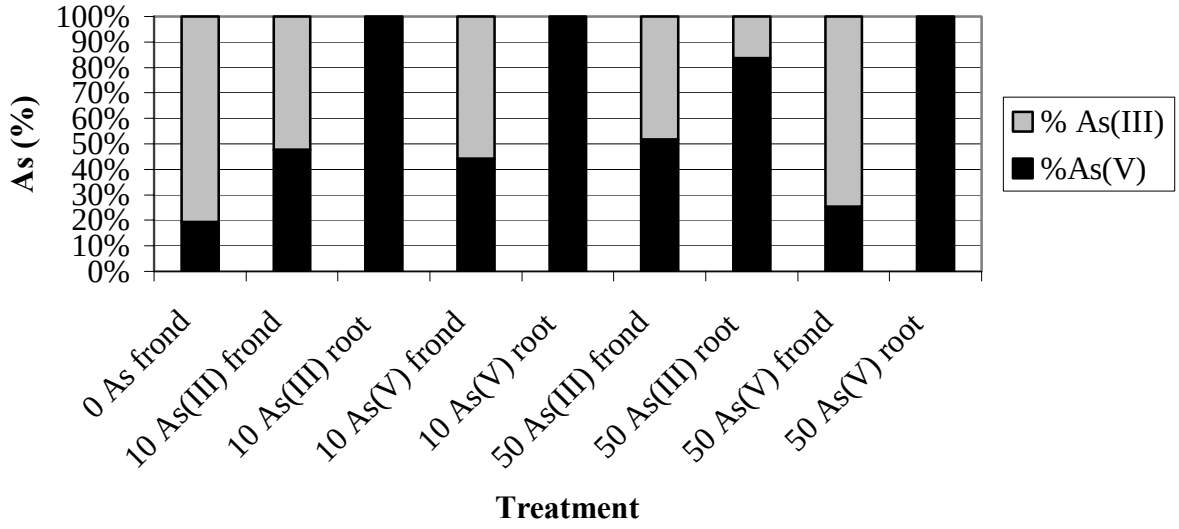


Figure 3-2. Percentages of As(III) and As(V) in the fronds and roots of *P. vittata* exposed to As(III) or As(V). No arsenic was detected in the roots of the control plants. Values represent means.

Almost all of the arsenic in the roots was present as arsenate, except in the 50 As(III) treatment (Fig. 3-2). Even when supplied with 50 mg l^{-1} As(III), 84% of the arsenic in the roots was present as arsenate. Root speciation data for the 0 As treatment are not presented in Figure 3-2 because the arsenic concentration was below the detection limits.

Arsenic Concentration and Speciation in Xylem Sap

As with fronds and roots, arsenic concentrations in hydroponics solution significantly ($P < 0.05$) affected the total arsenic concentrations in the xylem sap, with greater treatment arsenic concentrations resulting in greater arsenic concentrations in the xylem sap. However, there were no significant differences in the total arsenic concentrations in the xylem sap of ferns treated with different arsenic species (Table 3-1). Although the total sap arsenic concentration was not affected by arsenic species, arsenic

concentration in the xylem sap was greatest when the fern was supplied with a concentration of 50 mg As l^{-1} in either experiment.

In experiment A, the total concentration of arsenic in the xylem sap for the arsenite treatments were 2.5 and 29 mg As l^{-1}, for the 10 and 50 mg l^{-1} treatments, respectively. The total arsenic xylem sap concentrations for the 10 and 50 mg l^{-1} As(V) treatments were approximately two times greater compared to the arsenite treatments.

For experiment B, the 10 mg l^{-1} As treatment concentrations yielded the same xylem sap total arsenic concentrations for both MMA and DMA. However, the 50 mg l^{-1} DMA treatment resulted in a 3.5 times greater xylem sap concentration compared to the 50 mg l^{-1} MMA treatment.

In the 50 mg l^{-1} As(V) treatment, the total arsenic concentration of the xylem sap was approximately 1.25-fold greater than that of the arsenic concentration of the treatment solution. However, the 50 mg l^{-1} MMA treatment xylem sap was only 0.26 that of the treatment solution.

Table 3-1. Total arsenic concentrations in xylem sap of P. vittata exposed to 0, 10 or 50 mg l-1 arsenic as As(III), As(V), MMA or DMA. Values represent means ± std. dev.

	Solution arsenic concentrations (mg l^{-1})	
Solution arsenic species	**10**	**50**
Control	0.6 ± 0.4	0.6 ± 0.4
As(III)	2.5 ± 0.8	29.4 ± 10.1
As(V)	4.1 ± 1.9	60.7 ± 25.4
MMA	5.4 ± 2.8	13.0 ± 6.5
DMA	5.6 ± 2.1	44.7 ± 18.3

In experiment A, no methylated forms of arsenic were found in the xylem sap of ferns exposed to arsenate or arsenite. Although more arsenic in these treatments was transported as arsenate, it was only significant in the 50 mg l^{-1} As(V) treatment, where the xylem sap consisted of 57 mg l^{-1} As(V) versus 3 mg l^{-1} As(III) (Fig. 3-3 A).

Pteris vittata exposed to MMA and DMA in experiment B transported arsenic primarily in the form it was supplied (Fig. 3-3 B). However, small concentrations of arsenate and arsenite were detected in the xylem sap of these ferns.

Phosphorus Concentration in Xylem Sap

Inorganic phosphorus concentrations in the xylem sap ranged from 5.2 to 13.4 mg l^{-1}. However, the phosphorus concentrations in the xylem sap were not significantly affected by arsenic concentration or arsenic species supplied in the nutrient solutions (Fig. 3-4), nor were the phosphorus concentrations significantly different between experiments A and B.

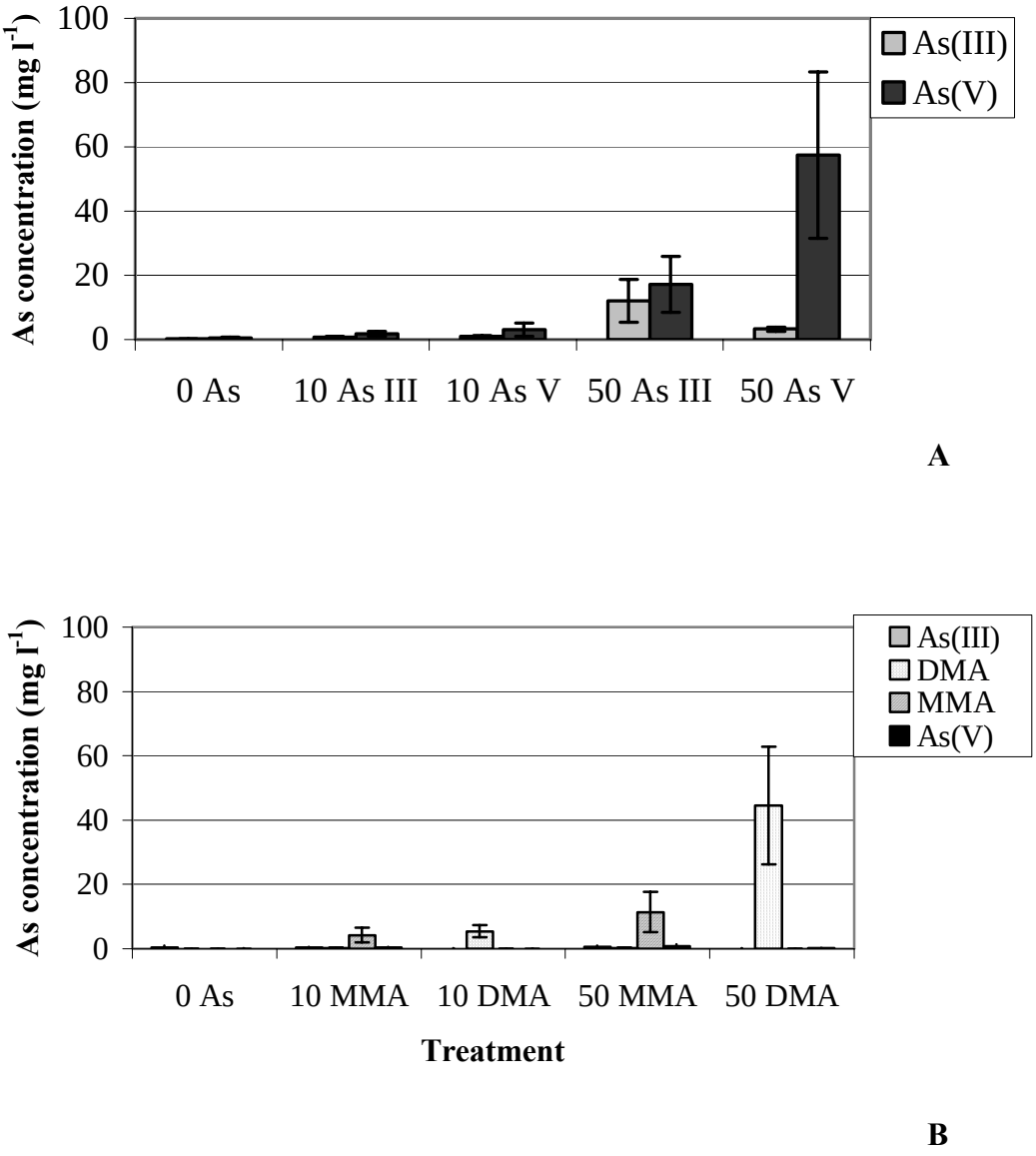
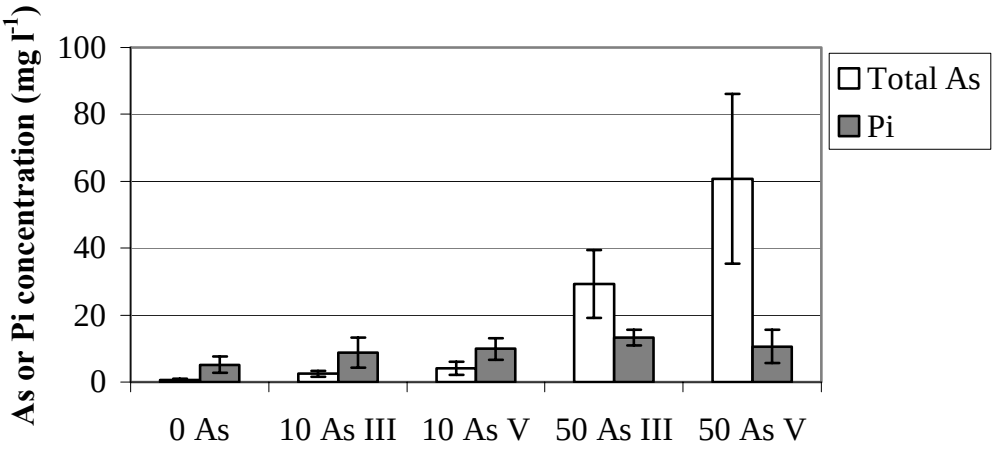
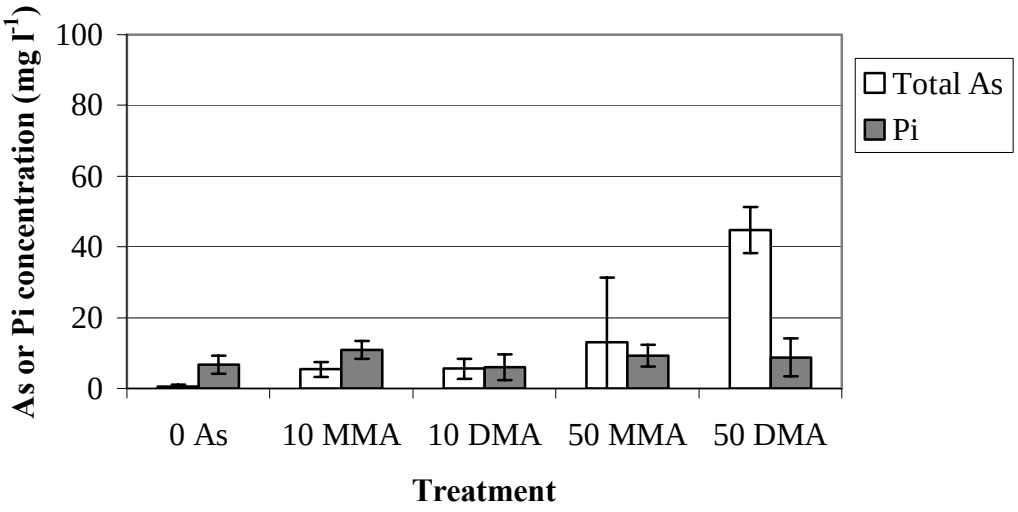


Figure 3-3. Concentrations of As(III), As(V), DMA and MMA in the xylem sap of *P. vittata* exposed to 0, 10 or 50 mg l^{-1} of (A) As(III) and As(V), and (B) DMA and MMA. No DMA or MMA were detected in the xylem sap of ferns exposed to As(III) or As(V). Values represent means ± std. dev.



A



B

Figure 3-4. Comparison of total arsenic and P_i (inorganic phosphorus) concentrations in the xylem sap of *P. vittata*. (A) arsenic supplied as As(III) or As(V) and (B) arsenic supplied as DMA or MMA. The P_i concentration in the xylem sap was not significantly affected by arsenic regardless of the form or concentration of arsenic supplied to the ferns. Values represent means ± std. dev.

Discussion

Previous studies (Ma et al., 2001; Tu et al., 2002; Wang et al., 2002; Zhang et al., 2002) have shown that arsenic concentrations in *P. vittata* increase with external arsenic concentrations, and the majority of the arsenic is concentrated in the fronds. This also was confirmed by these experiments.

Research by Tu et al. (2003) showed arsenite was the predominant species present in the fronds, and arsenate was the predominant species in the roots, with little organic arsenic being detected in the fern. Similar results were obtained in this experiment. Inorganic arsenic species found in the fronds and roots of *P. vittata* from experiment A were not significantly affected by the arsenic species supplied to the fern. With the exception of the 50 As(III) treatment, root arsenic was present entirely as arsenate despite that different forms of arsenic were supplied to ferns (Fig. 3-2). However, fronds contained 50-80% arsenite. Again, these results confirm those of previous studies (Zhang et al., 2002; Tu et al., 2003; Webb et al., 2003), where the predominant forms of arsenic in *P. vittata* fronds and roots are arsenite and arsenate, respectively. Similarly, in a study involving *Pityrogramma calomelanos*, another arsenic-hyperaccumulating fern, most of the arsenic found in its fronds was arsenite. Only trace amounts of MMA and DMA were found in a few samples (Francesconi et al., 2002). *Pteris vittata* is apparently reducing arsenic at some point between its presence as arsenate in the roots until it is stored as arsenite in the fronds.

Arsenic speciation analysis showed that 56-60% of the arsenic was present as arsenate in the hydroponics solution treated with 10 or 50 mg l^{-1} As(III) after the 3-d experiment (data not shown). Although this indicates that significant arsenic oxidation occurred in the hydroponics solution, a substantial level (40-44%) of arsenic was present

as arsenite at the end of the experiment. Assuming arsenite and arsenate were taken up by the plant at the same rate, as suggested in Figure 3-1, then 56-60% of the arsenic should be present as arsenate in the roots. The fact that 84-100% of the arsenic was present as arsenate in the roots treated with 10 or 50 mg l^{-1} As(III) suggests that either arsenic oxidation occurred inside the roots and/or arsenite was preferentially transported from the roots to the fronds.

A study by Wang et al. (2002) determined that arsenite was translocated more efficiently than was arsenate from *P. vittata* roots to its fronds. Similar results were found in *Arabidopsis thaliana* using phosphate mutants, *pho1* and *pho2*. A study of arsenic uptake and translocation in these mutants suggests that arsenite is the form preferentially loaded into the xylem (Quaghebeur and Rengel, 2004). This may not be the case in *P. vittata,* considering these findings of a slightly greater concentration of arsenate in the xylem sap.

However, it may not be feasible to assume that uptake of arsenite and arsenate by *P. vittata* roots is equal because the species may be taken up through different systems in the roots. Wang et al. (2002) found that arsenate was taken up more quickly by *P. vittata* than was arsenite, especially in the absence of phosphate. The authors suggest that this is due to arsenate being taken up via phosphorus-suppressible uptake in the roots. Meharg and Jardine (2003) suggest that aquaglyceroporins are the main inlet for arsenite into rice. Therefore, the root uptake rates of arsenite and arsenate into the plant roots are likely different.

In experiment A the arsenic concentration in the xylem sap was greatest when the fern was supplied with 50 mg l^{-1} As(V); therefore, it is possible that arsenic is more

readily concentrated in the xylem sap when the fern is supplied with arsenate. Such a finding would disagree with previous conclusions for *P. vittata* (Wang et al., 2002) and *Arabidopsis thaliana* (Quaghebeur and Rengel, 2004). However, if this were the case, i.e., arsenite, was not preferentially transported in the xylem sap, then the fact that greater arsenate was observed in the roots than that in the hydroponics solution may suggest oxidation of arsenite to arsenate inside the roots. Also because arsenate and phosphate are similar, it is conceivable that this difference may be due to arsenate being taken up via the phosphate uptake system.

A weak correlation was found between arsenic concentrations in the xylem sap and arsenic concentrations in the fronds ($r = 0.50$). This implies that the amount of arsenic accumulated in the fronds was affected by arsenic species, in addition to arsenic concentration. A slightly stronger correlation was found between arsenic concentrations in the xylem sap and arsenic concentrations in the roots ($r = 0.66$), i.e., greater root arsenic concentrations, resulting in greater xylem sap arsenic concentrations.

Interestingly, the arsenic concentration in the fronds treated with 50 mg l^{-1} MMA was the greatest (627 mg kg^{-1}, Fig. 3-1). However, the arsenic concentration in the xylem sap of the ferns treated with 50 mg l^{-1} As(V) was the greatest (60.7 mg l^{-1}, Table 3-1). Therefore the highest arsenic concentration in the xylem sap of ferns treated with arsenate did not translate into the highest arsenic concentration in the fronds.

In experiment A, the predominant form of arsenic transported in *P. vittata* xylem sap appeared to be arsenate, regardless of the species supplied in the external nutrient solution (Fig. 3-3 A). This was consistent with the root data, where most of the arsenic was present as arsenate, i.e., 84-100% , even in plants treated with 50 mg l^{-1} As (III) (Fig.

3-2). The arsenate concentrations in the roots were correlated with the arsenate concentrations in the xylem sap with r = 0.74. This suggests that greater arsenate concentrations in the roots resulted in greater arsenate in the xylem sap.

Although 84% of the arsenic was present as arsenate in the fern roots treated with 50 mg l^{-1} As(III) (Fig. 3-2), only 59% of the arsenic in the xylem sap was present as arsenate (Fig. 3-3 A), indicating that proportionally more arsenite than arsenate was present in the xylem sap. This, however, contradicted the fact that the highest arsenic concentration was observed in the xylem sap of plants treated with 50 mg l^{-1} arsenate instead of arsenite (Table 3-1). This may be explained by the fact that some of the arsenate was reduced to arsenite during the transport. This is supported by the fact that, though all of the arsenic in the roots treated with 50 mg l^{-1} As(V) was present as arsenate (Fig. 3-2), approximately 5.3% of the arsenic in the xylem sap was present as arsenite (Fig. 3-3 A); this suggests that a small amount of arsenic reduction occurred during the transport in *P. vittata*. However, most of the arsenic reduction occurred mainly in the pinnae of the fern. Because arsenate can compete for phosphate sites, such as ATP, within the plant, it is important for the arsenic reduction to occur. It is thought that thiol-containing compounds may sequester some arsenite and shuttle it to the frond vacuoles to limit the arsenic toxicity (Lombi et al., 2002; Webb et al., 2003). No methylated forms of arsenic were detected in the xylem sap when arsenite or arsenate was supplied. Therefore, arsenic methylation does not occur prior to or during arsenic transport in *P. vittata*.

In experiment B, the species of arsenic transported was strongly correlated with the arsenic species that was supplied to the fern (Fig. 3-3 B). For example in those ferns supplied with DMA, arsenic was transported mainly as DMA. There were also low

concentrations of arsenite and arsenate detected in the sap when DMA and MMA were fed to the fern. In general, inorganic forms of arsenic, such as arsenite and arsenate, are considered more toxic than organic forms (Tamaki and Frakenberger, 1992). Also, monosodium methanearsonate (MSMA) has been shown to be quickly absorbed by plant leaves and move into the symplast (Wauchope, 1983). Therefore, it may be easier for the fern to transport arsenic in methylated form, rather than to demethylate it for transport. Overall, the fern appears to transport arsenic in the form least harmful to itself, regardless of the species in which it is supplied. In previous studies, little or no methylated species have been detected in *P. vittata* fronds when supplied with DMA or MMA (Tu et al., 2003), suggesting that demethylation of the arsenic may be occurring in the pinnae.

Since phosphate and arsenate are chemical analogues, it is reasonable to expect competition between the two during their transport in the fern (Tu and Ma, 2003). The study by Wang, et al. (2002) showed that arsenic concentrations in the fronds and roots of *P. vittata* decreased with increasing phosphate concentrations in the nutrient solution. Therefore, it was thought that a similar phenomenon would take place with phosphorus the in the xylem sap, when various concentrations of arsenic were supplied to the fern. Tu and Ma (2003) found that phosphate might mitigate the phytotoxicity of arsenic in the fern. At a concentration of 2.67 mM As kg^{-1} of soil, arsenate even increased phosphate uptake. At a higher arsenic concentration, 5.34 mM As kg^{-1} of soil, phosphate concentrations decreased. However, in the xylem sap, the presence of arsenic did not seem to affect phosphorus concentration, and vice versa (Fig. 3-4). Therefore, phosphorus and arsenic are probably not competing for transport within the xylem sap at the concentrations used in this study. The concentration of phosphorus in 0.20-strength

Hoagland-Arnon solution is 120 mg l^{-1}. Therefore, the ratio of phosphorus to arsenic was 12:1 and 2.4:1 for the 10 and 50 mg l^{-1} arsenic treatments, respectively. Lower ratios of phosphorus to arsenic may have resulted in competition between the two elements and lower concentrations of phosphorus in the xylem sap.

CHAPTER 4
EFFECTS OF ARSENIC ON GLUTATHIONE REDUCTASE AND CATALASE IN THE FRONDS OF *Pteris vittata* L.

Introduction

It has been well established that *P. vittata* is able to accumulate very high concentrations of arsenic in its fronds. However, it is presently unclear as to how this fern tolerates such high concentrations of arsenic. Arsenic-exposure in plants may result in the production of reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicals, which can generate significant cell damage (Weckx and Clijsters 1996; Conklin, 2001; Hartley-Whitaker et al., 2001b).

A study on enzymatic antioxidants found that catalase (CAT), the antioxidant enzyme that converts H_2O_2 to water and oxygen, was induced in the fronds of *P. vittata* exposed to arsenic. However, under the same conditions they are not induced in the fronds of *Pteris ensiformis*, a non-arsenic hyperaccumulator (Srivastava et al., 2005). This further suggests a role for antioxidant enzymes in arsenic tolerance and/or hyperaccumulation by *P. vittata*. Also, the reduction of arsenate to arsenite can result in the production of ROS, such as H_2O_2, possibly resulting in the need for increased CAT activity.

Glutathione reductase (GR) is the enzyme that, in conjunction with NADPH, catalyzes the reduction of glutathione disulfide (GSSG) to glutathione (GSH) (Carlberg and Mannervik, 1985). This antioxidant enzyme has been detected in bacteria, yeast, plants and animals. It is essentially responsible for maintaining cellular GSH levels.

Glutathione is composed of the amino acids, glutamate, cysteine and glycine. Its significance lies mostly in its role as a reductant, as well as in its ability to detoxify harmful components within a cell. Glutathione is a source of non-protein thiols, and it is also a precursor for phytochelatins (PC) (Kneer and Zenk, 1992; Zenk, 1996; Pawlik-Showronska, 2001). Therefore, GSH has been implicated in aiding plants to cope with various environmental stresses, either directly by binding and detoxifying, or indirectly through conversion into PCs.

Glutathione reductase and GSH have both been the subject of numerous experiments investigating their roles in plant tolerance of various environmental conditions, such as photooxidation, water, temperature and heavy metal stresses (Aono et al., 1997; Gupta et al., 1999; Vitoria et al., 2001; Jiang and Zhang, 2002; Keles and Oncle, 2002; Piquey et al., 2002). A study conducted by Aono et al. (1997) indicated that transgenic tobacco (*Nicotiana tabacum* L.) plants with high GR activity exhibited a decreased sensitivity to photooxidative stress as a result of exposure to the herbicide paraquat. Similarly, *Zea mays* L. subjected to water stress showed higher GR activity, as well as other antioxidant enzymes, when compared to non-stressed plants (Jiang and Zhang, 2002). Vitoria et al. (2001) studied the effects of cadmium on radish (*Raphanus sativus* L.) GR activity. It was determined that GR activity increased in the radishes after 24 h exposure to cadmium. The authors concluded that the main response of the radish to cadmium was in its activation of the ascorbate-GSH cycle to remove H_2O_2, and that an alternative response may be to make GSH available for cadmium-binding protein synthesis. Glutathione reductase activity was found to also increase with exposure to copper in *Phaseolus vulgaris* L. (Gupta et al., 1999). Temperature stress (Keles and

Oncle, 2002) and salt stress (Bor, et al., 2003) were also shown to result in an increased response of GR.

Because of the production of ROS in the presence of arsenic, it is essential to understand the role that these important antioxidants play in *P. vittata* plants subjected to arsenic stress. An increase or stimulation of GR activity may lead to an increase in the GSH levels in cells, thereby contributing to the ability of a plant to interact with free radicals, detoxify heavy metals or contaminants and/or overcome other generally unfavorable environmental conditions. However, it is critical to know that there are many antioxidants and antioxidant enzymes, such as ascorbate, α-tocopherol, catalase, xanthophylls and carotenoids, which also aid plants in dealing with environmental stresses.

The objectives of this study were 1). to determine and compare the apparent enzyme kinetics (K_m and V_{max}) of antioxidant enzymes GR and CAT in the fronds of *P. vittata* and *P. ensiformis*; 2). to determine if the presence of arsenic inhibits the activities of these enzymes in both *Pteris* species; and 3). to determine if these enzymes are induced in the fronds of *P. vittata* upon arsenic exposure.

Materials and Methods

Plant and Chemical Materials

Pteris vittata and *P. ensiformis* ferns were used in the following experiments. All ferns were produced in the laboratory to ensure uniformity. All chemicals were supplied by Fisher Scientific (Pittsburgh, PA USA) or Sigma (St. Louis, MO USA), unless otherwise stated.

Enzyme Extraction

Fresh frond material was homogenized in a chilled mortar containing sea sand and extraction buffer (100 mM *Tris*-HCl pH 8.0, 2 mM EDTA, 5 mM DTT, 10% glycerol, 100 mM sodium borate, 4% w/v insoluble PVPP and protease inhibitors: 0.5 mM leupeptin, 20 mM AEBSF, 100 μM pepstatin A, 100 μM bestatin, 100 μM E-64 and 100 mM 1, 10 phenathrolin). The homogenate was filtered through cheesecloth and centrifuged for 20 min at 20,000g and 4^{o}C. The crude supernatant was collected, its volume estimated and 2 ml were reserved. To the crude supernatant, 5% PEG (w/v) was added. The solution was incubated for 20 min and centrifuged for 20 min at 20,000g and 4^{o}C. The 5% PEG supernatant was collected and its volume recorded. Additional PEG was added to obtain a final concentration of 20% (w/v). After incubation for 20 min, the 20% PEG fraction was centrifuged for 40 min at 20,000g and 4^{o}C. The 20% PEG fraction pellet was redissolved in redissolve buffer (50 mM *Tris*-HCl pH 8.0, 5 mM DTT and 10% glycerol). All protein fractions were stored at -80^{o}C until analysis.

Protein and Enzymatic Activity Determinations

Protein concentrations were estimated in the various fractions using the method of Lowry et al. (1951) as modified by Peterson (1977). Bovine serum albumin (BSA) was used as a standard.

Glutathione reductase (EC 1.6.4.2) activity was assayed by following NADPH activity at 340 nm on a UV-spectrophotometer (Beckman DU®-520 UV/VIS Spectrophotometer, Beckman Coulter, Inc. Fullerton, CA, USA) for 5 min in 1 ml of an assay mixture. The assay contained 50 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 0.15 mM NADPH, 0.5 mM GSSG and 50 μM of enzyme extract. The reaction

was initiated by the addition of NADPH. Glutathione reductase activity was calculated using the extinction coefficient of NADPH at 340 nm (6.2 mM^{-1} cm^{-1}) (Jiang and Zhang, 2002).

Catalase (EC 1.11.1.6) activity was assayed by following the decrease in absorbance, or degradation of H_2O_2, at 240 nm for 3 min with a UV-spectrophotometer. The assay contained 50 mM potassium phosphate buffer (pH 7.0), 88 μM H_2O_2 and approximately 50 μg protein. The reaction was initiated by the addition of H_2O_2. Catalase activity was calculated using the extinction coefficient of H_2O_2 at 240 nm (40 mM^{-1} cm^{-1}) (Chance and Maehly, 1955).

Enzyme Induction Study

Pteris vittata ferns of similar age/size (approximately 90 d old plants) were placed in a hydroponics system and acclimated for 7 d using 0.2 strength Hoagland-Arnon solution (Hoagland and Arnon, 1938). The ferns were kept in a controlled environment with 65% humidity and day and night temperatures of 25°C and 20°C, respectively. The ferns were subjected to an 8 h light period with a light intensity of 350 μmoles m^{-2} s^{-1}. Arsenic in the form of sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$), was added to a 0.2 strength Hoagland-Arnon solution with final concentrations of 0 and 10 mg l^{-1}. The experimental design was a randomized complete block which consisted of three replications.

After 3 d, fern fronds were harvested, flash frozen in liquid nitrogen and stored at –80°C until analysis. Protein extraction, determination and GR and CAT enzymatic assays (same as above) were performed on the 20% PEG fractions of the 0 and 10 mg L^{-1} treatments. Activities of both GR and CAT were also determined in mixed samples using approximately equal amounts of frond tissue from both 0 and 10 mg l^{-1} plants.

The induction or lack of induction of GR was further confirmed through immunoblotting. A SDS-PAGE was performed in a 12% (w/v) separation gel using the methods of Laemmli (1970). Coomassie Brilliant Blue stain was used to visualize proteins. Following activity staining, proteins were transferred from the 12% SDS-polyacrylamide gel to nitrocellulose electrophoretically (Mini Trans-Blot® Electrophoretic Transfer Cell, Bio-Rad Laboratories). After blocking, the blot was incubated with a 1/1500 dilution [IgG fraction diluted in Tris-Buffered Saline + Tween 20 (TBST)] of a *Zea mays* L. cystolic GR antibody (Pastori et al., 2000) for 15 h at 4^{o}C. The blot was then washed four times with TBST and incubated for 1 h at 4^{o}C with a 1/3000 dilution of alkaline phosphatase conjugated with of anti-rabbit IgG. The phosphatase conjugate was detected by nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP).

Determination of Apparent Kinetics

The apparent Michaelis-Menten enzyme kinetic parameters, V_{max} and K_m were determined for both GR and CAT in *P. vittata* and *P. ensiformis* using the 20% PEG fractions. The assay procedures for GR and CAT were similar to those described above except that substrate concentrations were varied as indicated. All assays were performed in triplicate.

The apparent kinetics for GR were determined for both GSSG and NADPH. The NADPH concentration was fixed at a saturating concentration during GSSG kinetics determination, and the GSSG concentration was fixed at a saturating concentration during NADPH kinetics determination. For CAT, the apparent kinetics of H_2O_2 were determined.

Data were plotted using Lineweaver-Burk plots (double reciprocal plots). The apparent kinetic parameters were derived from *x* ($-1/K_m$) and *y* ($1/V_{max}$) intercepts of the plots.

Determination of Arsenic Effects on Enzyme Activities

Inhibition and/or activation of GR and CAT activities by arsenic in *P. vittata* and *P. ensiformis* were examined. Various concentrations of arsenic were added directly to assays immediately prior to initiation of the enzymatic reaction. For GR, both arsenate, as sodium arsenate, and arsenite, as sodium arsenite, were examined. However, for CAT, only arsenate, in the form of sodium arsenate, was used to examine inhibition or activation. This is because arsenite will be oxidized to arsenate upon exposure to H_2O_2 (Aposhian et al., 2003). Therefore, the addition of arsenite would give false activities and/or yield similar results to arsenate. Effects of arsenate on the CAT activity of purified protein CAT positive control (bovine liver) was also examined for comparison.

Results

Glutathione Reductase and Catalase Induction Study

Spectrophotometric assays of GR activity indicated that GR in *P. vittata* was not induced upon exposure to arsenic (Fig. 4-1). These results were confirmed with the GR immunoblot (Fig. 4-2). However, CAT activity increased approximately 1.5 times when ferns were exposed to arsenate (Fig. 4-3).

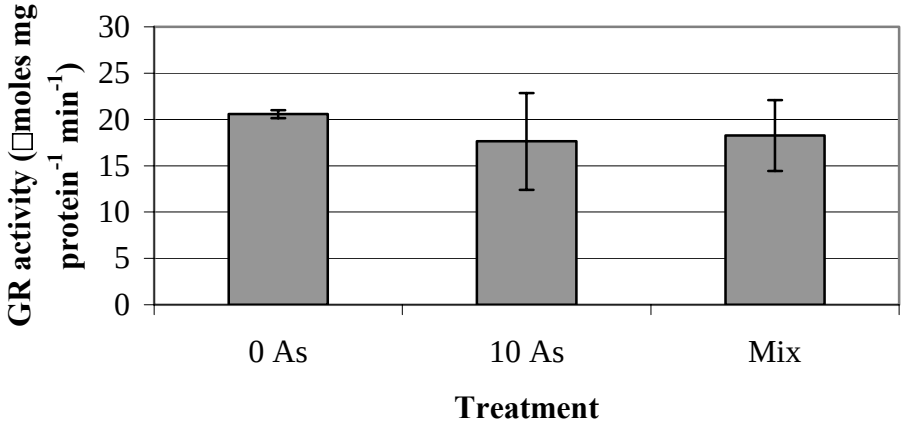


Figure 4-1. Glutathione reductase activity in *P. vittata* plants exposed to 0 and 10 mg l^{-1} arsenic, and the GR activity of an extraction mixture of consisting of equal amounts of frond tissue of both arsenic treatments. Values represent means ± std. dev. (n = 3).

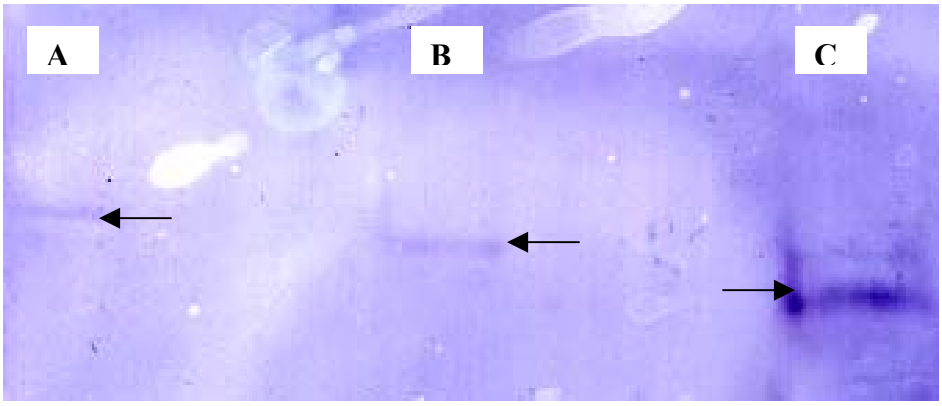


Figure 4-2. Immunoblot of GR activity in (A) crude extract of arsenic treated *P. vittata*, (B) crude extract of control *P. vittata* and (C) crude extract of *Zea mays*. Arrows indicate GR bands.

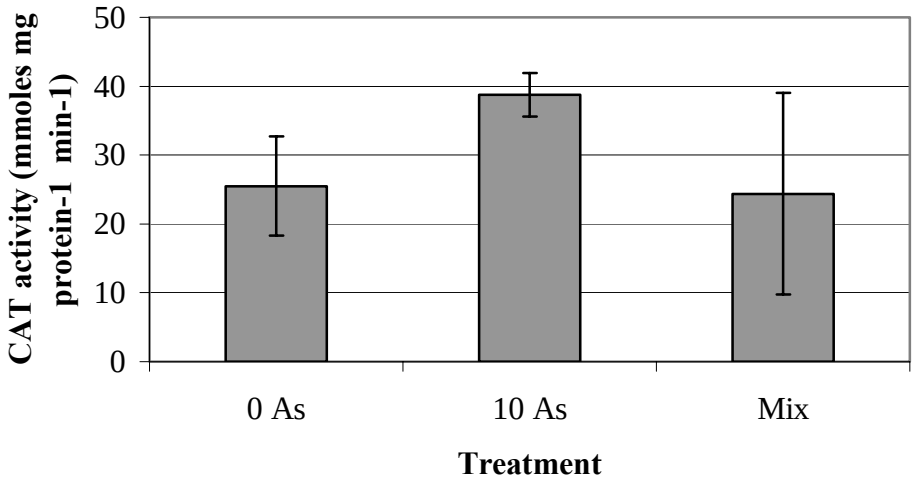


Figure 4-3. Catalase activity in *P. vittata* plants exposed to 0 and 10 mg l^{-1} arsenic, and the CAT activity of an extraction mixture of consisting of equal amounts of frond tissue of both arsenic treatments. Values represent means ± std. dev. (n = 3).

Glutathione Reductase and Catalase Apparent Kinetics

The GR activities exhibited Michaelis-Menten kinetics with respect to the substrate saturation response. The responses for varying concentrations of GSSG and NADPH are shown in Figures 4-4 A and 4-6 A for *P. vittata* and Figures 4-5 A and 4-7 A for *P. ensiformis*, respectively. Although the reactions catalyzed by H_2O_2 for CAT did appear to exhibit Michaelis-Menten kinetics for the substrate concentrations used, substrate saturation was not reached for either species (Fig. 4-8 A and 4-9 A). Higher H_2O_2 concentrations could not be used accurately in the spectroscopic assays.

There were no significant differences found between the apparent kinetic constant, K_m, of *P. vittata* and *P. ensiformis* for the substrates, as determined from the Lineweaver-Burk plots (Fig. 4-4 B, 4-5 B, 4-6 B, 4-7 B, 4-8 B and 4-9 B). The values for V_{max} of GR were also comparable between the two species. However, the V_{max} of CAT activity in *P.*

ensiformis was approximately an order of magnitude greater than that in *P. vittata*. The K_m and V_{max} values are summarized in Table 4-1.

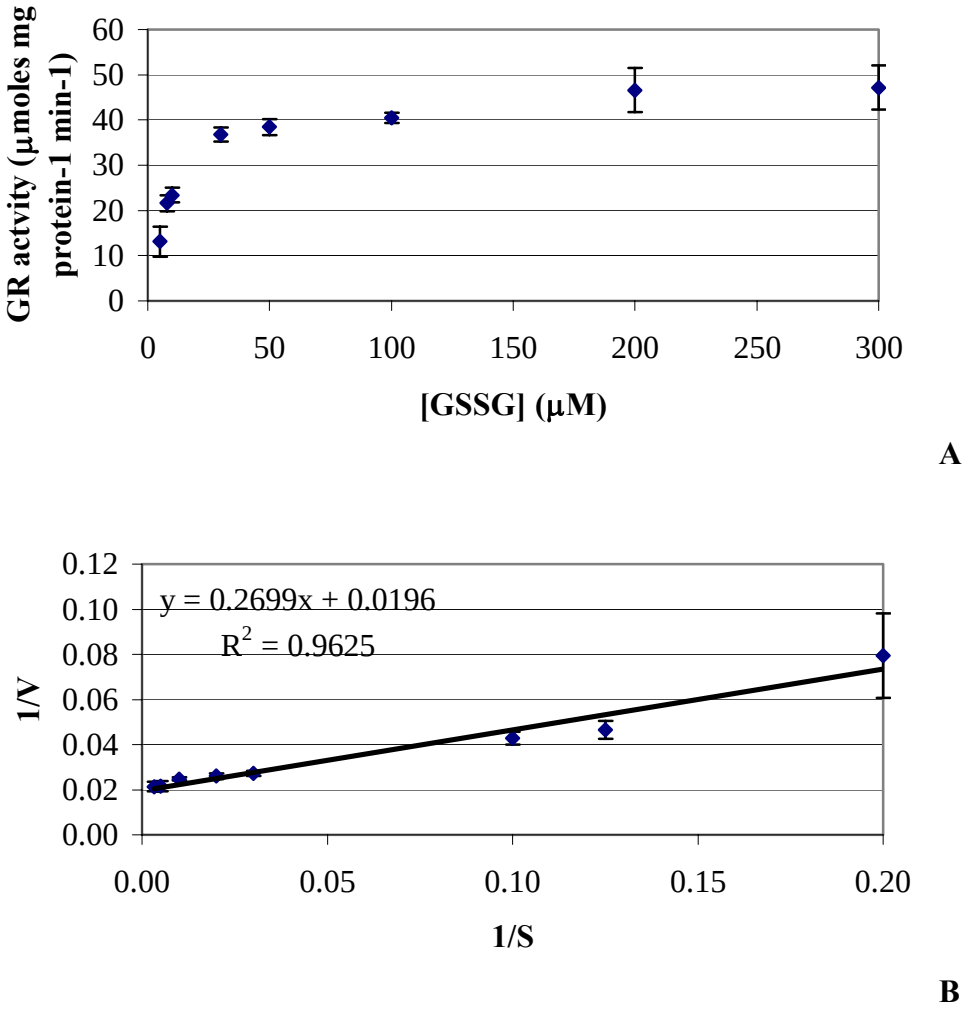
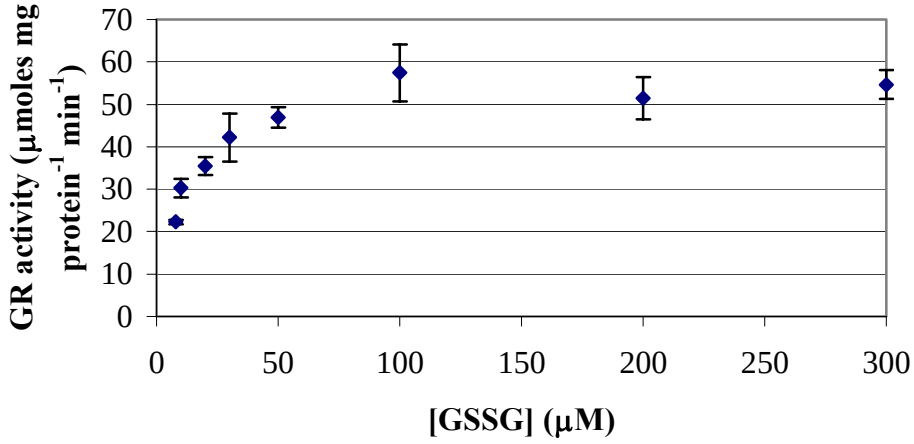
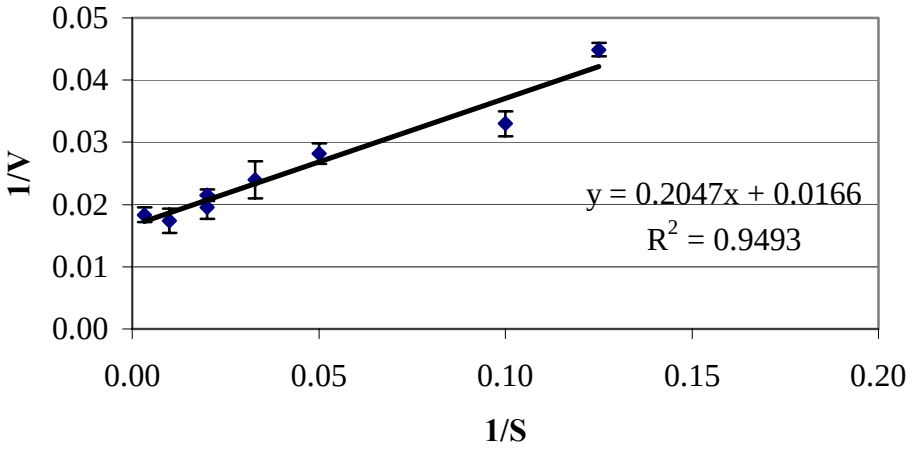


Figure 4-4. Apparent kinetic analysis of substrate, GSSG, for GR activity in *P. vittata.* (A) Direct plot showing the dependence of GR velocity on GSSG concentration. (B) Lineweaver-Burk (double reciprocal) plot. Substrate GSSG concentrations varied between 5 and 300 µM. The NADPH concentration was maintained at 200 µM. Values represent means ± std. dev. (n = 3).



A



B

Figure 4-5. Apparent kinetic analysis of substrate, GSSG, for GR activity in *P. ensiformis.* (A) Direct plot showing the dependence of GR velocity on GSSG concentration. (B) Lineweaver-Burk (double reciprocal) plot. Substrate GSSG concentrations varied between 8 and 300 µM. The NADPH concentration was maintained at 50 µM. Values represent means ± std. dev. (n = 3).

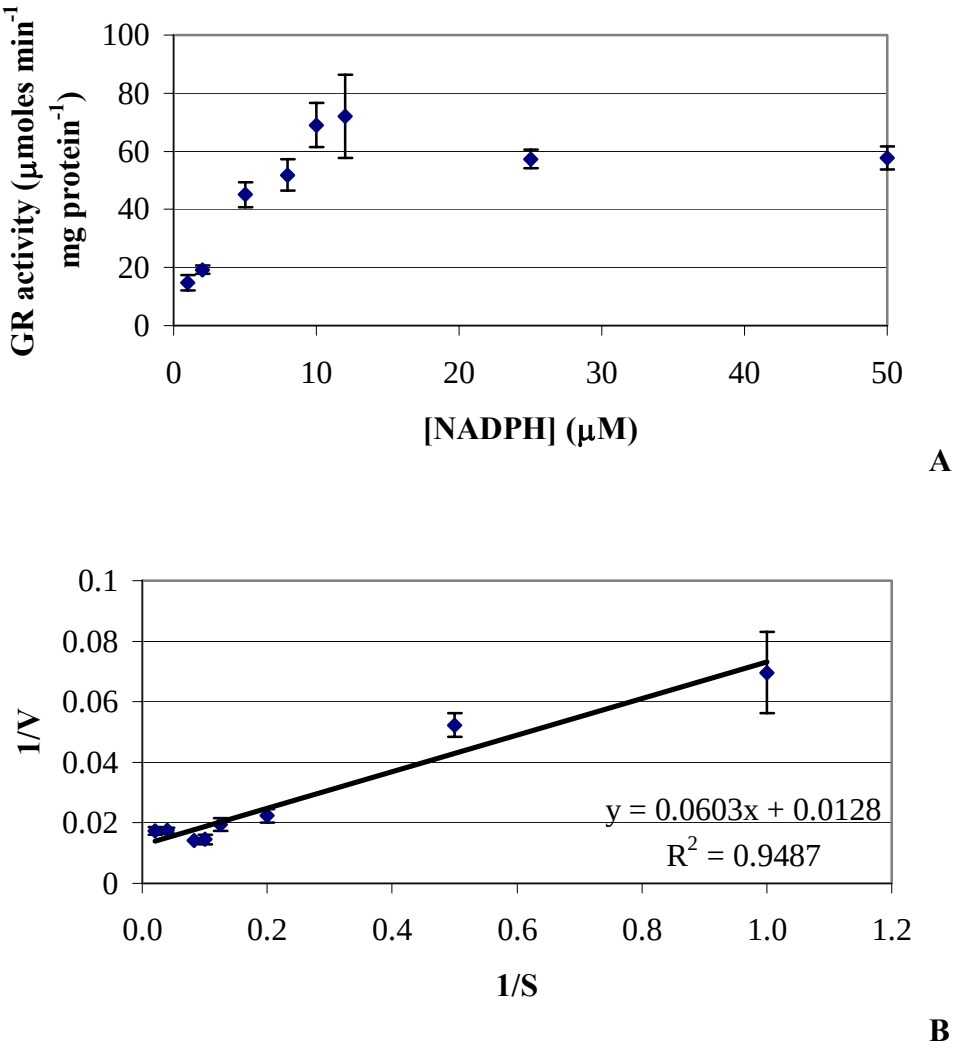


Figure 4-6. Apparent kinetic analysis of substrate, NADPH, for GR activity in *P. vittata.* (A) Direct plot showing the dependence of GR velocity on NADPH concentration. (B) Lineweaver-Burk (double reciprocal) plot. Substrate NADPH concentrations varied between 1 and 50 μM. The GSSG concentration was maintained at 100 μM. Values represent means ± std. dev. (n = 3).

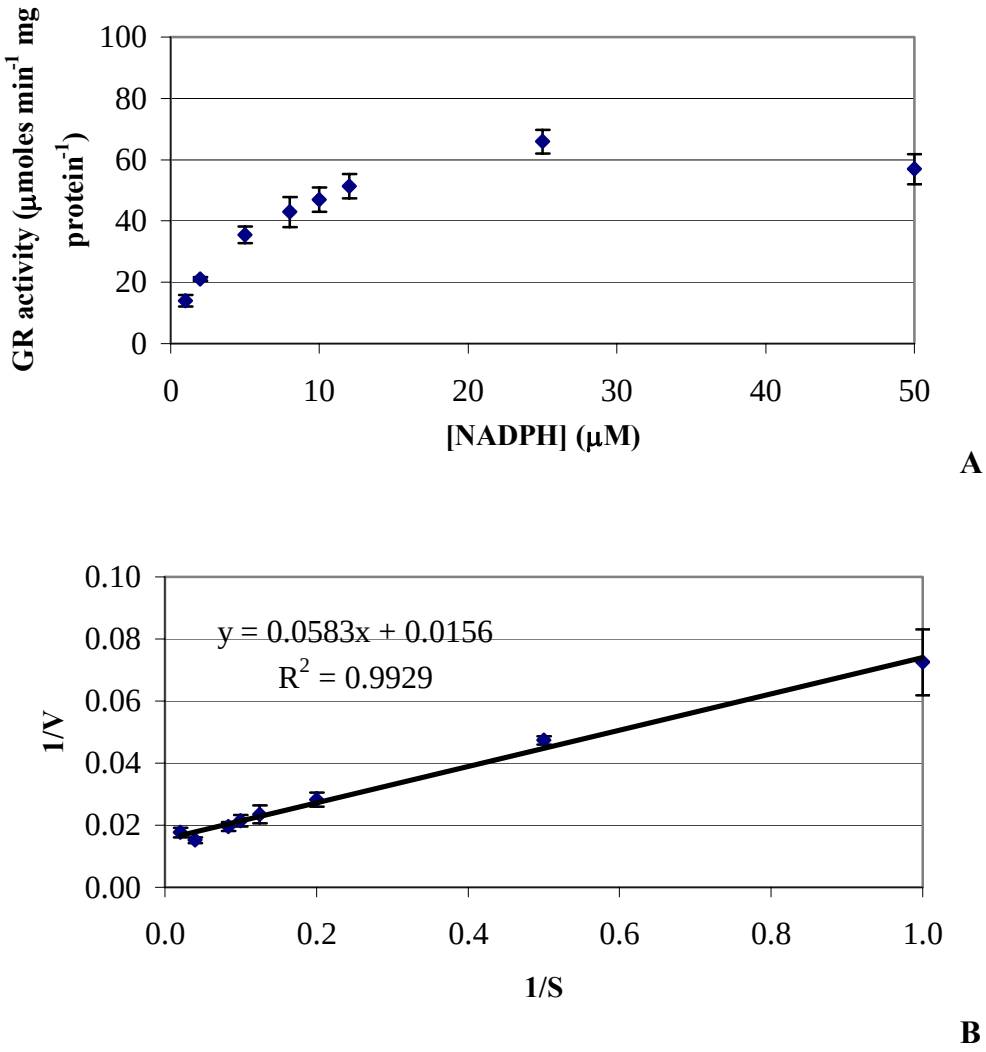
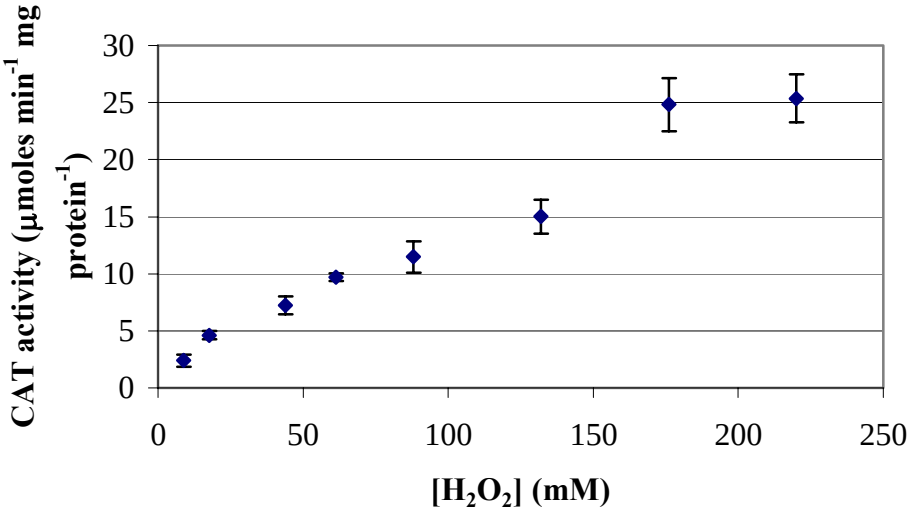
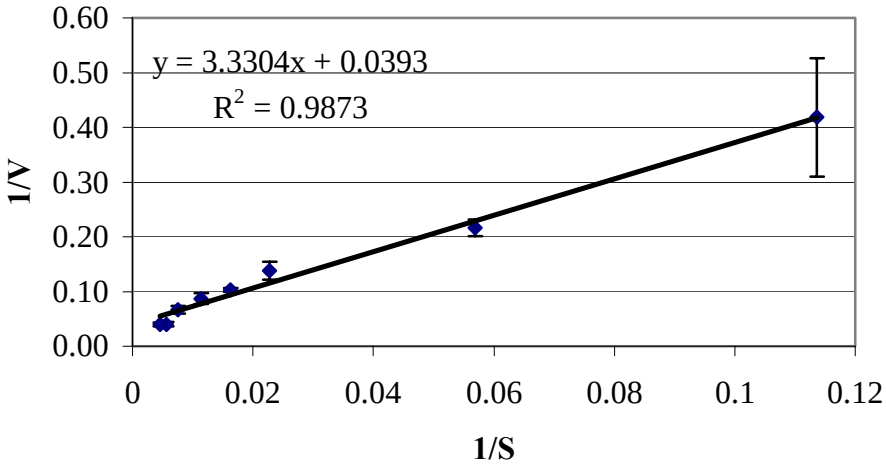


Figure 4-7. Apparent kinetic analysis of substrate, NADPH, for GR activity in *P. ensiformis*. (A) Direct plot showing the dependence of GR velocity on NADPH concentration. (B) Lineweaver-Burk (double reciprocal) plot. Substrate NADPH concentrations varied between 1 and 50 μM. The GSSG concentration was maintained at 100 μM. Values represent means ± std. dev. (n = 3).

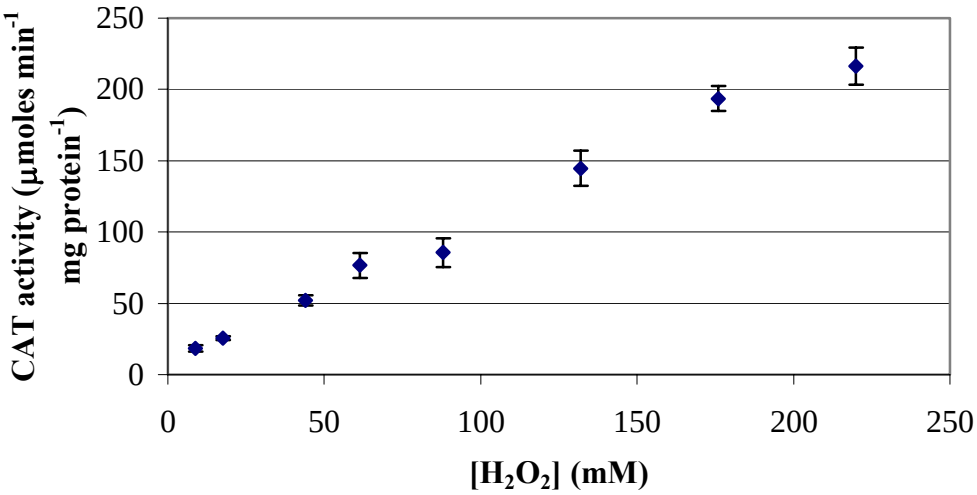


A

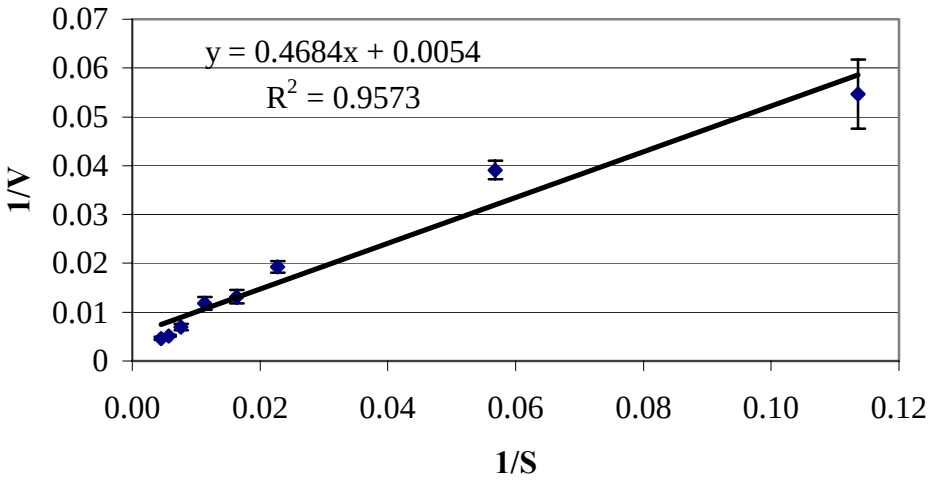


B

Figure 4-8. Apparent kinetic analysis of H_2O_2 for CAT activity in *P. vittata*. (A) Direct plot showing the dependence of GR velocity on H_2O_2 concentration. (B) Lineweaver-Burk (double reciprocal) plot. Substrate H_2O_2 concentrations varied between 8.8 and 220 mM. Values represent means ± std. dev. (n = 3).



A



B

Figure 4-9. Apparent kinetic activity of H_2O_2 for CAT activity in *P. ensiformis*. (A) Direct plot showing the dependence of GR velocity on H_2O_2 concentration. (B) Lineweaver-Burk (double reciprocal) plot. Substrate H_2O_2 concentrations varied between 8.8 and 220 mM. Values represent means ± std. dev. (n = 3).

Table 4-1. Summary of apparent kinetic parameters for GR and CAT. Values were derived from Lineweaver-Burk (double reciprocal) plots for GR substrates (GSSG and NADPH) and CAT substrate (H_2O_2) measured in *P. vittata* and *P. ensiformis*.

	Pteris vittata		***Pteris ensiformis***	
Glutathione reductase	**K_m** (μM)	**V_{max}** (μmoles mg protein^{-1} min^{-1})	**K_m** (μM)	**V_{max}** (μmoles mg protein^{-1} min^{-1})
GSSG	13.8	51.0	12.3	60.2
NADPH	4.7	78.1	3.7	64.1
Catalase				
H_2O_2	84.7	25.4	86.7	185.2

Effect of Arsenic on Enzyme Activities

Single replicate assays over a range of arsenate and arsenite concentrations, 0 to 500 mM, did not reveal inhibition or activation of GR activity in either *P. vittata* or *P. ensiformis* fronds (data not shown). Significant inhibition was not observed in either plant species until 1 mM arsenite was added to the assay. At 1 mM arsenite, GR activity was inhibited approximately 64% in both *P. vittata* and *P. ensiformis*. Arsenate concentrations up to 3 mM did not inhibit GR activity. To briefly confirm the lack of inhibition by arsenite, triplicate values of three concentrations, 0, 25 and 250 μM, were assayed for both species (Fig. 4-10).

Arsenate did not inhibit CAT activity in *P. vittata*, *P. ensiformis* or bovine liver positive control (Fig. 4-11, 4-12 and 4-13). However, the addition of arsenate did appear to activate CAT activity in *P. vittata* (Fig. 4-11). Catalase activity increased 175%, relative to the control in *P. vittata*, at 10 μM sodium arsenate (Fig. 4-14). Activity returned to a similar velocity as the control assay when a concentration of 20 μM sodium arsenate was added. Activity increased again and reached a maximum, approximately 300% that of the control, at a concentration of 200 μM sodium arsenate. The CAT

activity returned to a similar level to the control upon the addition of 500 μM sodium arsenate.

Pteris ensiformis (Fig. 4-12) and the bovine liver (CAT positive control) (Fig. 4-13) showed similar patterns as *P. vittata*. However, *P. ensiformis* and bovine liver maximum relative increases in activity were only 133% and 120%, respectively (Fig. 4-14). The maximum activity for *P. ensiformis* was obtained at 200 μM sodium arsenate, which was also the case for *P. vittata*. The bovine liver reached its maximum CAT velocity with the addition 100 μM sodium arsenate.

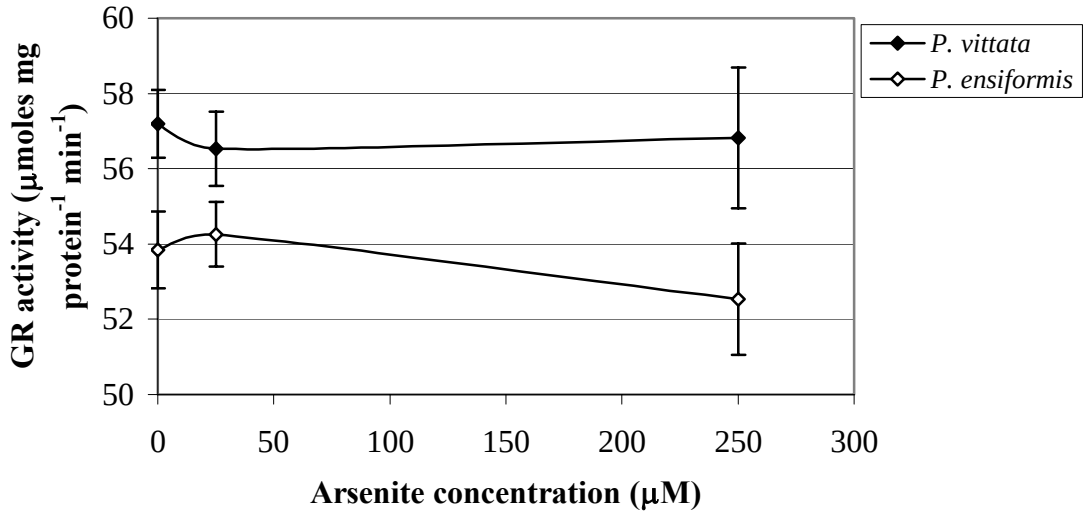


Figure 4-10. Effect of arsenite on GR activity in *P. vittata* and *P. ensiformis*. Values represent means ± std. dev. (n = 3).

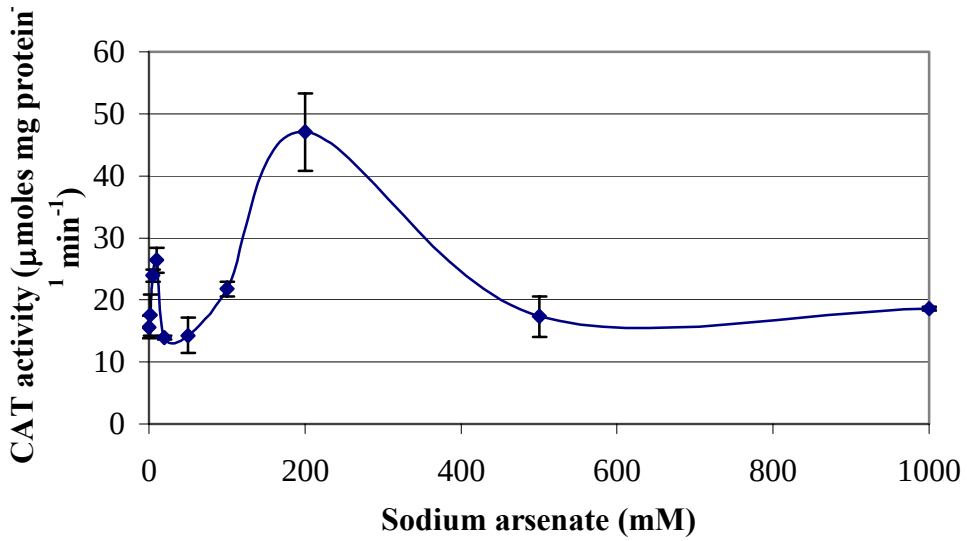


Figure 4-11. Effect of sodium arsenate on CAT activity in *P. vittata* 20% PEG protein fraction. Values represent means ± std. dev. (n = 3).

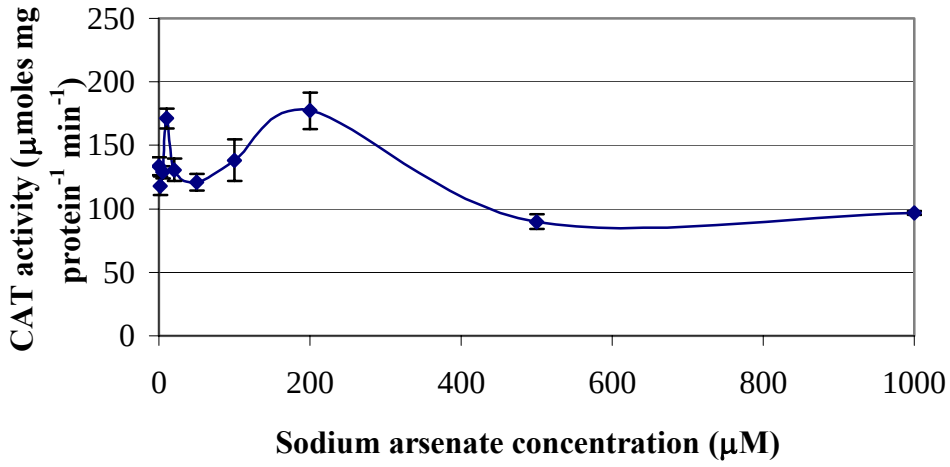


Figure 4-12. Effect of sodium arsenate on CAT activity in *P. ensiformis* 20% PEG protein fraction. Values represent means ± std. dev. (n = 3).

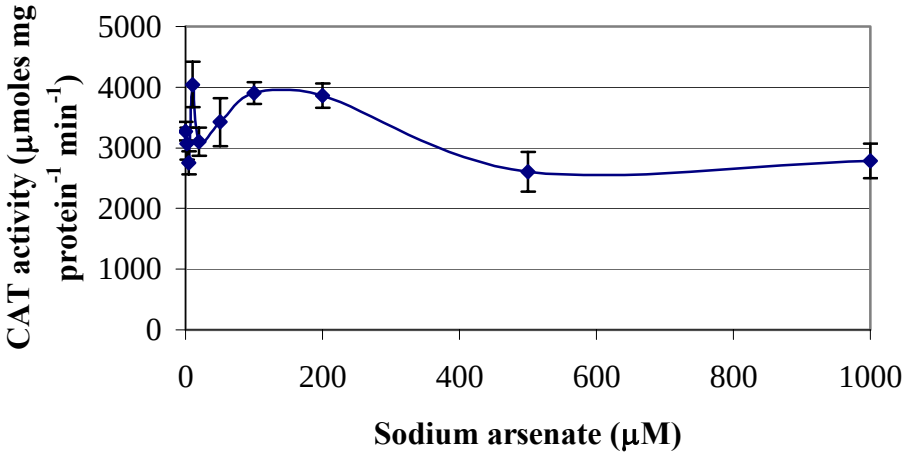


Figure 4-13. Effect of sodium arsenate on CAT activity in bovine liver (CAT positive control). Values represent means ± std. dev. (n = 3).

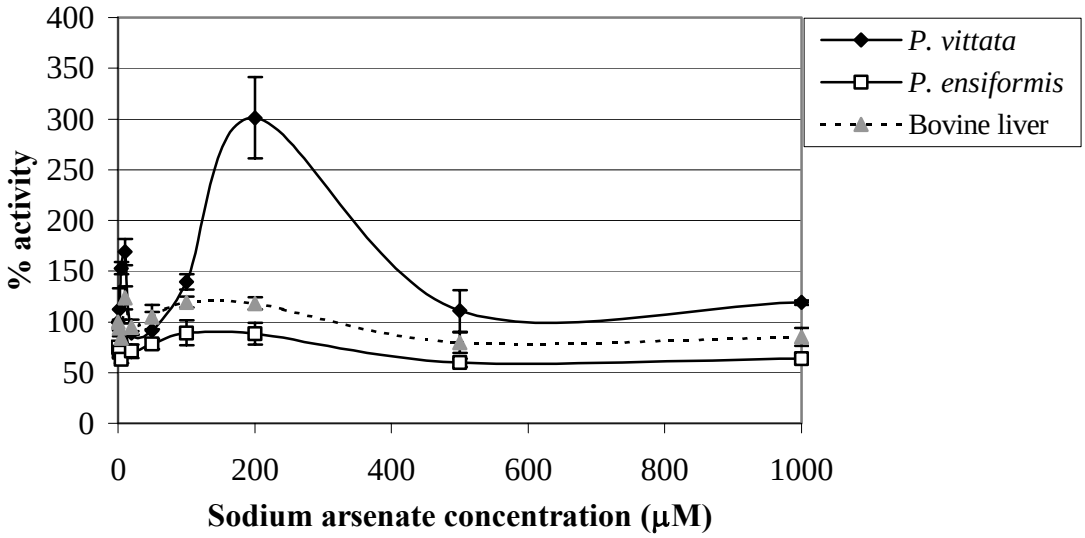


Figure 4-14. Comparison of the percent change in CAT activity in *P. vittata*, *P. ensiformis* and bovine liver (CAT positive control) upon exposure to arsenate. Percent change is based on the control (no sodium arsenate) assays for each protein. The control CAT activities for *P. vittata*, *P. ensiformis* and bovine liver were 16, 134 and 3271 µmoles min^{-1} mg $protein^{-1}$. Values represent means ± std. dev. (n = 3).

Discussion

Antioxidant enzymes play an important role in plant responses to environmental stress, such as exposure to arsenic. Changes in antioxidant enzyme activities upon stress can give insight into a plant's ability to tolerate stress and mediate its effects. Singh et al. (2005, unpublished data) found that the GSH:GSSG ratio did not change significantly in *P. vittata* exposed to arsenic. One reason for this was thought to be the induction by or the efficiency of GR. However, GR activity was not induced in *P. vittata* upon arsenic exposure. These results indicate that while GSH is an important antioxidant, the recycling of it from GSSG to GSH by GR does not play an important role in *P. vittata*'s ability to maintain the GSH: GSSG ratio. The same study indicated that GSH concentrations also increase in *P. vittata* exposed to arsenic (Singh et al., 2005, unpublished data). Therefore, it is possible that one or both of the GSH synthesizing enzymes (gamma-glutamyl cysteinyl synthetase and glutathione synthetase) may be induced by arsenic, causing an increase in GSH concentration and maintaining the GSH:GSSG ratio.

It was originally thought that although GR may not be induced in *P. vittata* that it may be more efficient (in terms of K_m) compared to a non-arsenic hyperaccumulator, *P. ensiformis*. However, kinetics studies produced K_m constants similar in both ferns. These K_m values were also comparable to those previously reported for GR in other plants (Hausladen and Alscher, 1994; Griffith et al., 2001). The direct plots for NADPH (Fig. 4-6 A and 4-7 A) indicated that higher concentrations of the substrate inhibits GR activity. This was more evident in *P. vittata*.

It is interesting that arsenic did not inhibit or activate GR activity in *P. vittata* or *P. ensiformis*. Because arsenite can readily bind to compounds with thiol groups, such as

GSH, it was thought that its presence could possibly impact GR activity by altering the GSH:GSSG ratio. Although arsenite did inhibit GR activity in both species, it did so at a concentration (1 mM) that was at least an order of magnitude greater than the substrates, GSSG and NADPH. A lack of inhibition of GR activity by arsenic suggests that GR may be compartmentalized away from arsenic, or that arsenic does not bind to GR and cause it to be inhibited. The results of the induction, kinetics and inhibition studies suggest that GR does not play an active role in the ability of *P. vittata* to hyperaccumulate arsenic.

Unlike GR, CAT was induced 1.5 times in the fronds of *P. vittata* plants exposed to arsenic. Catalase activities were also found to be induced in the fronds *P. vittata* and *P. ensiformis* in a study conducted by Srivastava et al. (2005). Catalase is the enzyme responsible for the degradation of the ROS, H_2O_2, to water and oxygen. *Pteris vittata* has been shown to contain mostly arsenite in its fronds (Chapter 3). This is in spite of the results shown in Chapter 3 that most of the arsenic, when supplied as inorganic arsenic, taken up by the fern is transported as arsenate. It is thought that the reduction of arsenate to arsenite in plants increases the concentration of ROS in plants (Hartley-Whitaker et al., 2001a; Meharg and Hartley-Whitaker, 2002). Therefore, it is possible that the induction of CAT activity may be a result of H_2O_2 produced, directly or indirectly, from arsenate reduction in the fronds. Superoxide dismutase (SOD) activity was also found to be induced in *P. vittata* fronds (Srivastava et al., 2005). The SOD enzyme dismutates superoxide anions, and it produces H_2O_2 in process (Fridovich, 1978; Fridovich, 1986; Fridovich, 1995). Therefore, the increase in CAT activity may also be affected by the induction of SOD.

Catalases have been known to be extremely efficient in the degradation of H_2O_2. The determination of the K_m constants from the CAT kinetics assays did not indicate that CAT enzymes in *P. vittata* were more efficient than *P. ensiformis*. The 7 to 8-fold difference in the V_{max} values for both species is interesting. This difference could be due to the presence of an inhibitor in the *P. vittata* extract. It may also be simply due to differences between the two plants. Switala and Loewen (2002) found that the observed V_{max} values of CAT in some bacteria varied 2 to 10 times for species within the same genus. The differences are thought to be a result of inactivation of smaller-subunit catalases by H_2O_2 damage. Small-subunit catalases reach their maximum velocity around 200 mM, which was similarly found in *P. vittata*. However, larger sub-unit catalases reached a maximum velocity around 1 M. *Pteris ensiformis* CAT activity still appeared to be fairly linear, but with some leveling off at a velocity of 220 mM H_2O_2. Determinations of CAT activity with H_2O_2 concentrations much greater than 200 mM are not possible using the spectrophotometric method, as effervescence by H_2O_2 does not allow for linear decrease in absorbance. It is presently unclear why *P. vittata* appears to be more sensitive to damage caused by H_2O_2. It would be expected that the arsenic hyperaccumulating fern would be less sensitive to such damage. However, because the proteins were not purified, the accuracy of these V_{max} values are rather uncertain and unreliable.

The study by Switala and Loewen (2002) also concluded that the traditional Michaelis-Menten kinetics terms, K_m and V_{max}, cannot be directly used for catalases. Catalases do not follow Michaelis-Menten kinetics over the H_2O_2 concentration range because of the two-step CAT reaction. Therefore, the kinetic parameters should be

considered to be theoretical. This is especially true for concentrations greater than 200 mM. The same study did find a better correlation for lower substrate concentrations, such as those used in this experiment.

Catalase activation by arsenate has not yet been reported in plants. However, sodium arsenate appears to activate CAT activity in *P. vittata* at two concentrations, 10 and 200 mM (Fig. 4-14). The percent activation at a concentration of 200 mM sodium arsenate was approximately 1.8 times greater than the activity found at 10 mM sodium arsenate. The increase in CAT activity observed at the two concentrations may be a result of the activities of two different CAT isozymes. Different CAT isozymes have been shown to respond differently to the same conditions or stresses (i.e., Horvath et al., 2002). As previously mentioned, almost all of the arsenate taken up by this fern is reduced to arsenite in the fronds. This reduction likely produces ROS. It is possible that the presence of arsenate activates some CAT isozymes in preparation for the pending arsenate reduction and the subsequent production of H_2O_2. Similar activation patterns, although not to the same extent, were observed in *P. ensiformis* and the CAT positive control. Therefore, these results suggest that activation of CAT by arsenate may constitute an important role in the ability of *P. vittata* to hyperaccumulate arsenic.

CHAPTER 5
PHYTOREMEDIATION OF AN ARSENIC-CONTAMINATED SITE USING *Pteris vittata* L.

Introduction

Remediation of contaminated soils has traditionally focused on engineering-related methods (Cunningham et al., 1997). Many of these methods, such as excavation, can be expensive, while containment remediation techniques, such as capping, do not actually remove the contaminant(s) from the soil. Recently, phytoextraction has emerged as a potential *in situ* remediation alternative to these traditional remediation methods.

Phytoextraction is the use of plants to remove pollutants from the soil and/or water matrices (Raskin and Ensley, 2000; Lasat, 2002; McGrath et al., 2002). Commonly, hyperaccumulating plants are employed for phytoextraction purposes. By definition, the aboveground dry matter of hyperaccumulators is comprised of greater than 0.1% of the element of interest. Ideally, a hyperaccumulator used for phytoextraction should have the following characteristics: high rates of accumulation and translocation, fast growth and a high production of biomass (Wantabe, 1997).

There is evidence that phytoremediation has a promising future role in soil and water remediation. As such, interest in phytoremediation as a viable remediation technology has significantly increased over recent years. Phytoremediation is still in its infancy, but it is being used in some in-field remediation. Currently there are no sites that have been completely remediated using phytoremediation (Schoor, 2002; USEPA, 2002a).

Therefore, this raises several issues. One of which is cost. It is widely claimed that phytoremediation is a much more economical remediation technology compared to most other remediation techniques. There are estimated costs for the use of phytoremediation. Using these figures, the costs do appear to be significantly lower than those for conventional techniques. However, the costs can vary widely depending on the site factors and the plant(s) being used to remediate the site. Another issue is time. The amount of time needed to fully remediate a site is, again, very dependent on the plant and site characteristics (Schoor, 2002)

Pteris vittata was the first arsenic-hyperaccumulating plant to be identified (Komar et al., 1998; Komar, 1999; Ma et al., 2001). It is a relatively fast-growing perennial plant that prefers alkaline soil. Most of the arsenic that is taken up by the fern is translocated and accumulated in its aboveground biomass. It was shown to have relatively high production of root and frond biomass. Further, *P. vittata* was found to have high bioconcentration factor (BF) and translocation factor (TF) of arsenic, indicating its ability to not only take up high amounts of arsenic, but also to translocate much of the arsenic to its fronds (Tu et al., 2002), which can subsequently be harvested and taken off-site for disposal. In a greenhouse study by Tu et al. (2002), 26% of the initial soil arsenic was depleted using *P. vittata* after 20 wk of growth.

Because of its fast growth, relatively large biomass production and ability to hyperaccumulate and translocate arsenic, *P. vittata* does exhibit the potential for use in phytoextraction of arsenic-contaminated soils. One study focused on using *P. vittata* and Indian mustard (*Brassica juncea*) to phytoremediate a soil contaminated with arsenic and lead (Pb) (Salido, et al., 2003). This study concluded that eight years would be needed to

decrease the acid-extractable soil arsenic concentration from an average of 82 to 40 mg kg^{-1}. However, additional studies and field-related data are needed before this fern can be used effectively for phytoextraction.

The main objectives of this field study were: 1) to determine the ability and efficiency of *P. vittata* in accumulating arsenic from an arsenic-contaminated site; 2) to determine the ability of *P. vittata* in decreasing total arsenic concentrations in the arsenic-contaminated soils; and 3). To determine the most appropriate harvesting practices in order to obtain maximum arsenic removal from the soil.

Materials and Methods

Experimental Site

The field site, located in North central Florida, was previously used to pressure treat lumber with CCA from 1951-1962. The lumber was pressure treated in a cylinder using a CCA-solution containing arsenic pentoxide, copper sulfate and either sodium or potassium chromate (Woodward-Clyde, 1992). Van Groenou, et al. (1951) found that this solution commonly had a composition of 11% As, 33% Cu and 56% Cr. The past pressure-treatment of lumber has lead to the current contamination found at this site.

The soil at the site is an Arrendondo-urban complex. The taxonomic classification is a loamy, siliceous, hyperthermic Grossarenic Paleudult. Previous analysis found the soil to have a pH of 7.5 and organic matter content of 0.5 to 0.8%. The particle size distribution of the soil at this site was 88% sand, 8% silt and 4 % clay-sized particles (Komar, 1999).

Planting and Plot Maintenance

Plot 1

In September 2000, a 30.3 m^2 plot was prepared at the site. The plot was hand-weeded, and non-porous black plastic mulch was placed on the experimental area. No tilling was performed prior to transplanting *P. vittata* into hand-excavated holes (10.2 cm wide by 10.2 cm deep). The planting density was 0.09 m^2 per fern, for a total of 324 ferns. At the time of planting each fern was supplied with 13 g of STA-GREEN® time-released fertilizer brand (12-4-8). Due to late planting, high mortality over the winter from frost and cold injury occurred, resulting in 314 ferns being replaced in April 2001.

The plot was hand-weeded approximately every two weeks as needed, and it was watered daily with spray irrigation. No additional fertilizers or soil amendments were added during the 2001 or 2002 growing seasons. During January and February 2002, the ferns were covered with black plastic to prevent frost injury. However, due to some frost injury and lack of water, 111 ferns died in 2001. They were replaced in April 2002 with ferns of similar size. This plot was discontinued in October 2002 due to expansion of the current business at the site.

Plot 2

In October 2002 plot 1 was essentially paved over. Therefore, another experimental plot was initiated in a different area at the same site. Both the size (30.3 m^2) and plant spacing (0.09 m^2) of plot 2 were identical to plot 1. Black plastic mulch was placed on the experimental area, and plants were directly transplanted from plot 1 to plot 2. No fertilizer was added at the time of planting. However, fertilizer (15-5-15) was

applied each year at a rate of 100 lb N yr^{-1}, in two split applications. The plot was hand-weeded approximately every two weeks, or as needed, and it was watered every other day with spray irrigation. Ferns were covered during the winter seasons using shade cloths. Approximately 6 ferns died each winter and were replaced the following April.

Plant Harvests

Plot 1

No plant harvests were performed during the 2000 growing season. However, four harvests were performed in 2001. Senescing fronds were removed at ground level by hand in August, September and October 2001. Frond samples were taken from each plant, and were grouped according to a pre-established grid of the site (36 total samples, 9 plants per sample). Fronds that were dead (brown and dry) and were close to senescence (little green-colored tissue or most of the frond area was yellow, brown with greatly mottled color) were removed from each plant at ground level by hand. In December 2001, all plants were harvested, totaling 324 samples. With the exception of fiddleheads and one to two live fronds, all fronds were removed from the ferns at ground level. These exceptions were made to help facilitate survival of the ferns during the winter season. Figure 5-1 A and B are photographs of the site during the 2001 season.

The ferns were harvested differently in 2002 to determine if harvesting frequency and/or method affected the amount of arsenic removed from the site. Three harvesting treatments were planned: senescing fronds harvested once a month (DD1); all fronds harvested once a year (A1x); and all fronds harvested twice a year (A2x). However, due to the termination of the plot in October 2002, the harvest treatments were not fully implemented. As a result, senescing fronds in 1/3 of the plot were harvested in August,

September and October, and all fronds in 1/3 of the plot were harvested in August. They were then extrapolated to the whole plot. Because of the very slow recovery of the ferns from the winter season and the replacement of the dead ferns, many of the ferns were not of adequate size to harvest until August.

The experimental design was a randomized complete block. The plot was divided into 3 blocks. There were 6 subplots per block, for a total of 18 subplots. Subplots contained 18 ferns. Each of the three harvest treatments was randomly assigned to two subplots in each block; therefore, each harvest treatment was replicated six times in the plot. Figure 5-1 C and D show photographs of plot 1 during the 2002 season.

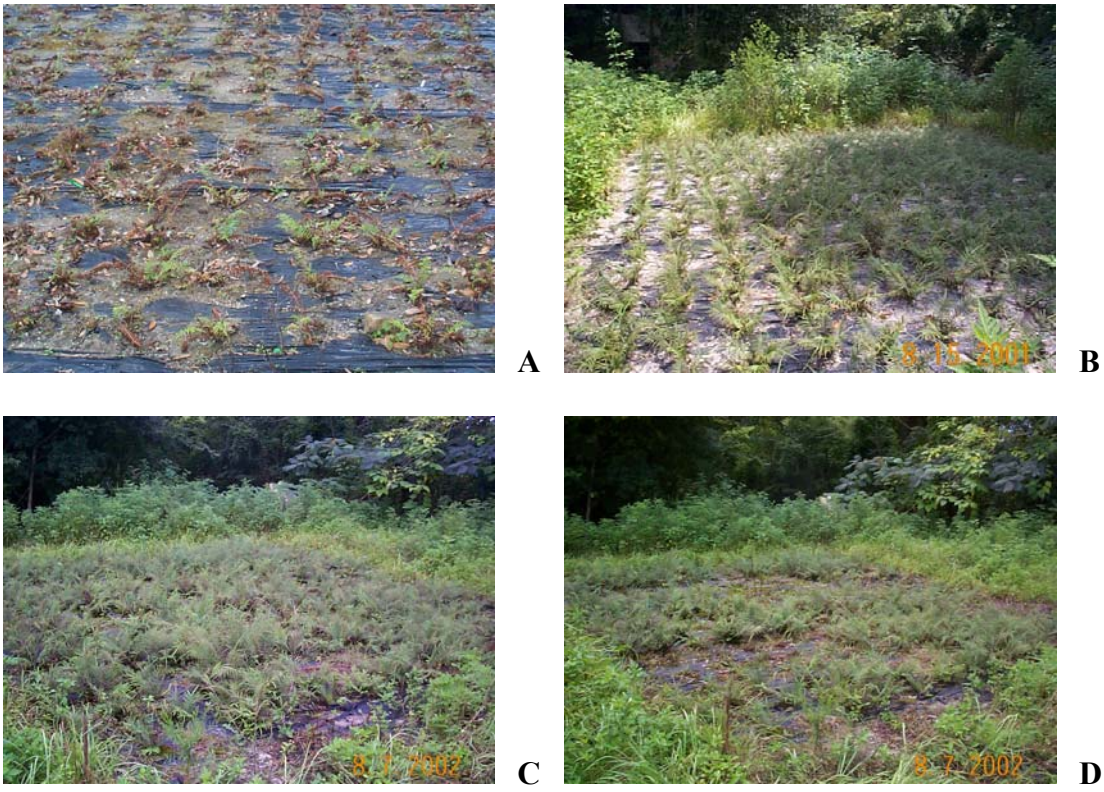


Figure 5-1. Photographs of *P. vittata* growing in the first experimental plot (2001 to 2002). (A) Photograph of plot 1 in April 2001. Ferns were recovering from the winter season. (B) Photograph of plot 1 in August 2001. (C) Photograph of plot 1 in August 2002 prior to harvest, and (D) immediately following harvest of the DD1 and A2x treatments.

Plot 2

No plant harvests were performed in 2002. In 2003, three plant harvests were made. All ferns were harvested to a height of 15 cm in July, September and November. Harvesting treatments were implemented in 2004 to evaluate the effects of harvesting frequency. The ferns were harvested to a height of 15 cm one (1x), two (2x) or four (4x) times that year. Fern borders were also in place around the harvesting treatments. The experimental design was a randomized complete block with four replications. Each replicate contained 20 *P. vittata* plants, for a total of 80 ferns per treatment. The borders consisted of a total of 84 ferns. Ferns were harvested in May (2x, 4x and borders), July, (4x and borders), September (4x and borders) and November (1x, 2x, 4x and borders).

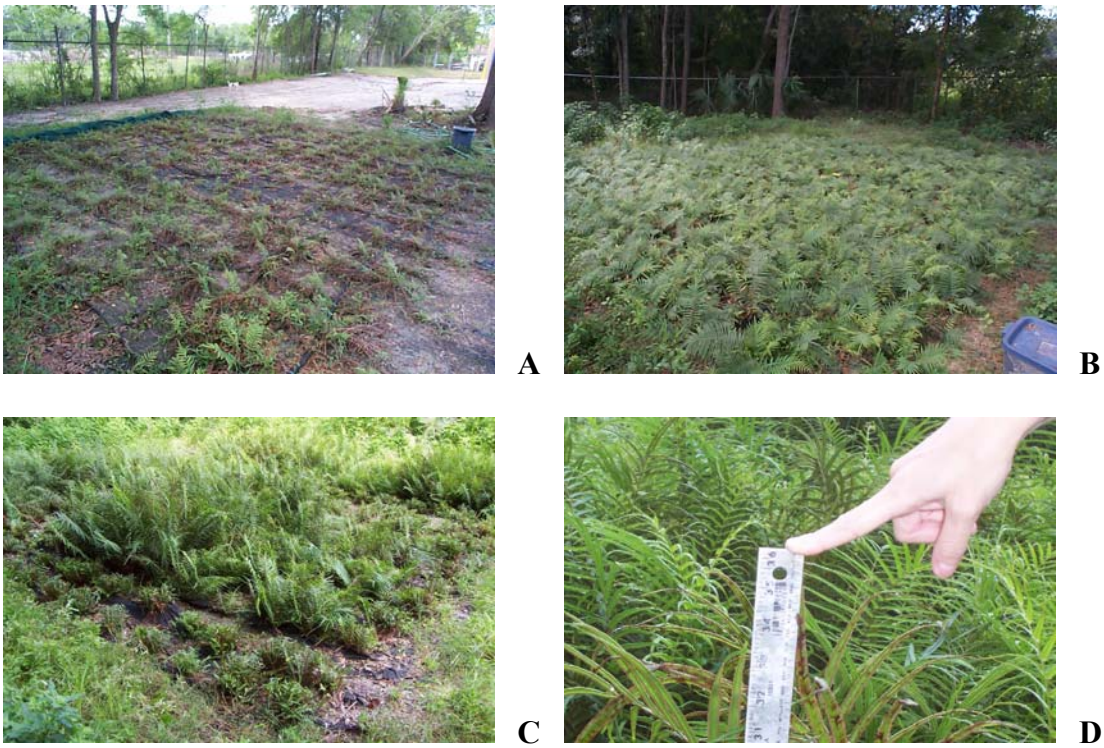


Figure 5-2. Photographs of *P. vittata* growing in the second experimental plot (2003 to 2004). (A) Photograph of plot 2 taken in April 2003. (B) Photograph of plot 2 taken in June 2003. (C) Photograph taken in 2004 after harvest in July 2004. (D) Close up of ferns showing that ferns were taller than 3 ft.

Determination of Frond Biomass and Arsenic Concentrations

For all harvests, frond samples were placed into a 60^{o} C oven for approximately 48 h. Soil particles were removed from the dried fern samples, as necessary. The samples were then weighed for dry biomass. Dried samples were ground through a 1 mm mesh Wiley Mill screen. The ground samples (0.25 g) were subjected to HNO_3/H_2O_2 digestion (USEPA Method 3051) on a hot block (Environmental Express, Ventura, CA). The digested plant samples were analyzed for total arsenic concentration using graphite furnace atomic absorption spectroscopy (GFAAS, Perkin Elmer SIMMA 6000, Perkin-Elmer Corp., Norwalk, CT).

In 2001 and 2002, all frond samples from the August to October dead and dying harvests were analyzed for total arsenic concentration. However, due to the large number of samples harvested in December 2001, only 72 of the 324 fern samples harvested were analyzed for total arsenic concentration. The samples that were chosen for analyses were those of the median dry mass weight. All frond samples collected in 2003 and 2004 were analyzed for total arsenic concentration.

Soil Sampling

Plot 1

Soil samples were extracted in September 2000, December 2001 and October 2002. In September 2000 and December 2001, 36 surface (0-15 cm) soil samples were systematically taken (1 sample per 0.09 m^2). In addition to the surface samples, 9 soil profile samples were extracted (15-30 cm and 30-60 cm). Three sets of profile soil samples were taken for every 12 surface samples. Due to extreme difficulty in extracting

the soil samples, only 10 random surface samples and 5 random profile samples at each depth were taken in October 2002.

Plot 2

Soil samples were extracted in December 2002, 2003 and 2004. Within the plot area, 49 surface (0-15 cm) and profile (15-30 cm and 30-60 cm) soil samples were taken systematically. In addition, 12 sets of soil samples were extracted from outside the plot area to compare the difference in soil arsenic concentrations inside and outside the plot, as affected by *P. vittata* (Fig. 5-3).

Determination of Total Soil Arsenic

All soil samples were air dried and sieved to pass through a 2 mm mesh screen. The sieved soil samples (0.5 g) were subjected to hot block (Environmental Express, Ventura, CA) digestion using USEPA Method 3051 (HNO_3/H_2O_2) for arsenic analysis. The digested soil samples were then analyzed for total arsenic concentration using GFAAS.

Sequential Soil Arsenic Fractionation

A sequential soil arsenic fractionation was performed on all soil samples extracted from plot 1 (2000 to 2002) using the method developed by Wenzel, et al. (2001). This sequential extraction method, which represents a functional fractionation, contains five arsenic fractions (decreasing in availability): non-specifically bound, specifically bound, amorphous hydrous oxide-bound, crystalline hydrous oxide-bound and residual.

Using approximately 1.0 g of air-dried soil from each soil sample, arsenic was extracted Wenzel, et al (2001). The non-specifically bound fraction was extracted by

shaking for 4 h at 20^{o}C with 25 ml of 0.05 M ammonium sulfate. The specifically bound fraction was extracted by 16 h of shaking at 20^{o}C upon the addition of 25 ml of 0.05 M ammonium phosphate. Twenty-five ml of 0.2 M ammonium oxalate buffer (pH 3.25) was then added. Samples were shaken in the dark for 4 h at 20^{o}C. The samples were then washed with 12.5 ml of 0.2 M ammonium oxalate buffer (pH 3.25) by shaking for 10 min in the dark. This fraction was labeled as the amorphous hydrous oxide-bound fraction. The crystalline hydrous oxide-bound fraction was extracted by mixing the samples with 25 ml 0.2 M ammonium oxalate buffer and 0.1 M ascorbic acid (pH 3.25) and placing them in a 96^{o}C water bath for 30 min. Each sample was washed by12.5 ml of 0.2 M ammonium oxalate buffer (pH 3.25) by shaking for 10 min in the dark. The residual fraction was extracted by acid digestion using USEPA Method 3051 (HNO_3/H_2O_2). Samples from each fraction, with the exception of the residual fraction, were centrifuged at 3500 rpm for 15 min and 20^{o}C after each extraction and/or wash. The supernatants were collected. The supernatants of each fraction were filtered through Whatman 42 filter paper and analyzed for arsenic concentration using GFAAS.

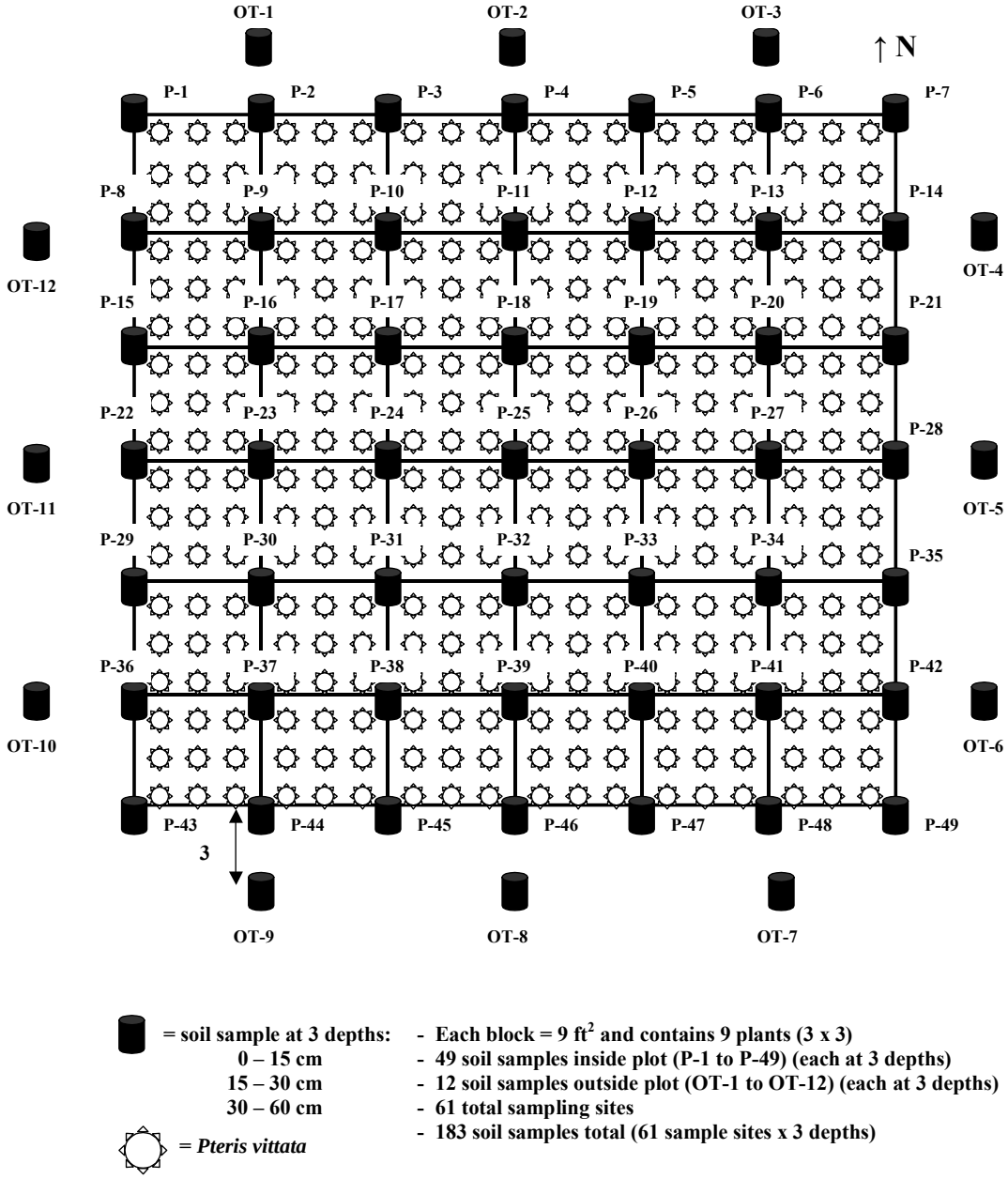


Figure 5-3. Soil sampling plan for experimental plot 2 in December 2002, 2003 and 2004.

Bioconcentration Factor

As previously mentioned, bioconcentration factor (BF) is the ratio of arsenic in the plant to that in the soil. The BF was calculated for the December 2001 total harvest and the sole A2x harvest in August 2002, as this was the only harvest containing both live

and dead fronds, giving a more representative average frond arsenic concentration. The BF was also calculated for 2003 and 2004 using the final harvest total arsenic concentrations for each treatment. The average total arsenic concentrations in the fronds from each harvest were divided by the corresponding average total soil arsenic concentrations.

Statistical Analysis

The soil data were tested for normality using Pro-UCL. The sample distributions were mixed, with data from year 2000 being lognormally distributed and data from year 2002, normally distributed. The data from year 2001 were neither normally nor lognormally distributed. Therefore, the minimum variance unbiased estimator (MVUE) of the median was used as a basis for statistical comparison for the three years. To test for significance among the three years, non-parametric tests were used (NPAR 1-Way) in SAS® (SAS Institute, 2001).

The soil data from plot 2 (years 2002, 2003 and 2004) were tested for significance using the general linear model (GLM) in SAS® (SAS Institute, 2001). All plant harvest data from both experimental plots were also analyzed using GLM in SAS.

Results

Arsenic Removal by Ferns

Plot 1

Of the three harvests of senescing fronds, significantly greater biomass was removed from the October harvest (Table 5-1). For the August and September harvests, a

total of 557 g of biomass were harvested, and for the October harvest, a total of 845 g of biomass was removed. However, there were no significant differences in arsenic concentrations in the senescing fronds among the three harvests (Table 5-1). The average arsenic concentration in the senescing fronds ranged from 2269 to 2403 mg As kg^{-1}. The amount of arsenic removed from the site in October (2150 mg) was significantly ($P < 0.0001$) greater than that removed in August (730 mg) or September (780 mg) due to the higher amount of biomass removed in October versus August and September.

As expected, compared to the harvests of senescing fronds, the harvest of all fronds in December had significantly ($P < 0.0001$) higher plant biomass and arsenic removal (Table 5-2). The December harvest yielded 2531 g of biomass and 12.1 g arsenic removed. The average arsenic concentration in the fronds was 4575 mg kg^{-1} dry biomass. Combined with the harvests of senescing fronds, a total of 3933 g biomass was removed from the site. On an acre basis, the biomass production for 2001 was 0.52 ton. In total, the ferns removed approximately 15.7 g of arsenic from this site during 2001 (Tables 5-1 and 5-2).

Table 5-1. A comparison of the total biomass removed, average frond arsenic concentration and amount of arsenic remediated from the senescing frond harvests in 2001 and (DD1) in 2002. The 2002 data are normalized to estimate for the entire year's harvests. Values represent means or totals ± std. dev.

	Average As concentration (mg kg^{-1})		**Total biomass (g)**		**Total As removed (g)**	
	Year					
Harvest	**2001**	**2002**	**2001**	**2002**	**2001**	**2002**
August	2403 ± 807	1992 ± 656	281	630	0.73 ± 0.01	1.26± 0.03
September	2389 ± 429	2560± 502	276	633	0.78 ± 0.01	1.62 ± 0.04
October	2269 ± 396	2569± 520	845	285	2.15 ± 0.03	0.73 ± 0.08
Total	--	--	1302	1548	3.66	3.61

Table 5-2. Comparison of average frond arsenic concentrations, total amount of biomass removed and amount of arsenic removed between the senescing fern fronds harvested in 2001 and 2002 (DD1), and all fronds harvested in December 2001 and August 2002 (A2x). The 2002 data are normalized to estimate for the entire year's harvests. Values represent means or totals ± std. dev.

	Frond Harvest			
	Senescing		**All**	
	2001	**2002**	**2001**	**2002**
Average As concentration (mg kg^{-1})	2354 ± 573	2374 ± 598	4575 ± 575	3186 ± 322
Total biomass (g)	1402	1548	2531	2244
Total As reduction (g)	3.6 ± 0.03	3.6 ± 0.11	12.1 ± 0.01	7.1 ± 0.46

Arsenic concentrations in the senescing fronds harvested in 2002, with an average arsenic concentration of 2374 mg kg^{-1}, were not significantly different compared to those in 2001 (Table 5-2). However, slightly more biomass was removed in 2002 (1548 g) than in 2001 (1402 g).

Although there were no differences in frond arsenic concentration, the October harvest of senescing fronds yielded significantly less ($P < 0.0001$) biomass than the August and September harvests (Table 5-1). In addition, more arsenic was removed from the site during the September harvest, and the least in October. However, in 2001, the October harvest yielded the most biomass and arsenic removed. Similar to 2001, arsenic concentrations and biomass from the harvest of all fronds were significantly higher ($P < 0.0001$) than those from the harvest of senescing fronds (Table 5-2).

During 2002, a total of 3792 g of plant biomass and 10.7 g of arsenic were removed from plot 1. Combined with 2001, approximately 26.3 g of arsenic was removed by the ferns during the two-year period (Tables 5-1 and 5-2).

Plot 2

Significantly more biomass was removed from plot 2 in 2004 than in 2003 (Table 5-3). The 1x harvest treatment in 2004 yielded significantly greater ($P < 0.0001$) biomass produced/removed compared to the 2x and 4x treatments. The amount of biomass removed from the single harvest was also approximately the same as the total biomass removed in three harvests of the entire plot in 2003. However, if the 1x harvest (a total of 80 plants) were extrapolated for the entire plot (324 plants), then approximately 32 kg of biomass would have been removed in a single harvest of plot 2 end of the year in 2004.

Frond arsenic concentrations for plot 2 in 2003 were similar to those found in plot 1. Although arsenic concentrations were not significantly different between the harvest treatments in 2004, the frond arsenic concentrations were approximately half of those found in 2003 (Table 5-4). Despite the lower arsenic concentration, the amount of arsenic removed from plot 2 was similar in 2003 (36.0 g) and 2004 (41.6 g). Again, if the 1x harvest were extrapolated for the entire plot, approximately 60.8 g of arsenic would have been removed in a final harvest of the entire plot. The 1x treatment also resulted in significantly more ($P < 0.0001$) arsenic removal from the site compared to the 2x and 4x harvesting treatments.

Table 5-3. Comparison of the total amount of biomass removed between the fronds harvested in 2003 and 2004. Values represent totals.

		2004				
		Harvest treatment				
	2003	1x	2x	4x	Borders	Total
Harvest	biomass (g dw)					
1	2252	--	1272	1363	857	3492
2	1938	--	--	859	549	1408
3	3421	--	--	1018	601	1619
4	--	7825	4168	577	463	13033
Total biomass (g dw)	*7611*	7825	5440	3817	2470	*19552*

Table 5-4. Comparison of the average frond arsenic concentrations and amount of arsenic removed between the fronds harvested in 2003 and 2004 in plot 2. Values represent means ± std. dev. or totals.

		Harvest treatment			
	2003	1x	2x	4x	Borders
Harvest	As concentration (mg kg^{-1})				
1	4576 ± 1600	--	3251 ± 695	2581 ± 376	2935 ± 261
2	5086 ± 963	--		1924 ± 349	2021 ± 570
3	4497 ± 668	--		2085 ± 405	2088 ± 482
4	--	2001 ± 381	2228 ± 262	2457 ± 467	2521 ± 507
As remediated	36.0	15.0	13.3	7.9	5.4

Soil Arsenic Concentrations

Plot 1

The MVUE of the median for the surface arsenic concentrations in the plots were 172, 162 and 129 mg kg-1 for 2000, 2001 and 2002, respectively. Non-parametric tests showed no differences among the years.

The average surface soil arsenic concentrations were not significantly different between years 2000 to 2002. In 2000, the average concentration of arsenic in the surface

soil was 190 mg kg^{-1}, while the 2001 average was 182 mg kg^{-1}, a decrease of 4% from 2000 to 2001 (Table 5-3). The lack of significance in the average arsenic concentration can be attributed to the extreme heterogeneous soil arsenic concentrations at the site (Fig. 5-4 A, B, C). Individual surface soil samples varied greatly in arsenic concentrations, within each year and between the years. However, the average surface arsenic concentration was reduced by 23% from 2001 to 2002. From 2000 to 2002, the total arsenic concentration in the 0-15 cm depth decreased from 190 to 140 mg kg^{-1} (Table 5-3).

Overall, the greatest decrease in soil arsenic concentration was found in the 15-30 cm depth. The arsenic concentration at this depth was 278 mg kg^{-1} in 2000 and 212 mg kg^{-1} in 2001. Soil samples taken in 2002 showed the average soil arsenic concentration to be 158 mg kg^{-1}, which was a 43% decrease in soil arsenic over two years. The soil arsenic concentrations in the 30-60 cm depth were decreased by 6% each year

Table 5-5. Average soil arsenic concentrations and arsenic depletion of soil samples taken in plot 1 in 2000, 2001 and 2002. Soil samples were taken at three depths. Values represent means ± std. dev.

	Average As concentration (mg kg^{-1})			**Total As depletion**	
Sample depth (cm)	**2000**	**2001**	**2002**	**mg kg^{-1}**	**%**
0-15	190 ± 89	182 ± 112	140 ± 81	50	26%
15-30	278 ± 138	212 ± 178	158 ± 31	120	43%
30-60	191 ± 125	180 ± 46	169 ± 79	22	12%

Plot 2

Soil arsenic concentrations did not significantly change in plot 2 during the 2002-2004 experimental period (Table 5-6). Variability in the total soil arsenic concentrations

was high. However, it was lower than in plot 1. The greatest overall net decrease in soil arsenic concentration was observed in the 0-15 cm depth. The other depths did not exhibit significant decreases in total soil arsenic concentrations over the two-year period. The soil in these depths also exhibited increases in total soil arsenic concentrations from 2003-2004.

The arsenic concentrations of the outside plot soil samples were not significantly different between the sampling years. The results showed similar changes in the total soil arsenic of the outside plot samples compared to the inside plot soil samples. The exception was that there was a greater total decrease at the 30-60 cm depth (Table 5-7).

Table 5-6. Average soil arsenic concentrations and net arsenic depletion of soil samples taken inside plot 2 in 2002, 2003 and 2004. Soil samples were taken at three depths. Values represent means ± std. dev. (n = 49).

	Year			Net As depletion	
Depth	**2002**	**2003**	**2004**	**mg kg^{-1}**	**%**
0-15	110 ± 33	111 ± 72	95 ± 38	15	14
15-30	130 ± 72	115 ± 55	133 ± 83	0	0
30-60	134 ± 60	124 ± 61	130 ± 74	4	3

Table 5-7. Average soil arsenic concentrations and net arsenic depletion of soil samples taken outside plot 2 in 2002, 2003 and 2004. Soil samples were taken at three depths. Values represent means ± std. dev. (n = 12).

	Year			Net As depletion	
Depth	**2002**	**2003**	**2004**	**mg kg^{-1}**	**%**
0-15	129 ± 45	141 ± 72	111 ± 42	18	14
15-30	137 ± 54	132 ± 56	144 ± 85	0	0
30-60	147 ± 102	131 ± 137	108 ± 111	39	27

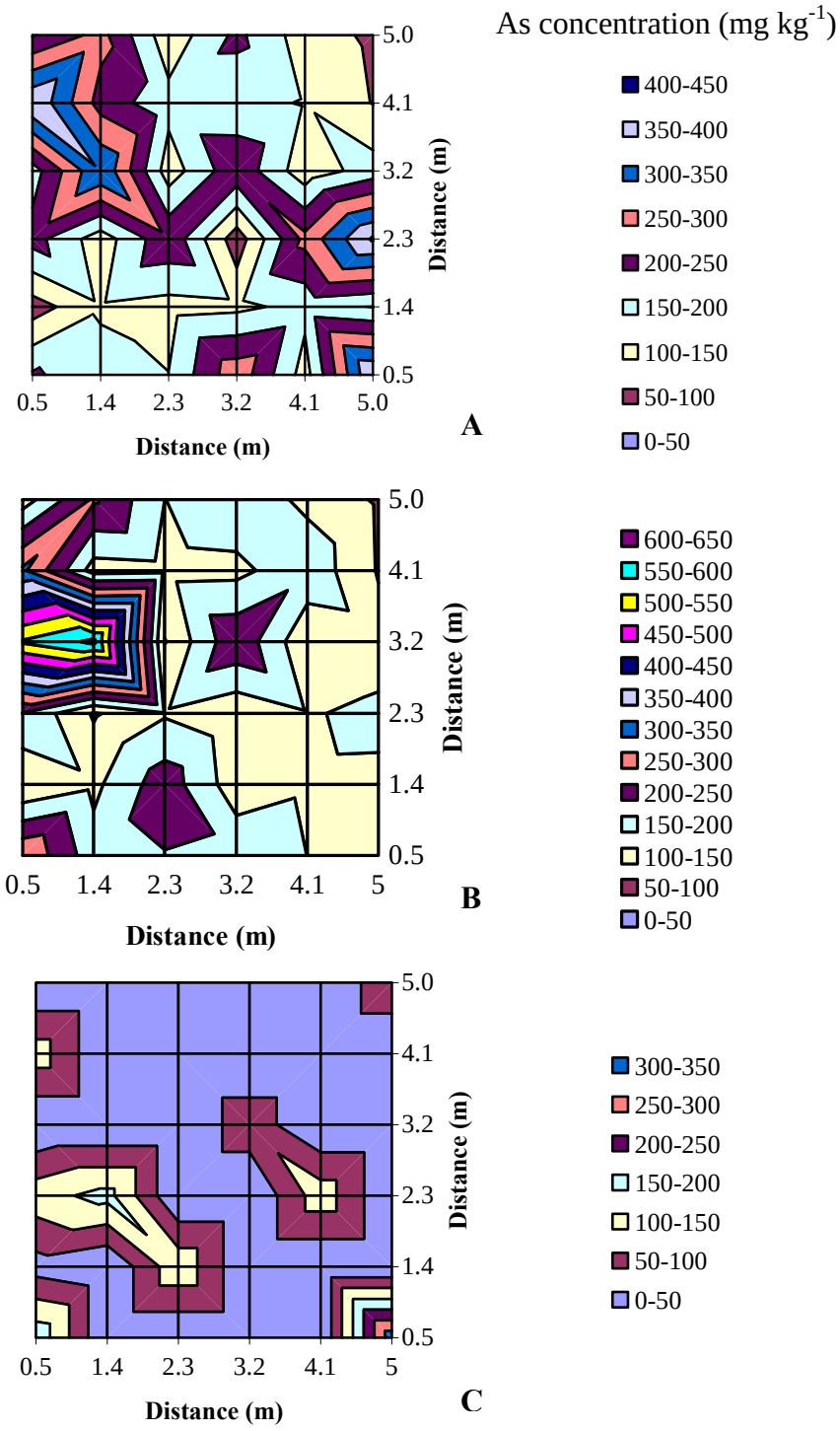


Figure 5-4. Area graphs of plot 1 showing the total soil arsenic concentrations in the top 15 cm of soil sampled in (A) 2000, (B) 2001 and (C) 2002. Graphs show the distribution and extreme variability in soil arsenic concentrations at the site.

Sequential Soil Arsenic Fractionation

The non-specifically and specifically bound arsenic fractions were the fractions most available to *P. vittata* for uptake. However, these fractions each made up a relatively low portion of the total concentration, and they did not change significantly over the time of the experiment (Fig. 5-5 A, B and C). If these two fractions were summed, together they would be similar to the amorphous hydrous oxide/Fe and Al bound fraction.

Of the total arsenic concentration, the amorphous Al and Fe-bound arsenic fraction constituted the greatest fraction at all sampling depths and years, (Fig. 5-5 A, B and C). This fraction, which is considered to be one of the more unavailable arsenic fractions, was also the one to show significant changes across different depths and years. At all three depths, the arsenic concentrations of the crystalline hydrous oxide/Fe and Al bound fraction remained relatively the same between 2000 and 2001. However, it was significantly lower in the 2002 soil samples. The residual arsenic fraction was the only fraction that did not significantly change over the course of the two-year experiment.

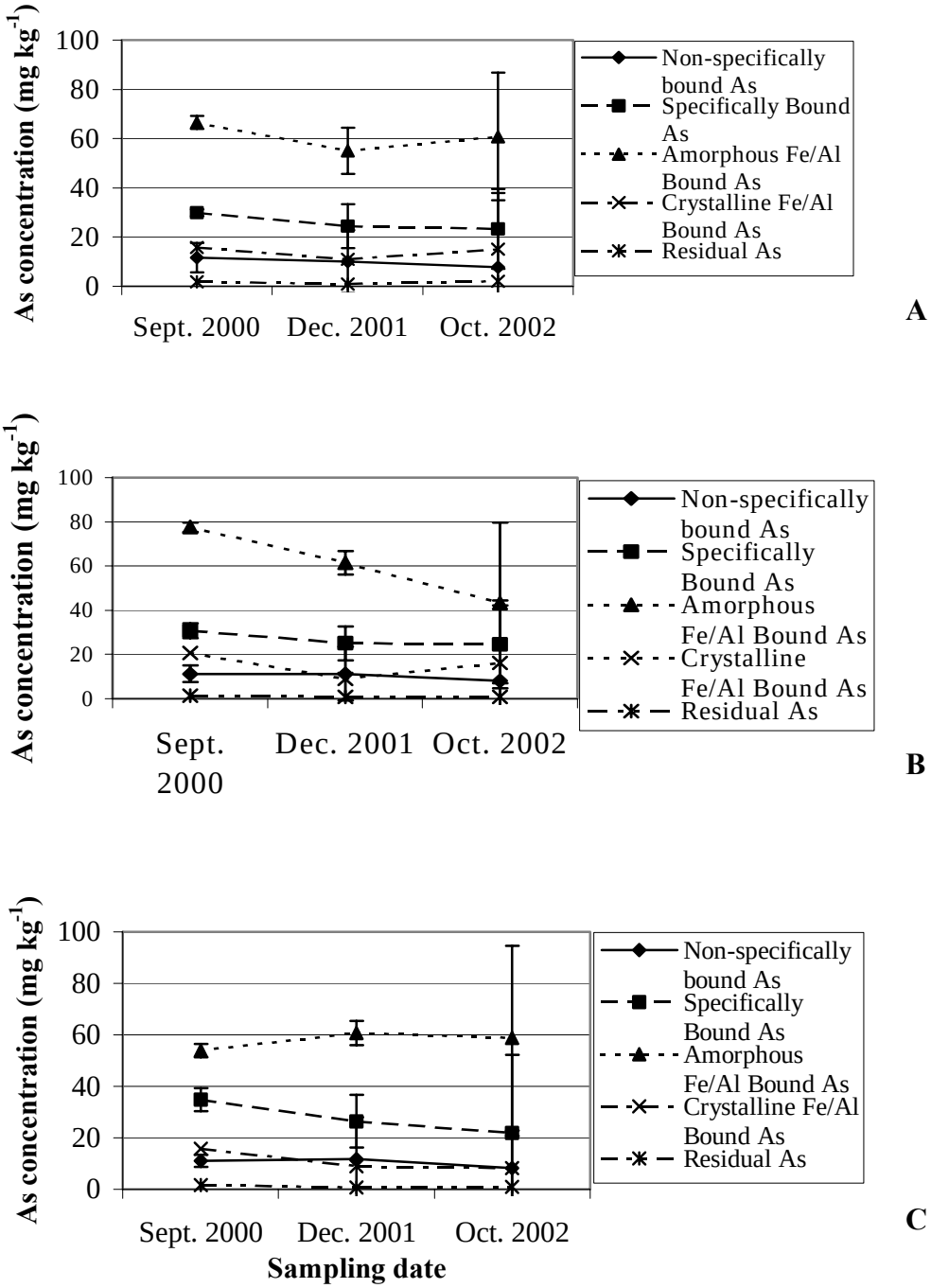


Figure 5-5. Sequential arsenic fractionation concentrations for soil sampled within plot 1 from 2000 to 2002 at depths of A). 0-15, B). 15-30 and C). 30-60 cm. Values represent average arsenic concentrations for each fraction ± std. dev. (where, n = 36 for the 0-15 cm depth in 2000 and 2001; n = 10 for the 0-15 cm depth 2002; n = 9 for the 15-30 and 30-60 cm depths in 2000 and 2001; and, n = 5 for the 15-30 and 30-60 cm depths in 2002).

Mass balance of Arsenic

Plot 1

Mass balance of arsenic in the soil-plant system over the two years of plot 1 showed large discrepancies between soil arsenic decreases and plant arsenic removal. In 2001, mass balance calculations using soil arsenic concentrations indicated that 648 g of arsenic were removed from the soil. However, the ferns accounted for only 15.7 g of arsenic. After two years, a total of 26.4 g of arsenic were removed from the soil by the harvesting and removal of *P. vittata* fronds. However, from 2000-2002, 1444 g of arsenic were calculated to have been removed from the soil (Table 5-8). This was 55 times more arsenic than was removed by the plant.

Table 5-8. Calculated mass balance of arsenic in the soil-plant system of plot 1 from 2000 to 2002.

	As depletion in soil (g)		
Soil depth (cm)	**2000-2001**	**2001-2002**	**Total**
0-15	54	283	337
15-30	445	365	810
30-60	149	148	297
Total	648	796	*1444*
As remediated by fern (g)	15.7	10.7	*26.4*

Plot 2

Mass balance calculations of plot 2 showed closer agreement in the values, compared to plot 1 (Table 5-9). However, there was still a discrepancy of 268 g of arsenic. Approximately 4.5 times more arsenic was depleted in the soil over the two years than the calculated removal via the fern fronds. The increase in total soil arsenic concentrations in the 0-15 cm depth from 2002-2003 and in the 15-30 and 30-60 cm

depths from 2003-2004 did not allow for calculation of soil arsenic depletion for those years at those depths

Table 5-9. Calculated mass balance of arsenic in the soil-plant system of plot 2 from 2002 to 2004.

	As depletion in soil (g)		
Soil depth (cm)	**2002-2003**	**2003-2004**	**Total**
0-15	0	108	108
15-30	102	0	102
30-60	135	0	135
Total	237	108	*345*
As remediated by fern (g)	36	41.6	*77.6*

Bioconcentration Factor

Using the total soil arsenic concentration, the BF was 1.5 times higher for the December 2001 harvest (45) compared to the August 2002 harvest (29).

The BF for plot 2 was 40 in 2003. The BF calculations for the 2004 harvests 1x (21), 2x (23), 4x (26) and borders (26) were approximately half of that calculated in 2003. Calculations are based on the final harvests of both years. Because the fern root arsenic concentrations were not determined, it was not possible to determine the TF for the ferns in either plot.

Discussion

The clean-up level of arsenic for Florida soils is 2.1 mg kg^{-1} in residential areas and 9.3 mg kg^{-1} in commercial areas, as regulated by the Florida Department of Environmental Protection (Florida DEP). However, background soil arsenic concentrations at uncontaminated sites may often exceed this regulated value, especially in urban areas (Chirenje et al., 2001; 2003b), likely due to non-point source anthropogenic activities. After evaluation of arsenic concentrations in the soils in two

Florida cities, Chirenje et al. (2003b) found the geometric means of soil arsenic concentration to be 0.40 and 2.81 mg kg^{-1} in Gainesville and Miami, respectively. However, the upper range of arsenic concentrations was 110 to 660 mg kg^{-1}. Soil arsenic concentrations worldwide average 5 mg kg^{-1} (Yan Chu, 1994). In 22 Superfund sites slated for arsenic remediation, the cleanup level ranged from 2 mg kg^{-1} to 305 mg kg^{-1}, with most of the sites falling into the 20 to 29 mg kg^{-1} cleanup range (Davis et al., 2001). Thus, in order to comply with regulations, there could exist an increased demand for efficient, cost-effective and reliable arsenic remediation strategies, such as phytoextraction.

Plant Arsenic Removal

Although there were not significant differences between the three harvests of senescing fronds of plot 1, the October harvest in 2001 resulted in the most arsenic removal by plants (Table 5-1). It was thought that the higher yield was possibly a result of a growth pattern or preferences of *P. vittata* (i.e., cooler temperatures and shorter days). However, in 2002, the October harvest yielded the lowest arsenic removal among the harvest of senescing fronds. The reasons for the difference were unclear. Closer observations should be performed to determine the peak growth period of this fern, as to maximize its phytoextraction potential and efficiency.

As expected, more biomass was removed when harvesting all fronds. The higher harvested biomass combined with the fact that arsenic concentrations in the live fronds were greater than those of the senescing fronds (Table 5-2), leads to the initial conclusion that *P. vittata* fronds should be harvested before they senesce. Tu et al. (2002) found that as fronds aged, arsenic concentration increased. The timing of harvest would allow the

maximum amount of arsenic to be removed from the site, and would therefore, minimize the length of time required for phytoextraction at a given site. However, it may not be practical to have several harvests of senescing fronds throughout a season, as their removal may not significantly affect soil arsenic concentrations.

Harvests of experimental plot 2 in both 2003 and 2004 yielded greater biomass compared to plot 1. This was especially true for the 2004 season. This difference is likely the result of better growing conditions and the fertilization of plot 2. Plot 2 had more shade and a more reliable water source for the ferns. Also, the increased shade afforded the ferns better protection over winter, allowing for quicker and better regrowth in the spring.

It was hypothesized that more frequent harvesting would stimulate the growth of *P. vittata*, thus yielding more biomass and, subsequently, more arsenic removal. This was not the case, as the 1x harvest yielded significantly greater biomass and arsenic removal than the other harvest treatments in plot 2 (Tables 5-3 and 5-4). Therefore, these results suggest that frequent harvesting does not stimulate growth of *P. vittata*, and in fact appears to decrease growth.

Despite a lower total soil arsenic concentration in plot 2 (Tables 5-5 and 5-6) the fern arsenic concentrations in plot 2 in 2003 were similar to those of plot 1. However, in 2004 the frond arsenic concentrations were significantly lower. This may be due to the increased growth of the ferns in 2004. It is hypothesized that the ferns took up arsenic at a similar rate, but the increased biomass production diluted the arsenic concentration in the fronds. Overall, the biomass (Table 5-3) and arsenic remediated (Table 5-4) results

from plot 2 suggest that only a single harvest is required at the end of the season to yield the best results for phytoextraction.

In the study by Salido et al. (2003) the average frond arsenic concentrations ranged from 1000 to 2740 mg kg^{-1}. However, the average frond arsenic concentrations in plot 1 of this study ranged from 1992 mg kg^{-1} for senescing fronds to 4575 mg kg^{-1} for live fronds (Tables 5-1 and 5-2) and from 1924 to 5086 mg kg^{-1} for plot 2 (Table 5-4). The differences found in the frond arsenic concentrations may lie in the fact that in their study, the experimental site was also contaminated with lead. The presence of the lead in soil may hinder the ability of *P. vittata* to remove arsenic from soil (Fayiga et al., 2004).

Salido et al. (2003) also estimated the amount of arsenic remediated per fern to be 24.3 mg. In this study, the amount of arsenic removed per fern plant was much greater, (47.8 mg in 2001, 33.4 mg in 2002, 37 mg in 2003 and 187.5 mg for the 1x treatment in 2004). The 1x treatment utilized in 2004 had greater arsenic removal per fern due to the significantly greater biomass harvested.

The better plant production and winter survival in plot 2 implies that the ferns need at least partial shade in order to reach maximum growth and, therefore, better remediation potential.

Because *P. vittata* prefers warmer climates, it is important to note that the harvest frequency could likely be lower if the ferns were employed in a cooler climate. However, the 2004 harvesting treatments employed in plot 2 strongly suggest that only a single harvest at the conclusion of the season appears to be the best harvesting frequency in order to produce more biomass and subsequently remove the most arsenic from the contaminated site.

The arsenic concentrations in live fronds were greater than those of senescing fronds (Table 5-2). Tu et al. (2003) found that during air-drying, the arsenic in the fern fronds leaches from the tissue. They found that a total of 15% of the total arsenic in the fronds leached, causing the leachate to contain 230 μg As L^{-1}. However, in 2001 and 2002 the senescing frond arsenic concentrations were 49% and 25% lower, respectively, than that of their live frond counterparts. This may be due to the differences in the frequency and intensity of the water applied to the fronds in the laboratory versus the field. Nevertheless, it is important to consider the potential leaching of arsenic from senescing fronds. The fact that arsenic leaches from senescing fronds as they age and/or dry highlights the need to properly handle arsenic-laden fronds as discussed by Tu et al. (2002). Regardless, arsenic-laden fronds are potentially hazardous, and should be treated as such during transportation and disposal.

Using the total soil arsenic concentration, the BF was 1.5 times higher for the December 2001 harvest compared to the August 2002 harvest, when all plants were harvested. Similar BF calculations were found in plot 2. In 2003, the BF was approximately 1.5 to 2 times greater for plants in plot 2 compared to 2004. Determining BF is valuable because arsenic concentrations in the fronds do not necessarily correlate to arsenic present in the soil. Some of the arsenic present in the soil is not readily available for plant uptake. The high BF indicates that *P. vittata* is very efficient in accumulating arsenic from the soil, even under the field conditions.

Beyond the typical concerns regarding the use of hyperaccumulators for phytoextraction, such as TF, BF and biomass, this fern seemed to be well suited for phytoremediation. Despite some climate restrictions, these ferns grow quickly and are

fairly easy to maintain. *Pteris vittata*, native to China, has been classified as type-II invasive plant species (i.e., its spread could be of concern in certain areas) [Southeast Exotic Plant Pest Council (SE-EPPC), 2004]. However, it was observed that over the four-year duration, only two volunteer ferns were found outside of the experimental plot perimeters.

Soil Arsenic Concentrations

Plot 1 total soil arsenic concentrations were extremely variable in the experimental site over the three years (Fig. 5-3 A, B and C). The average soil arsenic concentration data from the three years had mixed distributions. As such, complications arose when performing statistical analyses on these data.

All soil sampling depths in plot 1 revealed overall decreases in total soil arsenic concentrations (Table 5-5). The smaller decrease seen from 2000 and 2001 in the top 15 cm, where the bulk of the root mass was located, may have been a direct result of leaching of arsenic from the senescing fronds back into the soil. Therefore, the actual arsenic uptake by the roots at this depth may have been greater. This further strengthens the notion that fronds should be harvested before they senesce.

Although the majority of the fern roots were located in the top 15 cm of the soil (data not shown), the greatest decrease in arsenic was found in the 15-30 cm depth. This decrease may have resulted from the mobilization of the arsenic by root exudates. However, it is unclear as to the fate of the solubilized arsenic. It may have diffused to the root zone of the fern and subsequently taken up and translocated into the fronds. It is also possible that the solubilized arsenic may have been leached through the soil profile. This possibility was addressed in Chapter 6.

Analysis of plot 2 soil samples revealed a much less dramatic decrease in soil arsenic concentrations over the two-year sampling period (Table 5-6). Overall, the largest decrease was observed in the 0-15 cm depth. This is in much better agreement, than results from plot 1, with the idea that the abundance of fern roots are located in this depth. However, when compared to the outside plot soil samples (Table 5-7) the pattern of change in arsenic concentrations is similar. Therefore, it is not clear if the decreases in soil arsenic concentrations inside plot 2 are due to the presence and harvest of the ferns. The fewer number of soil samples taken outside the plot, their larger variability and the less exact location of these samples year to year may cast some doubt on the accuracy of their values over the seasons. While the outside soil samples suggest that the change in soil arsenic concentrations inside plot 2 may not be due to arsenic uptake by *P. vittata*, the questionable accuracy of the outside plot samples causes the comparison between the samples to be flawed. Therefore, most, but not all, of the change in arsenic concentrations in the soil at 0-15 cm depth of plot 2 from 2002-2004 is likely due to the presence of the fern.

Compared to plot 1, the soil arsenic concentrations of plot 2 had less overall variability. However, the changes from year to year were still not significant. The soil arsenic concentrations from plot 2 appear to be a more accurate testament to the results of phytoextraction using *P. vittata* than those results obtained in plot 1. This conclusion is based on the somewhat smaller variability, better mass balance agreement and the greatest decrease occurring in the 0-15 cm depth, where the majority of the roots are located.

Sequential Arsenic Fractionation

Although small decreases in the non-specifically, specifically and crystalline Fe and Al bound fractions were seen at all sampling depths, the soil arsenic fractionation analysis revealed that the greatest decrease in soil arsenic was from the amorphous Fe and Al bound fraction in the top two sampling depths (Fig. 5-5). This indicates that, to some extent, the fern roots may be able to solubilize arsenic in this fraction, which is generally not readily available to all plants.

Tu et al. (2004) studied the production of root exudates by *P. vittata*. They determined that the roots produced an abundant amount of exudates that could be used to solubilize nutrients in the soil. Compared to a non-arsenic accumulating fern, Boston fern (*Nephrolepsis exaltata* L.), *P. vittata* roots produced two times more dissolved organic carbon. Its roots also produced up to five times more oxalic acid than Boston fern roots.

The higher production of oxalic acid may help to explain the decrease in the amorphous Fe and Al bound fraction, as ammonium oxalate buffer was used to extract this fractionation the laboratory. Oxalic acid root exudates may allow *P. vittata* to solubilize this fraction. The ability of *P. vittata* to solubilize arsenic from more unavailable fractions would be considered an advantage for its use in phytoextraction. This ability would allow the fern to take up arsenic, even as the concentration of available arsenic decreases, thus making it a more efficient hyperaccumulator.

The residual arsenic fraction was the only fraction over all sampling depths that never significantly changed during the course of the experiment. It is hypothesized that the residual arsenic fraction may be too insoluble, even for the root exudates produced by *P. vittata*.

The non-specifically bound arsenic fraction, which is the one of the most available arsenic fraction, decreased significantly between the years 2001 and 2002. The fern probably took up this fraction. Because the non-specifically and specifically bound fractions are most available for uptake, they were expected to exhibit more significant decreases in concentration over time. Because it appears that the less available amorphous Fe and Al bound fraction has decreased in concentration, this fraction may have buffered any significant changes in the concentrations of the non-specifically and specifically bound arsenic fractions.

Aside from the production of root exudates, the availability of arsenic in soils can be affected by soil factors, such as pH and texture (Adriano, 2001). Generally, soil pH is critical because the speciation and subsequent leachability of trace elements is considerably affected. Arsenite absorbs optimally around pH 7.0; however, arsenate adsorbs optimally at pH 4.0 (Pierce and Moore, 1982). Therefore, arsenic is less mobile at lower a pH because most of the arsenic is present as arsenate in (aerobic) soils. There are high concentrations of arsenic-binding species, such as iron and aluminum at low pH (Sposito, 1989). As the pH increases there are fewer protonated sites, which allows arsenic to become more mobile. However, arsenic does have the ability to form a strong association with calcium, allowing it to possibly be retained at higher pH. This may be found under high arsenic concentrations, where arsenic has a secondary preference to calcium over aluminum (Woolson, 1983).

Soil texture affects the soil surface area, with finer textured soils having much more surface area and being more reactive. Therefore, these soils are more likely to retain higher amounts of trace elements than sand or coarse textured soils (Berti and Jacobs,

1996; Chen et al., 1999). Conditions in fine textured soils are also more conducive to organic matter (OM) accumulation and retention. Organic matter increases retention of both cationic and anionic species. The retention occurs by cationic bridging by Al and Fe, leading to anion retention, and the dissociation of edges of organic complexes in response to changes in pH. Also, the presence of iron and aluminum oxides is important in the ability of a soil to retain arsenic (Adriano, 2001; Jacobs et al., 1970; Lumsdon et al., 1984).

Therefore, it would be expected that more coarse-textured soils (i.e., sand) would result in greater arsenic availability or higher arsenic concentrations in the more available fractions. However, despite the neutral pH, low clay and OM contents of the soil at the site, much of the arsenic was associated with amorphous iron and aluminum (Fig. 5-5 A, B and C). This may be due to the presence of clay coatings in the sand. Rhue et al. (1994) showed that some horizons that are highly weathered retain their clay coatings and exhibit high retention. However, those that did not retain their coatings exhibited lower retention of elements.

Mass Balance

Considering mass balances further complicates the determination of the time necessary to remediate a site. The large divergence found between the amounts of arsenic removed using *P. vittata* and the mass balance of arsenic in plot 1 needed to be addressed. Although the roots of the ferns planted at the experimental site were not sampled, the roots of *P. vittata* generally have very low arsenic concentrations when compared to the aboveground biomass (Ma et al., 2001; Tu et al, 2002; Tu and Ma, 2003). Therefore, it is unlikely that any significant amount of the arsenic was stored in

the roots. An estimate of the root arsenic can be calculated using the average root biomass and root arsenic concentrations as determined by Tu et al. (2002) for *P. vittata* grown for 20 wk under greenhouse conditions using the CCA-soil. After 20 wk, average root biomass was about 14 g DW plant^{-1}; and root arsenic concentrations were 200 to 300 mg As kg^{-1}. Therefore, the fern roots would contain approximately 3.5 mg As plant^{-1}, and a total of 1.13 g As for the 324 ferns at the experimental site. This does not account for the missing arsenic. It is possible that the fern root biomass at the experimental site was much larger due to the length of time the ferns were growing. Casual observations of the root masses of few ferns at the site in 2002 revealed that the vast majority of the roots were located in the top 15 cm soil depth. Also, these fern root masses did not appear to be much larger than the root biomass determined by Tu et al. (2002). Regardless, due to the low arsenic concentration of the roots, the total root mass would need to be greater than 4000 kg in order to account for the remaining arsenic that was absent from the mass balance of the experimental site.

Plot 2 mass balance, although not exact, was much closer than that of plot 1. If it is assumed that no real changes in soil arsenic concentrations took place in the 15-30 and 30-60 cm depths over the 2002-2004 seasons, and only arsenic removal from the 0-15 cm depth, where most of the roots are located, was estimated then the mass balance of plot 2 would be in much better agreement. Such an assumption would show that the difference in the total amount of arsenic depleted (108 g) and the amount removed by *P. vittata* (77.6 g) was only 30.4 g, or 28%. It is believed that the results of the harvests, soil arsenic concentrations and the subsequently calculated mass balance of plot 2 are much

more indicative of what is truly taking place as a result of phytoextraction of an arsenic contaminated site using *P. vittata*.

However, it is necessary to address the discrepancies of the mass balances in both plots 1 and 2. The most likely explanation of the mass balance discrepancy is the extreme heterogeneous soil arsenic concentration at the site. Slight variations in sampling places may have resulted in large variations in total soil arsenic concentrations (Fig. 5-4 A, B and C). This idea is supported by the fact that despite sizeable decreases in soil arsenic concentrations, these decreases were never deemed to be significantly lower, due to the substantially large standard deviations. Such deviations should cast considerable doubt on the validity of any mass balance. However, in order to fairly address the topic of the mass balance, additional explanations as to why the calculated mass balance resulted in such gross discrepancies is presented in the following paragraphs.

The production of root exudates by *P. vittata* (Tu et al., 2004) coupled with excess watering could be one possible cause for arsenic removal from the soil (0-60 cm). If the ferns were able to solubilize large quantities of arsenic from the soil, as is suggested by the sequential arsenic fractionation (Fig. 5-5 A, B and C) it is possible that some of that arsenic could be leached before the fern roots are able to take it up. Again considering the arsenic concentrations in the 15-30 cm depth between 2001 and 2002, it would be expected that the root exudates solubilized some arsenic (Fig. 5-5 A, B and C). Thus, it would reason that there would be an increase in soluble arsenic in the soil. Because the majority of the root mass was observed to be in the top 15 cm of the soil, if the arsenic

was leached to lower depths, the ability of the ferns to retrieve and take up this leached arsenic would be compromised.

The movement of arsenic can be directly related to its precipitation, dissolution and complexation in the soil (Kabata-Pendias and Pendias, 2001; Bhattacharya et al., 2002). Likewise, soils with greater clay content tend to have higher arsenic concentration (Galba and Polacek, 1973). Typical Florida soils contain a high amount of sand (Brown et al., 1990); the soil at this site was only 4% clay and 88% sand. Therefore, it is possible that leaching of arsenic occurred at the site. Allinson et al. (2000) studied the leachability of CCA constituents when applied to a soil composed of 81% sand and 9% clay. They found that 10 d after a high concentration of CCA was applied to the soil, the arsenic concentrations in the leachate steadily increased. The leachate arsenic concentration subsequently leveled off after 30 d. Even low doses of CCA resulted in arsenic leaching through the soil. Therefore, coarse soil texture coupled with high root exudates production by the fern roots and daily watering regime, the arsenic present in the soil may have had opportunity to leach. However, the arsenic contamination has been present in the soil for over 50 years.

The lack of correlation found between the variable soil arsenic concentrations throughout the experimental site and arsenic frond concentration or arsenic removed via fronds from the site raises an interesting question pertaining to the physiological capability of a plant. It may be assumed that there exists a direct correlation between the soil arsenic concentration and frond arsenic concentration. Ma et al. (2001) found direct correlations between arsenic concentrations in the soil and ferns. However, because no such correlation was found for *P. vittata* growing in this experimental site, there is a

possibility that there is an arsenic-uptake threshold for this plant. Perhaps only a certain amount of arsenic can be loaded and/or taken up by the fern at a given point in time. If this scenario were true, then it would strengthen the possibility that all of the arsenic that is solubilized by the roots may not be taken up by the fern before it is leached. An arsenic-loading threshold would not mean that the *P. vittata* does not accumulate high amounts of arsenic. However, such a threshold would dictate the amount of time required for the uptake and accumulation of the arsenic in the plant.

It is also possible that the lack of correlation between the total soil arsenic concentrations and the frond biomass harvested or frond arsenic concentrations may be due to lower soil phosphate being available for uptake by the roots. Tu and Ma (2003) found that low to medium concentrations of arsenate in soil increased the phosphate uptake by the fern. The authors attributed an increased biomass production by *P. vittata* to this increased phosphate uptake. They further discussed that soil phosphate applications may be important in order for *P. vittata* to be more efficient in arsenic hyperaccumulation and, therefore, in phytoextraction of arsenic contaminated soils. Although the actual soil phosphorous concentrations were not determined in this study, no soil amendments were added to plot 1 during the first two-year study. It is likely that the soil phosphate concentrations were low, especially at the conclusion of the study. Therefore, the absence of a phosphate fertilizer application in plot 1 may account for lack of the correlation between soil arsenic concentrations and frond biomass, which was found in other studies involving *P. vittata* (Ma et al., 2001).

As previously stated, it was determined that the lower concentration of arsenic in the dead and dying fronds is a result of arsenic leaching from the fronds as they senesce.

It may be assumed that the arsenic that leaches from the fronds as they senesce would be a water-soluble fraction. This means that the arsenic would be in a form that could be easily taken up by the roots of *P. vittata*. However, if the fern has a certain threshold concentration for uptake or the amount of water caused the arsenic to move through the soil too quickly, it is possible that the arsenic would leach down through the soil profile.

Other possible explanations for the discrepancies in the mass balance are arsenic volatilization by *P. vittata* or by microorganisms at the site. If either or both of these possibilities were true, it would be a rather disturbing and dangerous scenario. When arsenic is volatilized from a matrix it may be transformed into arsine gas or trimethylarsine gas (Frakenberger, 1998). *Pteris vittata* has not yet been shown to volatilize arsenic through its fronds. However, some fungal species have been shown to produce trimethylarsine gas (Cox and Alexander, 1973; Frakenberger, 1998). Specifically, *Candida humicola*, *Gliocladium rosem* and *Penicillium* spp. can produce trimethylarsine gas from monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). Further, *Candida humicola* can convert arsenite and arsenate into trimethylarsine gas (Cox and Alexander, 1973). Chilvers and Perterson (1987) estimated that 28,000 t As y^{-1} is added via anthropogenic sources. However, 45,000 t As y^{-1} is the amount of arsenic that is naturally cycled into the atmosphere. It is possible that some or all of these fungal species are present in the soil at the experimental site. Although the presence and the extent of the fungal populations in the soil at the experimental site are unknown, it is doubtful that they constituted as a significant source of arsenic removal from the soil over the course of the study, as the arsenic contamination has been present since 1951. However, this is an aspect that may merit further research.

Estimated Time of Remediation

Using the total soil arsenic data from the 2002-2004 seasons, it is estimated that 14 years would be needed in order to completely remediate the top 15 cm of soil using *P. vittata* to meet the residential site and/or commercial site Florida DEP requirements, 2.1 and 9.3 mg As kg^{-1}, respectively (Table 5-10). This is almost twice the amount of time that was estimated by Salido, et al. (2003). However, their estimates are based on achieving a soil cleanup goal of 40 mg As kg^{-1}.

Remediation estimates were made using the results obtained in experimental plot 2, because it is believed that these more accurately represent the phytoextraction of arsenic by *P. vittata.* Also, only the changes in the top 15 cm of plot 2 were considered for the estimates, because the decreases in soil arsenic concentrations in this depth and this plot seemed to be the most reliable. Remediation of the topsoil may be more of an immediate concern, in terms of their likelihood being accidentally ingested by humans or animals or being taken up by other plants, which may be used as a food source by animals.

The amount of time that would be required to cleanup the site based on the USEPA requirements was also determined (Table 5-10). As previously stated, the USEPA does not have a maximum concentration limit for soil arsenic. Instead, they determine the arsenic remediation goals based on land use, site factors, background arsenic concentrations and the cancer risk level. Therefore, for the purposes of this study the geometric means of the residential and commercial cleanup for a cancer risk factor of 10^{-6} was used to determine the amount of time that would be required to remediate this site. These levels are, as stated in the study by Davis et al. (2001), 23 mg kg^{-1} for residential sites and 50 mg kg^{-1} for commercial sites. Based on these guidelines, the time that would be required for remediation was only shortened by 2 to 5 years, depending on depth and

land use, compared to the amount of time to meet the State of Florida's requirements (Table 5-10).

This remediation estimate may not be accurate because it is based on the total soil arsenic concentrations. Two complications in the remediation estimate arise from using the average total soil arsenic. First, those data were variable, causing the estimate to be rather flawed. Because the average soil arsenic concentrations were so variable, some areas of the site would be fully remediated before 14 years. Similarly, some areas of the plot would need longer than 14 years to meet the State of Florida's requirement for soil arsenic concentrations.

The second complication was that the determined remediation estimates are based on the total arsenic concentration in soils, which does not necessarily determine arsenic phytoavaliability (Adriano, 1986; Lasat, 2002). A small fraction of the soil arsenic is readily available to plants, while the rest is not (Kabata-Pendias and Pendias, 2001), thus resulting in diminished returns, in terms of remediation, as the phytoextraction progressed. Therefore, it is also important to consider the fractions in which arsenic is present in the soil. This fern appears to be able to solubilize some less available forms of arsenic (Fig. 5-5 A, B and C). However, it is unlikely that it could take up all of the arsenic present in the soil (i.e., the arsenic present in the residual fraction).

Based on all of the data, overall, it appears that the use of *P. vittata* in the phytoextraction of arsenic-contaminated soils may be feasible option for remediation. However, its use would be much better suited for sites with low-level arsenic contamination and those that are not in immediate need for remediation, as the time to successfully remediate a site may take several years.

Table 5-10. Estimated time for phytoextraction of plot 2 with *P. vittata.* Estimates are for residential and commercial land uses, based on the State of Florida regulations and USEPA average arsenic cleanup goals.

		Regulating body	
		Florida[a]	USEPA[b]
Land use	**Depth (cm)**	**Remediation time (y)**	
Residential	0-15	14	12
Commercial	0-15	13	8

[a]Based on Florida DEP 2.1 mg kg^{-1} for residential and 9.3 mg kg^{-1} for commercial sites
[b]Based on the geometric mean of cleanup goals at residential (23 mg kg^{-1}) and commercial (50 mg kg^{-1}) sites as stated in Davis et al. (2001).

Estimated Remediation Cost

It is rather difficult to accurately assess the cost of this phytoremediation operation from start to finish. The cost of operation would vary depending on the concentration of the contaminant, total length of the operation and the number of crops grown on the area of concern (USEPA, 2002a). One estimate is that the planting of phytoremediation crops costs $10,000 to $25,000 per acre (Schnoor, 2002). However, this cost estimate does not include preparation, monitoring, design maintenance, etc. Salido et al. (2003) estimated that the total cost to adequately phytoremediate the area in their study (1 ha) would range from $40,000 to $200,000. This estimate varied depending on the cost to purchase *P. vittata*. However, the estimate considers only the purchase price of the ferns. Again, it does not take into consideration labor, maintenance, fertilization, site planning, etc. Using the $5 per fern cost estimate presented in the study by Salido et al. (2003), and the 0.09 m^2 plant spacing that was used in our study, the approximate fern cost would be

$538,000 ha^{-1} or $215,200 acre^{-1}. This cost estimate is much higher than that in the comparison phytoextraction study (Salido et al. 2003) because the planting density of that study was approximately 2.5 times lower than the planting density used in this study.

Salido et al. (2003) state that their cost estimates do not consider the replacement cost of the ferns. Both their estimate and the estimate given above assume a 0% mortality rate of the ferns for the life of the remediation project. However, this is likely an unrealistic assumption, especially considering that *P. vittata* are partial to warmer climates. As previously stated, in plot 1 over the first winter, the ferns had an astonishing 97% mortality. Due to much better care and preparation, only 34% of the ferns perished from the second winter season. Only 2% of the ferns needed to be replaced in plot 2 each spring. However, mortality rate is unlikely to be 0%.

Very little information is available related to the total cost of using phytoextraction to remediate soil from start to finish. This is probably due to the fact that few, if any, sites that have yet to be completely remediated using the phytoextraction technique and to the need for site specific costs. However, a few studies attempt to estimate the cost based on the contaminant. To remediate one acre of soil to a 50 cm depth using phytoextraction, Salt et al. (1995) estimated a cost of $60,000 to $100,000. They estimate that the same level of cleanup using soil excavation would cost more than $400,000. Glass (1999) estimated a range of $25 to $100 per ton of soil remediated using phytoremediation. This cost range is 2 to 5 times lower than that of more conventional remediation techniques, which may cost from $50 to $500 per ton of soil remediated (Cunningham and Ow, 1996).

Suggested Phytoextraction Setup

Based on the results obtained from this field study, phytoextraction using *P. vittata* appears to be more practical for sites with low-level arsenic contamination. If a site is deemed suitable for phytoextraction, the following setup and maintenance recommendations, which are based on the results from this study, may be useful for successful implementation.

Prior to planting ferns at a contaminated site, the soil should be plowed, in order to homogenize it. Homogenization will allow for less variability in soil arsenic concentrations at the site. This will result in a more accurate evaluation of the success of the phytoextraction system from year to year, and it will help to ensure that the entire site will be remediated uniformly. The soil should also be fertilized in order to promote plant growth. Some form of mulch (i.e., black plastic) should be placed onto the soil surface for weed control.

Plant spacing is recommended to be no less than 1 ft^2, but *P. vittata* may be planted as much as 2.5 ft^2 apart. Although *P. vittata* is tolerant to sun, it appears to grow better when in at least partial shade. Therefore, if the site is not shaded, it is recommended that some form of shade be added (i.e., a constructed cover or inter-planting with trees). The watering regime will be highly dependent on the soil and the climate of the region. For plot 2 experimental site in this study, approximately 30 min of spray irrigation was performed every second day.

Fertilizer (15-5-15) should be applied each year, based on a rate of 100 lb N yr^{-1}, in two split applications. Single harvests appear to yield the greatest amount of arsenic removal from the soil, via the ferns. Therefore, it is recommended that only one harvest be conducted at the end of each year to a height of 5 to 15 cm.

Over wintering of the ferns will also depend on the climate/region. In any case, it is recommended that the ferns be covered with a form of shade cloth in order to help them survive the winter. If *P. vittata* is to be used in areas with more severe winters (i.e., Midwestern and Northeastern United States) it is possible that many of the ferns may need to be replaced after the winter season.

Yearly soil sampling should be conducted, in order to assess the progress of the remediation. Sampling should be conducted systematically with profile samples to a depth of at least 60 cm. However, the depth of sampling will be dependent on soil and site factors.

Lastly, it may also be advisable for the soil to be plowed or homogenized during the course of the remediation as the soil arsenic levels decrease. Plowing would bring soil from the subsurface to the surface. This would allow *P. vittata* to extract arsenic from depths its roots are not able to access.

CHAPTER 6
EFFECT OF *Pteris vittata* L. ON ARSENIC LEACHING AND ITS POTENTIAL FOR THE DEVELOPMENT OF A NOVEL PHYTOREMEDIATION METHOD

Introduction

Arsenic contamination of soil can arise through a variety of sources. Therefore, it is important to address the soil contamination and target it for appropriate remediation to prevent possible impacts on the ecosystem. Arsenic is often used in chromated copper arsenate (CCA) wood preservatives, pesticides and glass manufacturing. However, the trend of arsenic use in industrial production and in agriculture has decreased (Adriano, 1986).

Data regarding study on phytoextraction of an arsenic-contaminated site using *P. vittata* showed discrepancies in the arsenic mass balance at the site (Chapter 5). Average frond arsenic concentration was approximately 4500 mg As kg^{-1}. Using *P. vittata* at the site, 26.4 g of arsenic was removed over a two-year period in plot 1, and 77.6 g were removed in plot 2 over two years (Chapter 5).

Soil arsenic concentrations at three depths (0-15 cm, 15-30 cm and 30-60 cm) were determined for the site. Total arsenic analysis of the soil samples showed an arsenic depletion of 12-43% over a two years in plot 1 and 14% over two years in plot 2. However, when a mass balance of plot 1 was performed it was shown that 1444 g of arsenic was removed from the site. This implies that only 1.3% of the arsenic removed from the soil was through removal by ferns. The mass balance of plot 2 did not show as large of a difference.

From the previously mentioned phytoremediation study, it was hypothesized that a combination of watering and solubilization of arsenic by exudates produced from *P. vittata* roots may have caused leaching of soil arsenic. In another study (Tu et al., 2004) *P. vittata* were shown to produce a high amount of root exudates, strengthening this hypothesis. However, soil arsenic fractionation analysis showed no increase in the non-specifically bound or specifically bound arsenic fractions. It is thought that these arsenic fractions may leach from the soil, eliminating any substantial changes. It was essential to determine if *P. vittata* increases the leachability of arsenic in soil, as this could pose a threat to groundwater if employed in the field.

However, if there is a promotion of leaching, it may be able to be harnessed for remediation as well. It is proposed that there exists the potential for development of a new *ex-situ* soil arsenic remediation method that is a variation of phytoremediation or phytoextraction. This new remediation technology, *phytoleaching*, would involve the excavation removal of arsenic-contaminated soil. This is essentially a combination of phytoextraction and soil washing. Arsenic in the soil would be removed through uptake by *P. vittata* and leaching from the soil. The ferns would remove the arsenic through uptake and storage in fronds, which would subsequently be harvested. Using water or chemical solution, arsenic may be leached from the soil and collected.

Therefore, the development of a phytoleaching system would harness both arsenic-removing processes of *P. vittata*, specifically, hyperaccumulation and solubilization, if it is occurring. The arsenic would be removed from the soil using *P. vittata* directly through the removal of the arsenic by harvesting fern fronds that have hyperaccumulated

arsenic. Arsenic would also be removed by *P. vittata* through the solubilization of arsenic in the soil and its subsequent leachate, which would be collected.

If properly developed and adequately functioning, this soil remediation technique could become a viable arsenic treatment option in the future. Possible advantages of this newly proposed remediation treatment are: 1). decreased time of successful remediation compared to phytoextraction alone; 2). reduced amount of contaminated material requiring hazardous disposal compared to excavation; 3). possible extension of regions where *P. vittata* can be used for remediation; and 4). easier plant maintenance, control and monitoring compared to traditional phytoremediation/phytoextraction. Some possible limitations are: 1). the cost for and need of soil excavation; 2). possible limitation to only one contaminant; and 3). depending on climate, the remediation effectiveness may be limited by temperature (i.e., freezing).

The objectives of this study were:1). to determine if *P. vittata* promote leaching of arsenic in soil; and 2). to develop a phytoleaching method/system that can be used for remediation of arsenic-contaminated soil.

Materials and Methods

Overview of Proposed Phytoleaching System

A schematic diagram overview of the proposed phytoleaching system is shown in Figure 6-1. Arsenic-contaminated soil was used for remediation by phytoleaching. The soil was contained and a leachate collection system installed underneath the soil. Each pot contained 6 kg of CCA-contaminated soil. One *P. vittata* was planted per pot in the contaminated soil. Soil and ferns were watered regularly (with or without chemical amendments) to encourage arsenic leaching from soil and uptake by *P. vittata*. The

leachate was collected and removed after each leaching event and preserved for subsequent analysis. Fern fronds were harvested and removed at the conclusion of the experiment.

Soil

The soil was obtained from a field site that was previously used to pressure treat lumber with CCA from 1951-1962. (Woodward-Clyde, 1992). The soil collected from the site is classified as a loamy, siliceous, hyperthermic Grossarenic Paleudult. The soil particle size distribution is 88% sand, 8% silt and 4 % clay. Previous analyses showed the soil to have a pH range of 7.4 to 7.6 and organic matter content of 0.5 to 0.8% (Komar, 1999). Soil was homogenized prior to packing it into the pots.

Treatments

Each treatment was applied to soil with ferns and to soil without ferns, in order to determine the role of *P. vittata* on arsenic leaching in the system. Prior to initiation of leaching, ferns were allowed four weeks to acclimate to the contaminated soil in order to minimize stress or death of ferns.

The watering intensity and frequency treatments were 1 pore volume, 1 time week^{-1} and 1 pore volume, 2 times week^{-1}. A pore volume was approximately equal to 1500 ml for each pot. Leaching was performed for four weeks, for a total of four and eight leaching events for the 1 time week^{-1} and 2 times week^{-1} frequency treatments, respectively.

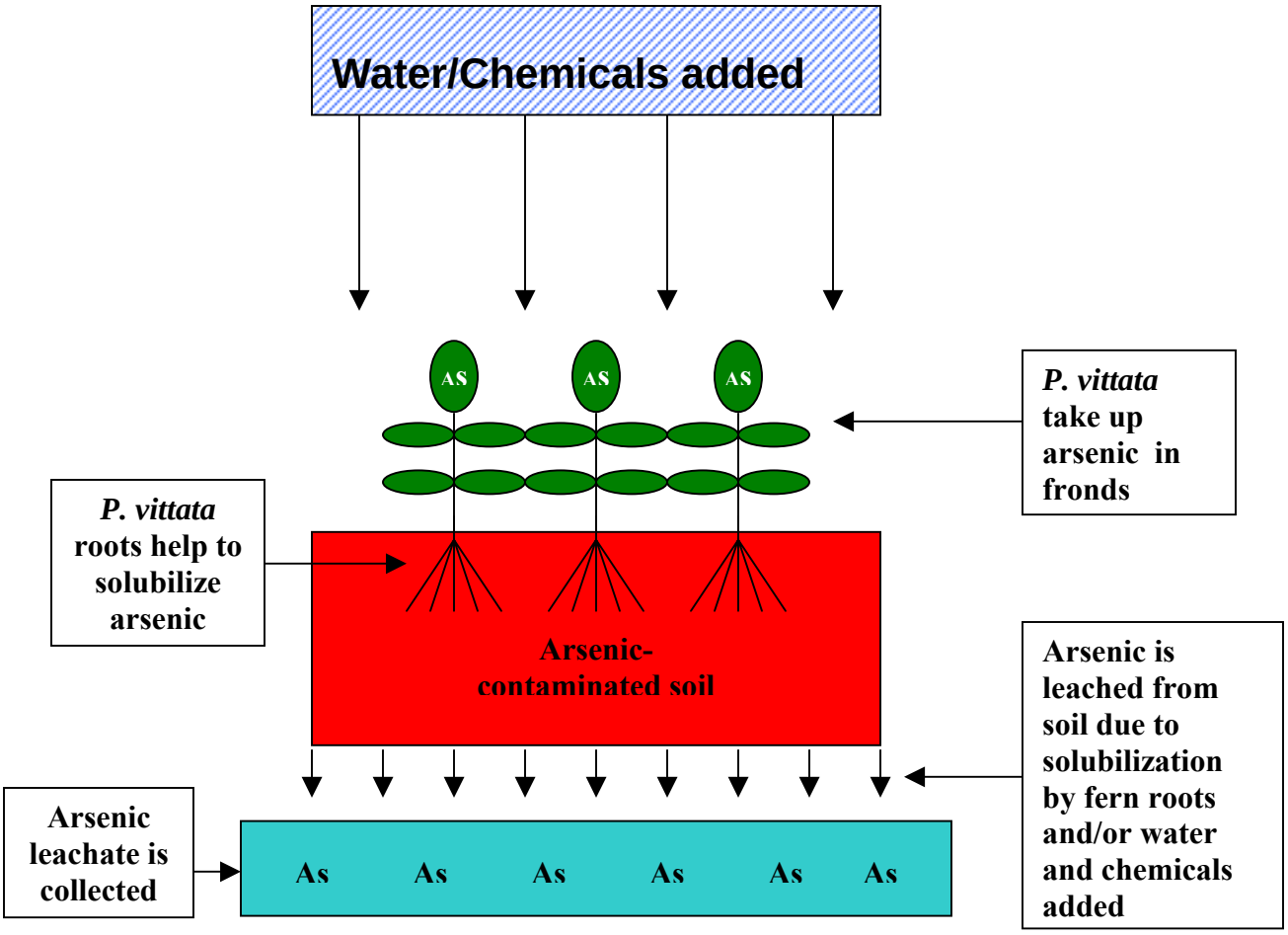


Figure 6-1. Schematic diagram of the phytoleaching system.

The chemical treatments were: 1). water only/no chemical additions; 2). 0.01 M ammonium phosphate; and 3). 0.01 M Ammonium oxalate buffer, pH 5.0. The no chemical treatment was used to determine if *P. vittata* roots produce enough exudates to cause arsenic leaching. Ammonium phosphate is used (at 0.05 M strength) in the sequential fractionation by Wenzel (2001) to extract the non-specifically-bound arsenic fraction. It was assumed that this should extract water-soluble arsenic as well. Also, since phosphate and arsenate are chemical analogues, the phosphate may help displace arsenate in the soil, causing it to leach. The 0.01 M strength solution was used to

minimize salts in the soil, as *P. vittata* is sensitive to salts. A 0.2 M ammonium oxalate buffer, pH 3.25, is used to extract the amorphous Fe and Al oxide bound arsenic in the sequential fractionation by Wenzel (2001). In the Chapter 5, this fraction was shown to constitute the highest percentage of total soil arsenic. Also, oxalic acid is a compound that can be used to remove chromium and arsenate from pressure-treated lumber. This is due to its ability to solubilize both chromium and arsenate (Clausen, 2000). For this experiment, a lower concentration (0.01 M) was used, again due to the sensitivity of *P. vittata* to salts. Also, the pH of the buffer was adjusted to 5.0, because the fern prefers an alkaline soil environment.

Chemical treatments were prepared fresh daily using deionized water. They were applied to each pot evenly and slowly in six- 250 ml increments. Each increment was added only after the previous one had adsorbed into the soil.

Fern, Soil and Leachate Analyses

After four weeks, the fronds of *P. vittata* were harvested at soil level, dried for 24 h at 65^{o} C, and ground in a Wiley Mill to pass through a 1 mm-mesh screen. The ground samples (0.25 g) were subjected to hot block (Environmental Express, Ventura, CA) digestion using USEPA Method 3051 for arsenic analysis. The digested plant samples were analyzed for total arsenic using graphite furnace atomic absorption spectroscopy (GFAAS) (Perkin Elmer SIMMA 6000, Perkin-Elmer Corp., Norwalk, CT).

Total soil arsenic concentrations were determined before and after the experiment. Five soil cores were randomly taken from each pot. Soil samples were air dried and subsequently digested via hot block using EPA method 3051. The acid-digestated soil samples were analyzed for total arsenic concentration by GFAAS.

Leachate samples were taken after each leaching event, and the total volume was determined. The leachate samples were filtered using Whatman 2 filter paper to remove any soil and debris. The total arsenic concentrations were determined using GFAAS.

Experimental Design and Statistical Analysis

The experiment was completely randomized block with a 2 (watering intensity/frequency) x 3 (chemical addition) x 2 (fern treatment) factorial with three replications. All data were analyzed using the General Linear Model (GLM) with the Statistical Analysis System (SAS Institute, 2001).

Results

Leachate

Overall, three times more arsenic was leached from pots without ferns (19 g), compared to those pots containing ferns (6 g). The arsenic concentration in the leachate of the non-fern treatments was significantly greater ($P < 0.0001$) than the fern treatments. The non-fern treatment leachates had 1.8 times higher arsenic concentration compared to those treatments with *P. vittata*.

The average arsenic concentration and total amount of arsenic removed in the leachate using the ammonium phosphate chemical treatments were significantly greater ($P < 0.01$) than either the ammonium oxalate buffer or water treatments, regardless of the fern treatment. The non-fern ammonium phosphate treatment, leached two times per week, yielded the greatest arsenic removal via leachate (Fig. 6-2). It yielded about 2.5 times more arsenic in the leachate than did its fern treatment counterpart. The no chemical and ammonium oxalate buffer treatments did show arsenic in the leachate.

However, the amount was far less than the phosphate treatments (with the exception of the fern + ammonium phosphate + 1 time per week treatment). This difference resulted from the average arsenic concentration in the leachate of the ammonium phosphate treatments being 11 to 27 times greater than the other two chemical treatments. Although both the ammonium oxalate buffer and no chemical treatments yielded leached arsenic, the ammonium oxalate treatments yielded slightly, but not significantly, more arsenic than their no chemical counterparts.

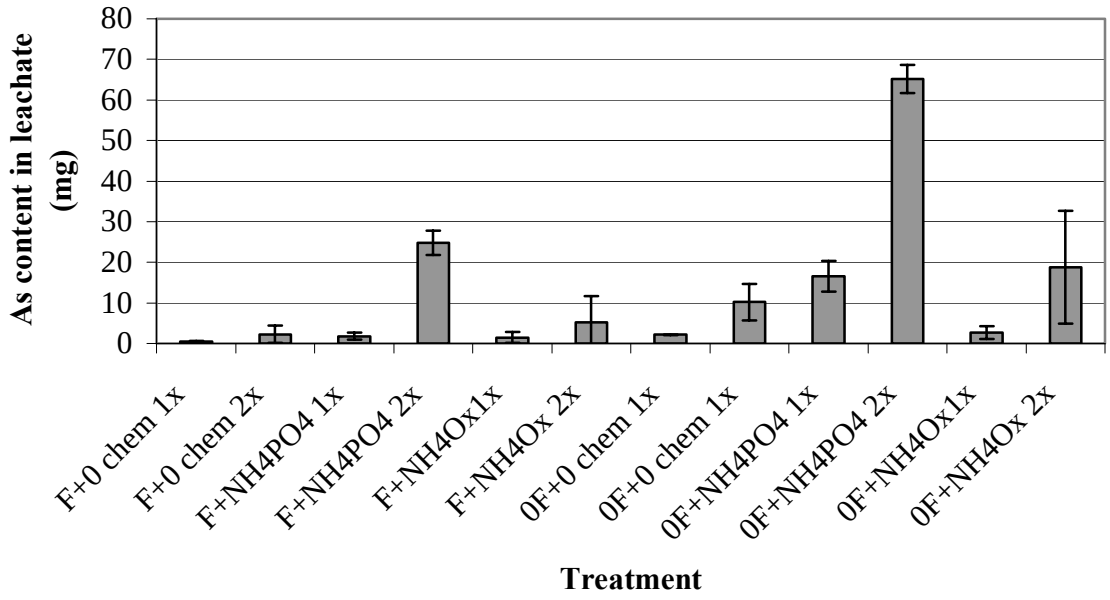


Figure 6-2. Total amount of arsenic removed from the soil through leaching for each chemical and frequency treatment (F, with fern and 0F, without fern). Values represent means ± std. dev. (n = 3).

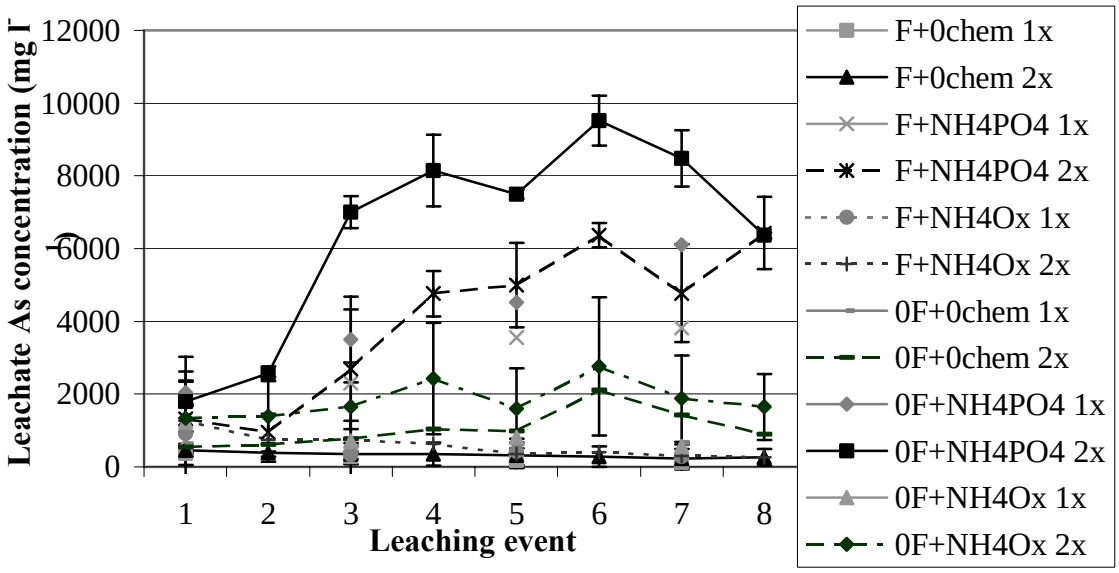


Figure 6-3. Leachate arsenic concentrations for every frequency, chemical and fern treatment of each leaching event (F, with fern and 0F, without fern). Values represent means ± std. dev. (n = 3).

All of the ammonium phosphate chemical addition treatments showed a trend of increasing arsenic concentration in the leachate as the experiment progressed (Fig. 6-3). However, the non-fern, two leaching events per week of ammonium oxalate buffer and water only treatments increased leachate arsenic concentrations slightly during the first half of the experiment and then decreased slightly. The leachate concentrations of the other no chemical addition and ammonium oxalate buffer treatments were constant or decreased slightly over the course of the experiment.

Ferns

Pteris vittata frond biomass for the 0.01 M ammonium phosphate chemical treatment (21 g) was significantly greater ($P < 0.05$) compared to the other chemical treatments (16 g). However, no interactions were found between the chemical treatments and frequency treatments; therefore, overall, frond biomass was the same (Table 6-1).

Although ferns treated with ammonium phosphate had greater biomass, this did not translate into higher frond arsenic concentration or amount of arsenic removed from the soil through fern uptake and storage. The ferns treated with no chemicals (water only) had significantly greater ($P < 0.0001$) frond arsenic concentrations (2060 mg As kg^{-1} dw). This resulted in a significantly greater ($P < 0.01$) amount of arsenic removed from the soil (33 mg) via uptake by the ferns in the no chemical treatments. Again, there were no interactions found between the chemical and watering frequency treatments.

Table 6-1. The effects of chemical treatment and leaching frequency on frond biomass, frond arsenic concentration and the amount of arsenic removed from the arsenic-contaminated soil. Values represent means ± std. dev. (n= 3)

Treatment	Frequency (per week)	Frond biomass (g dw)	Frond As concentration (mg kg^{-1} dw)	As removed by fern (mg)
No chemicals	**1**	16 ± 1	2448 ± 291	38 ± 2
	2	16 ± 1	1671 ± 263	27 ± 4
0.01 M ammonium phosphate	**1**	22 ± 4	1204 ± 404	25 ± 5
	2	19 ± 3	1017 ± 142	19 ± 2
0.01 M ammonium oxalate buffer, pH 5.0	**1**	16 ± 1	1454 ± 72	24 ± 3
	2	17 ± 4	1304 ± 247	22 ± 6

Overall, significantly more ($P < 0.01$) arsenic was removed from the soil when ferns were present. However, the non-fern, ammonium phosphate two leachings per week treatment resulted in 1.5 to 2.5 times more arsenic removed from the soil than any treatment containing a fern (Fig. 6-4).

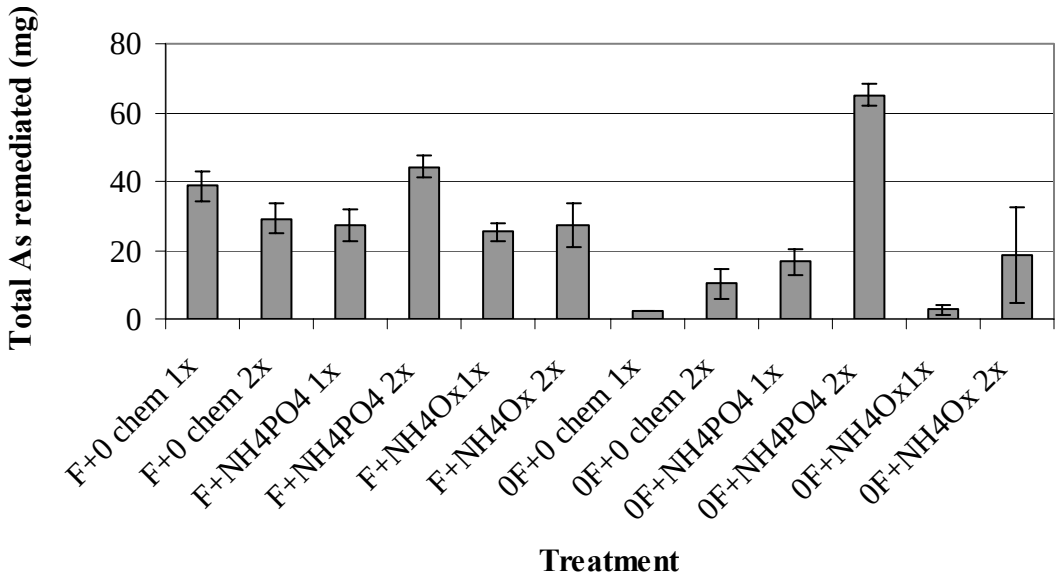


Figure 6-4. Total arsenic removed from the arsenic-contaminated soil via the phytoleaching (leaching and fern) system (F, with fern and 0F, without fern). Values represent means ± std. dev. (n = 3)

Soil

The average soil arsenic concentration for all treatments before the experiment was 140 mg As kg^{-1} soil. Post-experimental soil arsenic concentrations were rather variable, ranging from 116 mg As kg^{-1} soil to 191 mg As kg^{-1} soil (Fig. 6-5).

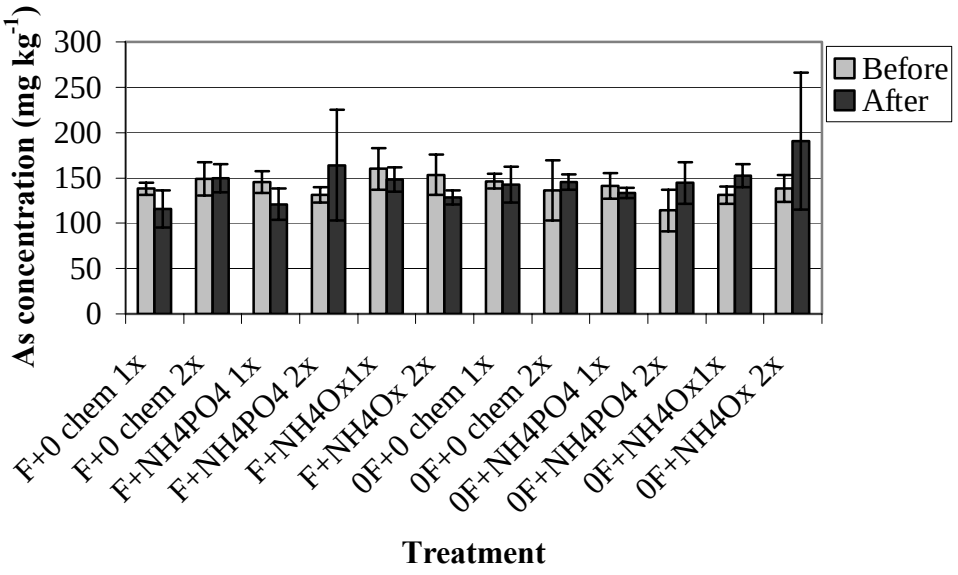


Figure 6-5. Total soil arsenic concentrations before and after the leaching treatments (F, with fern and 0F, without fern). Values represent means ± std. dev. (n = 3)

Discussion

The original hypothesis suggested that more arsenic would be leached from those treatments containing ferns. The hypothesis was based on the fact that the ferns have high production of roots exudates, specifically oxalic and phytic acids, which help to solubilize arsenic (Tu et al., 2004). However, this was not the case, as more arsenic was leached from pots without ferns. Therefore, *P. vittata* does not promote arsenic leaching from soil.

The greater arsenic in the leachate of non-fern treatments is likely due to the overall volume of leachate collected from the pots. Soils with ferns dried much more in between leaching events, due to water uptake and transpiration by the plant, compared to those soils with no ferns. This was especially true for chemical treatments with ferns that received only one leaching event per week. Because of this, significantly less ($P <$

0.0001) volume of leachate was collected, thus less arsenic removed from the soil. However, the difference in volume between the non-fern and fern treatments was only 1.5 times (data not shown).

The phosphate, especially in the first half of the experiment, may cause more arsenic to leach from the soil as the phosphorus remains in the soil. Therefore, subsequent leachings remove arsenic that was made available from the one or two previous leachings. The other chemical addition treatments may be simply removing what arsenic is already available in the soil. With subsequent leachings, this pool becomes smaller; therefore, the arsenic concentration in the leachates of these treatments is also lower.

It was expected that the treatments leached two times per week would yield approximately two times greater arsenic in the leachate than those same treatments leached once per week. However, this was not the case. The leachate of those treatments that were leached two times per week contained 3.5 to 14 times more arsenic (Fig. 6-2). This is due to both slightly higher arsenic concentrations (1.2 to 2.2 times greater) and total volume leached (2.5 for the non-fern treatments and 3.2 to 6.2 for the fern treatments) (data not shown). The largest discrepancy in the total arsenic remediated in the leachate was found in the fern treatments.

It would not be surprising for greater biomass to result from the ammonium phosphate chemical treatments, as the added phosphate would enhance the fern's growth. However, it is unclear as to why no interactions were found. Although care was taken to choose ferns of equal size prior to the experiment, perhaps there was a difference in the starting biomass. This could have translated into the differences seen at the conclusion of

the experiment. It is suggested that a longer experimental leaching period (8 weeks versus 4 weeks) in addition to accounting for the pre-experimental fern biomass may help to better identify any possible biomass differences. However, the four-week fern acclimation period seemed more than adequate, as all of the ferns appeared healthy throughout the experiment.

The lower arsenic concentrations in the fern fronds treated with ammonium phosphate or ammonium oxalate could be the result of either directly or indirectly decreasing arsenic uptake by the fern roots. In the case of ammonium phosphate, it is likely that *P. vittata* roots preferentially took up the phosphate. It has been suggested that ferns take up arsenate via the phosphate transport system (Wang et al., 2002). In this experiment, the phosphate was readily available for uptake, as it was soluble. In addition, the ammonium phosphate treatment resulted in much greater arsenic concentrations in the leachate, possibly decreasing the amount of soluble arsenic immediately available for uptake by fern roots.

It is doubtful that ammonium oxalate directly decreased arsenic uptake through competition for root uptake. However, it is not clear as to why this treatment did result in significantly lower frond arsenic concentrations versus the water only treatments. Although there was a slightly higher concentration of arsenic in the ammonium oxalate leachate compared to the water only treatments, the difference was not significant. Therefore, it cannot be stated that the difference in frond arsenic concentrations is due to the lower amount of soluble arsenic available to the roots. However, perhaps through indirect effects on fern growth (i.e., root growth) due to the addition of ammonium oxalate decreases in arsenic uptake could have occurred. Also, the lower pH of the

ammonium oxalate solution may have affected the fern growth. It is suggested that root biomass be accounted for in any future experiments to help determine its role.

The data suggest that the addition of a fern into the soil system is more effective in removing arsenic from the soil than leaching alone. However, it appears necessary to adjust for the lower volume of leaching in order to normalize for the amount of arsenic leached or made available from the soil when ferns are present. This normalization will allow for better comparison between the fern and non-fern treatments.

Soils in the fern treatments tended to be drier than those without ferns, especially the once per week treatments. However, the soils of all the twice per week leachings stayed wetter than their once per week counterparts. Allowing the water and/or chemicals to remain in the soil for a longer period of time, greater exchange of arsenic soil sites may have take place. Therefore, in the subsequent leachings, the arsenic was more easily leached from the soil. It was also observed that the solutions, when applied to the non-fern treatments, did not infiltrate the soil as quickly as it did in the fern treatments. The fern drying out the soil quicker and possibly making or keeping the soil less compact likely caused the slower infiltration. This could have had a significant impact on the amount of arsenic leached from the soil by allowing for greater exchange of arsenic from the soil.

In soil, the movement of arsenic is often directly related to its precipitation, dissolution and complexation (Kabata-Pendias and Pendias, 2001; Bhattacharya et al., 2002). Soils with greater clay content tend to have higher arsenic concentration (Galba and Polacek, 1973), however, in Florida a typical soil contains a high amount of sand (Brown et al., 1990). The soil used for this experiment was 88% sand and 4% clay.

Allinson et al. (2000) examined the leachability of CCA constituents in a soil composed of 81% sand and 9% clay. They found that 10 d after a high concentration of CCA was applied to the soil arsenic concentrations in the leachate steadily rose, but subsequently leveled off after 30 d. Even low doses of CCA resulted in arsenic leaching through the soil.

Therefore, the difference in volume was not the only factor contributing to the large difference in the amount of arsenic remediated in the leachate. Again, this difference in leachate arsenic concentration is in opposition to the original hypothesis. But the roots of the ferns likely take up a portion of the arsenic that becomes available as a result of the leaching treatments. It is not known how much this portion constitutes from the entire pool of available arsenic. Therefore, although the total amount of arsenic leached was not greater in the treatments containing ferns, the total amount of arsenic made available in the soil due to the chemical treatments may be greater.

The soil data indicated that there was a decrease in soil arsenic concentration in the soil of some treatments, while other treatments resulted in an increase in soil arsenic concentrations, although not significantly. These results were obtained despite the fact that arsenic was leached and/or removed via the fern from every treatment. Although the soils were mixed prior to initiation of treatments, the soils may still have been heterogeneous. The leaching events may have caused arsenic to concentrate in certain areas of the soil (i.e., preferential flow). Also, the soil likely requires a much higher leaching volume and/or leachate arsenic concentration before any real decrease in soil arsenic concentration is observed.

Most importantly, the results from this study indicate that *P. vittata* roots do not cause or promote significant leaching of arsenic in soil. This information was vital for determination of the missing arsenic in the mass balance of the arsenic phytoextraction pilot site (Chapter 5). Overall, the method of phytoleaching may be efficient and useful in certain situations, such as highly contaminated site, sites where arsenic is a threat to groundwater or sites where *P. vittata* cannot be successfully implemented *in situ* due to site or growing season restrictions. However this method requires further study and refinement before any serious implementation.

Future Directions

1). Because the ammonium oxalate buffer chemical additions showed little promise for use in this potential remediation technique, it is suggested that it be excluded from any future experiments. The water only treatments should remain and serve as a control for comparison with the ammonium phosphate treatments.

2). The fern acclimatization period of four weeks was more than sufficient for the ferns. However, the four week leaching period did not seem lengthy enough to elicit significant change in the soil arsenic concentrations of the various treatments. Therefore, the leaching period should be extended to a minimum of eight weeks. This longer leaching period will allow for more definitive differences in all factors (plant, leachate and soil) between the treatments.

3). It seems necessary to estimate the fern frond and root biomass prior to the start of the experiments. Also, accounting for the post-experimental root biomass should be considered to determine the effects of the treatments on their growth, as well as the frond growth.

4). Better care needs to be exercised when packing the pots with contaminated soil to ensure even packing of all pots. As such, pots should be packed to a bulk density between 1.1 and 1.4 Mg m^{-3}. This will allow for more even infiltration of water between the treatments.

5). The discrepancies in leaching volumes recovered between the non-fern and fern treatments needs to be eliminated and/or adjusted in order to better compare these treatments. One suggestion is to plant a non-arsenic hyperaccumulating plant species in the non-fern treatments (i.e., *Pteris ensiformis* or *Nephrolepis exaltata*). The roots of these plants may allow for better infiltration and may also cause the soils to dry out enough for comparison between treatments. Another suggestion is to estimate the amount of water lost in the fern treatments to transpiration (assuming that evaporation between the fern and non-fern treatments is equal). This can be accomplished by weighing the pots. It would have to be assumed that water lost to transpiration would have had equal arsenic concentration as the leachate collected from the pot. This assumption is may or may not be correct. It would not, however, adjust for the retention time of solution in the non-fern versus fern treatments. This may be a problem, because it is suggested that the retention time may significantly impact the amount of arsenic dissolved from the soil. However, it is hoped that with more attention to the packing of columns, such differences in solution infiltration would be much smaller.

6). Small experiments concentrating on the root exudates (i.e., phytic acid) identified by Tu et al. (2004) could be performed to understand how to better enhance leaching of arsenic from the soil. The fern root exudates may be more efficient in promoting dissolution of arsenic from the soil, resulting in greater leaching and/or fern

uptake of arsenic. This may allow for more arsenic to be leached in a smaller volume of solution, decreasing the amount of waste for disposal. In the same respect, various concentrations of ammonium phosphate can be examined to promote greater leaching of arsenic, especially with a smaller volume of solution. However, it necessary to mention that increasing phosphate concentration may decrease the amount of arsenic taken up by the fern.

CHAPTER 7
CONCLUSIONS

The practical use of *P. vittata* in the remediation of soils is vital. However, attempting to understand its ability to hyperaccumulate arsenic is also important in order to fully understand its capabilities. Elucidating the transport of arsenic in the fern allowed some insight into its efficiency. The data from the xylem sap study indicated that arsenic is stored mostly as arsenite in the leaflets of the fern and is predominately transported as arsenate. However, if arsenic was supplied in as MMA or DMA it was transported mainly in that form. This suggests that the majority of the arsenic reduction and demethylation takes place in the fern pinnae. When supplied as arsenate or arsenite, *P. vittata* did not clearly transport the arsenic in only that one form, but it appears to favor transporting arsenic in the form of arsenate over arsenite. Because arsenic in the fronds of *P. vittata* is almost exclusively arsenite, the demethlylation and arsenate reduction is taking place in the fronds, which may aid in the fern's arsenic hyperaccumulating efficiency.

The transport of arsenic in the xylem sap did not affect the phosphorus transport in the xylem sap. Therefore, arsenic may be simply moving with the transpiration stream of the plant, and is not competing for loading into the xylem sap. Regardless, the fact that arsenic uptake, at least at the concentrations evaluated in this study, did not affect phosphorus transport may be a key in the ability of *P vittata* to hyperaccumulate arsenic. Because arsenate and phosphate are chemical analogues, they may compete chemically. The ability of *P. vittata* to allow arsenic transport without interfering with phosphate

transport may be important in allowing for the plant to hyperaccumulate arsenic without sacrificing its health.

Once arsenic is present in the fronds of *P. vittata*, its presence can affect antioxidant enzymes. Because of the ability of *P. vittata* to maintain its GSH:GSSG ratio when exposed to arsenic, it was initially thought that GR may play an important role in arsenic hyperaccumulation in the fern. However, GR was not induced in *P. vittata* upon exposure to arsenic, nor was it directly inhibited or activated by the presence of arsenic. In addition its kinetics of GR were similar to the arsenic non-hyperaccumulator, *P. ensiformis*. Therefore, GR did not appear to play an important role in the arsenic-hyperaccumulation ability of *P. vittata*. However, investigation into the GSH synthesizing enzymes, gamma-glutamyl cysteinyl synthetase and glutathione synthetase, may yield interesting results, as GSH is an important compound in the detoxification of arsenite in *P. vittata.*

The results suggested that CAT may be vital in arsenic-hyperaccumulation. Catalase was not only induced in the fronds of *P. vittata*, but it was also activated, up to 300% of the activity of the control, in spectroscopic assays, upon the addition of sodium arsenate. Although a similar activation pattern was observed in the non-hyperaccumulator, *P. ensiformis,* the percentage increase of the activation was not as high as in *P. vittata.* The activation of CAT at two different concentrations of sodium arsenate may be the result of different CAT isozymes present in the fern. It is hypothesized that the induction serves to prepare *P. vittata* for the increase in production of ROS species that will result from arsenate reduction that is occurring in the fronds.

This preparation may allow the fern mediate the stresses caused by the hyperaccumulation of arsenic.

Arsenic contamination of soils is a concern worldwide. Most of these concerns arise from the health problems associated with arsenic in the environment. Because of this, arsenic contaminated soils often require remediation. Although numerous remediation options currently exist, the evaluation of the arsenic-hyperaccumulating fern, *P. vittata*, for use in the phytoremediation of arsenic-contaminated soils is an important aspect to developing effective, efficient and economical remediation options.

Based on the data obtained from the phytoextraction pilot studies, *P. vittata* does exhibit the ability to accumulate and remove arsenic from arsenic-contaminated sites. Results from the sequential arsenic fractionation indicate that *P. vittata* may be able to take up arsenic that may be somewhat unavailable to many other plants. This attribute can contribute to its efficiency of phytoextraction of arsenic contaminated soils. However, the data from the field studies suggested that the use of this fern for phytoremediation may be better suited to sites with low-level arsenic contamination, as the required remediation time can be extensive. Although this may be a cost-effective alternative to more traditional remediation methods, all factors must be considered to determine the most suitable method for each site.

If *P. vittata* is used in the phytoextraction of an arsenic-contaminated soil, results suggest that the regular harvesting of senescing fronds, although possibly beneficial, may not be effective enough to merit the time, labor and expense of their removal. However, a single harvest at the conclusion of the growing season will yield the greatest results from the ferns in terms of biomass and arsenic removal from the site. In addition, site

conditions, such as shading and protection from frost injury, need to be considered in order to maximize *P. vittata* productivity. Further investigations into determining the most appropriate agronomic practices are also needed to enhance plant growth and arsenic uptake in order to obtain a maximum soil arsenic removal by this fern.

The results of the phytoleaching experiment indicated that, most importantly, *P. vittata* does not promote arsenic leaching in soils. This was initially a concern because of the discrepancy of the mass balance calculations from data collected in the phytoextraction field study. Secondly, the use of phytoleaching as a remediation method for arsenic-contaminated soils exhibited some potential for success. It may aid in the use of *P. vittata* in situations that may not be suited for phytoextraction, such as those with high arsenic concentrations, threats of contamination to groundwater and areas that have a shorter growing season. However, these preliminary data are certainly not conclusive, and they cannot be used to make definite recommendations for the employment of this method. Based on the data obtained, refinement of the treatments and methods used is required.

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BIOGRAPHICAL SKETCH

Gina M. Kertulis-Tartar, the youngest of four children, was born on January 2, 1975, in Mechanicsburg, Pennsylvania, to Mr. and Mrs. Anthony S. and Barbara E. Kertulis, Sr. After receiving her high school diploma in 1993 from East Pennsboro Area High School in Enola, Pennsylvania, Gina attended Harrisburg Area Community College in Harrisburg, Pennsylvania.

In 1995, Gina transferred to the Pennsylvania State University, University Park, Pennsylvania. It was there that she received a Bachelor of Science degree in soil science and a Bachelor of Science degree in agronomy in 1998. During that time, she was employed as a science intern for the Pennsylvania Department of Transportation and as a crop scout for the Pennsylvania Crop Management Association of Franklin County.

In 2001, Gina received her Master of Science degree in agronomy from West Virginia University, Morgantown, West Virginia. During that time, she spent two years as a graduate teaching assistant in the laboratory of Principles Plant Science course. Her master's thesis title was "Effects of Nitrogen and Cutting Management on Root Growth and Productivity of a Kentucky Bluegrass and White Clover Pasture."

Immediately after completing her master's degree, Gina attended the University of Florida, Gainesville, Florida. Employed as a graduate research assistant, she completed this current work on the investigation of *Pteris vittata* L. It was also during this time that she married her husband, Kenneth T. Tartar.