

IMPROVED ARSENIC ACCUMULATION IN *PTERIS VITTATA* AND ITS UNIQUE
ABILITY TO ACQUIRE PHOSPHORUS

By

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The fern, *Pteris vittata* L. (Chinese brake), is capable of accumulating arsenic (As) and storing it in the aboveground biomass. The physiological aspects pertaining to the connection between As and phosphorus (P) acquisition were studied along with plant mediated effects on soil As mobilization.

The role of phytase enzymes in As tolerance and P acquisition by *P. vittata* were studied. Enzyme-mediated hydrolysis of phytate in *P. vittata* extracts was not inhibited by As at 5 mM or by heating at 100°C for 10 min. Phytase in root exudates of *P. vittata* allowed its growth on media amended with phytate as the sole source of P. Phosphorus in *P. vittata* tissue grown on phytate was equivalent to control plants with an increase in As uptake. In three soils, *P. vittata* phytase retained significantly more activity compared to phytase from wheat and an As-sensitive fern, *Pteris ensiformis*.

Since P and As compete for uptake by *P. vittata* roots, we hypothesized that the physiological responses of plant roots in a P-limiting environment would increase As uptake. We evaluated this by growing *P. vittata* in three As-contaminated soils amended with phosphate rock (PR) over two years. Phosphate rock, which provides a

long-term supply of sparingly-soluble P, did not limit plant growth compared to the fertilized control. To our knowledge, frond weights in this study are the largest reported and the first to increase over subsequent harvests. Frond As concentrations grown increased in PR amended soil, allowing for significantly more As removal than the control.

Soil arsenic sorption during *P. vittata* growth was examined by sequentially fractionating soil As by decreasing availability: soluble, exchangeable, amorphous, crystalline, and residual. Soluble As declined slightly with no change in the exchangeable fraction. The amorphously bound fraction in all three soils accounted for the majority of As loss. Since plant As uptake arises from the soluble and exchangeable fractions, a model based on the ratio between available and amorphous fractions were used. This model takes into account the redistribution of As to more available fractions during phytoremediation and allowed for accurate prediction of As uptake by *P. vittata* over two years.

CHAPTER 1 INTRODUCTION

During the last century, vast portions of the earth's natural resources have been appropriated to sustain its growing population. A chief consequence of this has been the deterioration of soil ecosystems from exposure to toxic chemicals. Yet even as the productivity of our soil declines, more is expected of it due to our growing population. In the United States alone, there are 1,210 registered superfund sites contaminated with arsenic (U.S. Environmental Protection Agency, 2004). Arsenic is a deadly toxin and is linked to cancer of the bladder, kidney, liver, lung, and prostate (ATSDR, 2009). Tens of millions of people are potentially exposed to excessive levels of arsenic (Ng et al., 2003). Due to its deadly toxicity and carcinogenicity, arsenic is ranked by the Agency for Toxic Substances & Disease Registry as the #1 most dangerous chemical in the environment (ATSDR, 2007a). Development of novel remediation techniques are required to safely meet the demands of a burgeoning society. Phytoremediation using *Pteris vittata* (Chinese brake fern) offers a simple, non-invasive, and cost-effective method to remediate arsenic contaminated soil compared to traditional clean-up techniques. This application is made possible by the unique capacity of *P. vittata* to hyperaccumulate arsenic in its aboveground biomass (Ma et al., 2001). This feature allows the plant to tolerate an otherwise lethal environment. The capacity to remove high concentrations of arsenic from soil and water offers an easy, non-invasive, and cost-effective remediation method compared to traditional clean-up techniques (U.S. Environmental Protection Agency, 2002).

The success of phytoremediation depends on several factors including the extent of soil contamination, metal availability for uptake into roots (bioavailability), and plants' capacity to mobilize, absorb, and accumulate metals in shoots. The complexity of this interaction, controlled by climatic conditions, argues against generic and in favor of site-specific phytoremediation approaches. This underlines the importance of understanding the mechanisms and processes that govern metal uptake and accumulation in plants. Due to its high rate of As-accumulation, fast growth and large production of biomass, *P. vittata* is ideal for phytoremediation. However, factors facilitating the uptake of arsenic into *P. vittata* are still in question. Normally, upon arsenic exposure, plant root growth is inhibited (Meharg and Hartley-Whitaker, 2002; Zhao et al., 2008). However, *P. vittata* roots respond by increasing biomass while mobilizing arsenic in the rhizosphere. One possibility is that the increased arsenic availability could be response to low available phosphorus (P) in the soil.

Despite being abundant in the lithosphere, P is one of the most limiting nutrients affecting agricultural production around the world (von Uexküll and Mutert, 1995). A large proportion of soil P is organic (P_o), which is stable, insoluble, and immobile, thus making it unavailable for root uptake (Holford, 1997). The predominant form of soil P_o is IPx inositol hexakisphosphate (IP_6), which exists in the myo- form (phytate; IHP), and is a stable compound highly invulnerable to chemical or enzymatic degradation (Turner et al., 2002). Within the soil fraction, phytate can constitute >50% of P_o and >25% of total P (Anderson et al., 1980). Hence, despite its abundance, P is one of the most unavailable and inaccessible macronutrients in the soil, and frequently limits plant growth (Holford, 1997). During periods of low availability of soil P, plants respond at the

morphological, physiological and molecular level. One such response is utilization and secretion of phosphomonoesterases into the rhizosphere which can improve phosphate availability (Raghothama, 1999; Vance et al., 2003). High-affinity transporters are generally accepted as entry points for phosphate, which is also the proposed route for arsenic uptake by *P. vittata* (Meharg and Macnair, 1992). Since phosphate and arsenic are chemical analogues, *P. vittata* must make efficient use of P due to arsenates inherent toxicity. Thus, root mediated responses of *P. vittata* during phosphate starvation may be unique, making it a potential model plant in understanding how roots can serve to enhance the availability and use of P.

Deducing the mechanisms behind arsenic tolerance and uptake would be potentially valuable for developing crops that tolerate inhospitable environments. Using *P. vittata*, the major objectives in this research were to 1) understand the role of phytase in phosphorus acquisition and arsenic-uptake, 2) develop plant management strategies to maximize growth and arsenic-uptake over several seasons and 3) evaluate the impact on soil arsenic mobilization during phytoremediation.

CHAPTER 2 REVIEW OF LITERATURE

Arsenic in the Environment

Arsenic is a ubiquitous element that ranks 20th in abundance in the earth's crust (Mandal and Suzuki, 2002). Arsenic occurs naturally in over 200 different mineral forms, of which approximately 60% are arsenates, 20% sulfides and the remaining 20% includes arsenides, arsenites, oxides, silicates and elemental arsenic (Wedepohl, 1969). Arsenate (AsV) and arsenite (AsIII) are the most common inorganic forms in the environment. Soil As concentrations in the USA are estimated to range between 0.1–55 mg kg⁻¹, with an average of 7.2 mg kg⁻¹ (Allard et al., 1995). However, arsenic concentrations in soil may be much higher, primarily due to anthropogenic contributions from arsenical pesticides, fertilizers, burning of fossil fuels; chromate copper arsenic (CCA) treated wood and disposal of industrial and animal wastes (Nriagu and Pacyna, 1988). For nearly five decades (1930 to 1980), arsenic-based pesticides were applied on agricultural land throughout the United States, adding >10,000 tons of arsenic to the soil each year (Welch et al., 2000). Arsenic does not breakdown and can readily accumulate in soil (Davenport and Peryea, 1991). Despite being a known issue, arsenic accumulation in soils is a continual problem. In the U.S., approximately 100 metric tons of arsenic-based feed additives (roxarsone[®]) are fed to chickens every year. Arsenic laced-laced poultry litter is later spread on agricultural fields at a rate between 9 - 20 metric tons ha⁻¹ yr⁻¹ (Cortinas et al., 2006; Hileman, 2007). By 2002, more than 90% of all outdoor wooden structures in the U.S. were treated with arsenate pesticide (Gray and Houlihan, 2002). With high concentrations of arsenic (~1,200 mg kg⁻¹), treated wood has a long life-span (20-50 years) and acts as a source of arsenic

contamination in the vicinity (Stook et al., 2004). Even though CCA wood was banned for residential use in 2004, $\sim 6.1 \times 10^6$ kg of As is used annually for wood treatments in the U.S. (Brooks, 2012). Normally, soil As concentrations exceeding the limit results in regulatory actions at industrial or hazardous waste sites, but no such protocols exist for residential and public spaces suggesting the presence of a widespread regulatory health crisis (Belluck et al., 2003).

Once introduced to the soil, factors such as climate, organic and inorganic soil components and redox potential status impact the level and species of As in a given soil (Mandal and Suzuki, 2002). Under aerobic oxidizing conditions, arsenates predominate, strongly sorbing to clays, iron, manganese oxides/hydroxides, and organic matter (Sun and Doner, 1996). Arsenite is found in reducing anaerobic conditions, which can be methylated by microorganisms, producing monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsine oxide under oxidizing conditions (Norman, 1998). Typical mineral soils can have pH values in the range 5 to 9 with Eh values of -300 (water-logged) to +900 (well aerated) millivolts (mV). In soil-based studies, redox conditions and pH significantly affect the availability and consequent toxicity of arsenic (Marin et al., 1992; Meharg and Hartley-Whitaker, 2002).

Arsenic Toxicity

Most cases of human toxicity from arsenic have been associated with exposure to inorganic arsenic (U.S. Environmental Protection Agency, 2001; ATSDR, 2009). In humans, exposure to As may lead to damages of internal organs, the respiratory, digestive, circulatory, neural, and renal systems; the most significant hazards are skin, lung and bladder cancers (Ng et al., 2003; Tchounwou et al., 2004). Arsenate is a molecular analog of phosphate and interferes with oxidative phosphorylation and ATP

synthesis (NRC, 2001). Arsenite is more broadly toxic because it binds to sulfhydryl groups or vicinal thiols in pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, impairing the function of many proteins and respiration (ATSDR, 2007b).

Inorganic arsenic species are highly toxic to plants. Arsenate is the dominant form of arsenic in aerobic soils and acts as a phosphate analogue, which enables transport across the plasma membrane via phosphate co-transport systems (Meharg and Macnair, 1992). Upon arsenic exposure, plants suffer from inhibition of root growth to death (Barrachina et al., 1995; Burlo et al., 1999; Zhao et al., 2008). Once inside the cytoplasm, arsenate competes with phosphate, replacing phosphate in ATP to form unstable ADP-As, and leading to the disruption of energy flows in cells (Meharg, 1994).

Arsenate can be reduced to arsenite by glutathione in plant tissue, which is also highly toxic to plants as it reacts with sulfhydryl groups (-SH) of enzymes and proteins, leading to inhibition of cellular function, generation of reactive oxygen species (ROS) and death (Ullrich-Eberius et al., 1989; Abbas and Meharg, 2008). Following the reduction of arsenate to arsenite in plants, arsenic may potentially be further metabolized to methylated species leading to further oxidative stress (Zaman and Pardini, 1996).

Remediation of Arsenic Contaminated Soil

According to the National Priorities List (NPL), there are 578 Superfund sites where arsenic is a concern, with As-contaminated soils accounting for ~372 (66%) of the sites (U.S. Environmental Protection Agency, 2004). Many engineering technologies have been developed for remediation of arsenic contaminated soils (U.S. Environmental Protection Agency, 2002). Remediation of arsenic contaminated soils is a costly endeavor. Conventional treatments often require the use of expensive equipment,

which has high operational costs. Furthermore, some require excavation of the soil, greatly altering the environment, while others (solubilization and stabilization) do not remove the arsenic, leaving the potential for future exposure. Alternatively, the use of phytoremediation preserves the topsoil while reducing hazardous contaminants. It is also cost effective, requiring no special equipment or operating costs. Finally, phytoremediation can also be aesthetically pleasing, garnering more public acceptance. Phytoremediation is plant-based technologies that degrade, extract, contain, or immobilize contaminants from soil or water. It has the potential to clean up waters, soils, slimes and sediments contaminated with pesticides, PAHs (polycyclic aromatic hydrocarbons), fuels, explosives, organic solvents, chemical manures, heavy metals, metalloids, and radioactive contaminants (Adams et al., 2000). In the process of phytoremediation, pollutants are taken up by plant roots and either decomposed to less harmful forms (e.g., CO₂ and H₂O) or accumulated in the plant tissues. Thus, phytoremediation is environmentally friendly, inexpensive (relative to other remediation techniques) and can be carried out in polluted places (in-situ). The success of phytoremediation depends on several factors including the extent of soil contamination, metal availability for uptake into roots (bioavailability), and plants' ability to intercept, absorb, and accumulate metals in shoots (Krämer, 2005). *Pteris vittata* is one such a plant that contains the qualities necessary for successful phytoremediation, specifically due to its ability to hyperaccumulate arsenic.

***Pteris vittata* L.**

Description and Distribution

Pteris vittata is a vascular fern within the Pteridaceae family as deduced by phylogenetic studies based on *rbcL* nucleotide sequences (Hasebe et al., 1995). The

complete taxonomic classification of *P. vittata* is: Kingdom – Plantae, Subkingdom – Tracheobionta (vascular plants), Division – Pteridophyta (True Fern), Class – Filicopsida, Subclass – Polypodiidae, Order – Pteridales or Polypodiales, Family – Pteridaceae (maidenhair), Subfamily – Pteridoideae, Genus – *Pteris* (derived from Greek word pteron, meaning wing or feather, for the closely spaced pinnae, which give the leaves a likeness to feathers), Species – *vittata* (ladder), Specific epithet: *vittata* – Linnaeus (U.S. Department of Agriculture, 2009).

Pteris vittata grows in tropical and subtropical regions. Originally native to Africa, China, Japan, Thailand, and Australia, it is currently found worldwide. In the U.S., *P. vittata* grows naturally in Hawaii, California, and the southeastern states; Texas to South Carolina and Florida (Jones, 1987). In Florida, they are often found on calcareous substrate, such as old masonry, sidewalks, building crevices, and nearly every habitat in southern Florida with exposed limestone, notably pinelands (Lellinger, 1985).

Pteris vittata are hardy, fast growing perennial forbs with characteristics of mesophytes. They prefer full to partial sun while their size varies depending on conditions, can reach heights of 1.2 m. Its stipe is stout with dense brown scales, which extend along the rachis. Fronds cluster from a horizontal rhizome and blades are green to pale brown, containing numerous pinnae, which separate proximally (Lellinger, 1985). The life cycle of *P. vittata* involves sporophytes where spores are produced on the margin of the lower side of the pinnae. The spores (used in sexual propagation) germinate and produce the heart shaped prothalli. Both male and female organs develop from the prothalli giving rise to the gametophyte stage. The most interesting characteristic about *P. vittata* is its extreme tolerance and ability to hyperaccumulate

arsenic, making it the first embryophyte to display an affinity for arsenic (Ma et al., 2001).

Arsenic Hyperaccumulator

Brooks et al. (1977) first coined the term “hyperaccumulators” to describe plants that uptake and accumulate metals more than $1000 \mu\text{g metal g}^{-1}$ dry mass, which is still in common use. Due to its high rate of arsenic accumulation, fast growth and a high production of biomass, *P. vittata* is ideal for phytoremediation. In both controlled and natural experiments, arsenic was found to accumulate in the fern’s fronds, reaching concentrations as high as $23000 \mu\text{g As g}^{-1}$ dry mass (Tu et al., 2002). Arsenic hyperaccumulation can also occur directly through foliage, suggesting that *P. vittata* foliage tissue has specific arsenic transporters (Bondada et al., 2004). Even the callus, gametophyte, and sporophytes tissue of this fern were effective in accumulating arsenic (Gumaelius et al., 2004; Yang et al., 2007).

Rhizosphere Modifications

At an arsenic concentration of 200 mg kg^{-1} , presence of phosphate was shown to have little effect on improving *P. vittata* growth; but when arsenic concentrations increased ($>400 \text{ mg kg}^{-1}$), P was critical for *P. vittata* growth (Tu and Ma, 2003). It has been suggested that minimum ratios of P/As of 1:2 in the fronds is required for normal growth of *P. vittata* (Tu and Ma, 2003). In order to achieve these ratios of P, *P. vittata* may utilize root exudates to maximize uptake and inadvertently increase arsenic availability in soil, leading to increased hyperaccumulation of As. Compounds released from plant roots can have a direct impact on the solubility of mineral elements or can indirectly influence turnover and availability of nutrients by interaction with soil microorganisms (Neumann, 2007). Despite their importance, little is known about the

role of rhizosphere processes in heavy metal hyperaccumulators including *P. vittata* (McGrath et al., 2001; Wenzel et al., 2003; Rathinasabapathi et al., 2006). It is possible that arsenic uptake in *P. vittata* is a byproduct of P mobilization and acquisition. Phosphate is considered one of the least available plant nutrients in the soil. High-affinity phosphate transporters are generally accepted as entry points for P, which is also the proposed route for arsenic uptake by *P. vittata* (Meharg and Hartley-Whitaker, 2002; Wang et al., 2002). The physiological, genetic, molecular and biochemical analysis of phosphate starvation response mechanisms highlight the ability of plants to adapt and thrive under P limiting conditions. These responses, particularly root exuded carboxylates and phosphatases, help enhance the availability of P, which increase its uptake and improve the use-efficiency of P within the plant (Raghothama, 2000). Root-induced mobilization of nutrients (e.g., P, Fe and Zn) requires the release of specific stable compounds (e.g., citrate, malate, oxalate, malonate, and phytosiderophores) in the root zone with highest nutrient uptake (e.g., apical root zones) (Jones, 1998; Neumann, 2007). Anion channels have also been implicated in exudation of citrate, which are responsible for mobilization of soluble Fe- and Al-phosphates (Neumann et al., 1999; Zhang et al., 2004). Porter and Peterson (1975) reported that a clear correlation exists between arsenic and Fe in plants growing on soils heavily contaminated with arsenic, with the results indicating that Fe plays a role in As accumulation in these plants. High Fe concentration in soil solutions in the rhizosphere of *P. vittata* have also been identified, suggesting the possible mobilization of Fe by the roots of *P. vittata* (Fitz et al., 2003). However, more studies are needed to explore the role of nutrient elements on the detoxification and accumulation of arsenic in *P. vittata*.

Root Induced Arsenic Mobility

Changes in the rhizosphere characteristics of *P. vittata* relevant to its use in phytoextraction have been examined. Arsenic depletion in its rhizosphere and limited resupply from less available arsenic pools were indicated by a 19% reduction in arsenic flux (Fitz et al., 2003). Gonzaga et al. (2006) evaluated the influence of arsenic uptake by *P. vittata* on different fractions of arsenic in the bulk and rhizosphere soil by fractionating by decreasing availability. *Pteris vittata* was more efficient than *Nephrolepis exaltata* to access arsenic from all fractions, though most of the arsenic taken up was from the amorphous hydrous oxide-bound fraction (67-77% of soil arsenic), the most abundant (61.5% of all fractions) instead of the most available (non-specifically bound fraction) (Gonzaga et al., 2006). Increased arsenic availability in the rhizosphere of *P. vittata* may be a byproduct of root mediated mechanisms to increase nutrient availability. Rhizosphere modifications are known to be important for the acquisition of various nutrients (Jones et al., 2004). Tu et al. (2004) examined root exudates of *P. vittata* in a hydroponic system and found dissolved organic carbon (DOC) in the root exudates increased from 19 to 30 mg kg⁻¹ (root dry weight) as arsenic increased from 267 to 1068 µM. A similar experiment was conducted by Lou et al. (2010), who found that DOC contents from *P. vittata* root exudates decreased with increasing P concentrations, regardless of whether arsenic was present or not.

Phosphorus deficient plants have been shown to enhance exudation of carboxylic acids (i.e., citric and malic acid), which are thought to change soil pH, displace P from sorption sites, and chelate metal cations that could immobilize P or to form soluble metal-chelate complexes with P (Kirk et al., 1999). Since As and P are chemical analogues, it is reasonable to assume that root exudation may also mobilize

arsenic in the rhizosphere. This may explain the phenomenon in *P. vittata* in that the presence of P decreases arsenic influx, whereas P starvation nets a arsenic influx by 2.5-fold (Wang et al., 2002).

Arsenic is also known to complex with Fe-oxide surfaces (Sun and Doner, 1996). The excretion of protons and/or the release of reducing and chelating compounds by *P. vittata* could result in co-dissolution of arsenic from Fe-oxides/hydroxides, although little is known about Fe nutritional aspects and related rhizosphere processes of *P. vittata* (Fitz and Wenzel, 2002). However, since *P. vittata* are known to grown on calcareous soil (Jones, 1987), they may share similarities with other acidifuge plants, which have been reported to effectively mobilize P and Fe from limestone (Ström et al., 1994). Plant Fe deficiency normally occurs on calcareous soils (pH > 7.0) (Marschner, 1995). Organic acids such as citrate and malate are known to be potent complexers of Fe in soil and induce the dissolution of unavailable insoluble ferric oxyhydroxides (Jones, 1998). Thus, *P. vittata* could be a key factor determining fluxes and pool sizes of arsenic in the soil. Most root studies have been performed in solution culture, which facilitates observations in soil environments. Especially because roots grown under hydroponic conditions may be morphologically and physiologically different from those growing in soil (e.g., no root hairs, no cortical degeneration, different branching patterns, no mechanical impedance or water stress). In addition, the aeration, microbial and nutrient status of these hydroponic cultures is often different from those in a typical soil environment.

Phytoremediation of Arsenic Contaminated Soils by *P. vittata*

Numerous studies have examined the use of *P. vittata* for phytoextraction (Tu et al., 2002; Salido et al., 2003; Kertulis-Tartar et al., 2006; Baldwin and Butcher, 2007;

Gonzaga et al., 2008; Santos et al., 2008). However, with the exception of Kertulis-Tartar et al. (2006), most of these experiments are conducted on a short time scale (months). Phytoremediation is expected to take several growing seasons (years) to effectively reduce soil arsenic concentrations. Furthermore, the use of arsenic spiked soil and hydroponic studies do not correlate well with hyperaccumulation in a natural soil environment.

Gonzaga et al. (2008) conducted a greenhouse experiment evaluating arsenic removal by *P. vittata* and its effects on arsenic redistribution in six arsenic-contaminated soils. They confirmed that it is possible to use *P. vittata* to remediate arsenic-contaminated soils by repeated frond removal. Over the course of one year (October 2003 – October 2004), three harvests were conducted and frond biomass along with arsenic accumulation was drastically reduced compared to the first harvest (Gonzaga et al., 2008). In a similar experiments by Shelmerdine et al. (2009) and Caille et al. (2004), growth and arsenic uptake of *P. vittata* were significantly reduced in subsequent harvests. Problems with this experiment include: use of small pots, which limited root growth, and during the first harvest, all the fronds were cut to the base of the rhizome, greatly inhibiting plant re-growth.

Phosphate fertilizer amendments have been shown to reduce arsenic concentration in the fronds of *P. vittata*, even though they increase arsenic concentration in the soluble soil fraction. However, phosphate fertilizers are known to increase arsenic uptake in other plant systems (Peryea, 1991, 1998). In a three month field study, Cao et al. (2003) showed that arsenic concentration in the fronds of *P. vittata* increased by 265% when 15 g kg⁻¹ phosphate rock was added to a contaminated

sandy soil, although no increase was observed from plants grown in soil spiked with soluble arsenic salts. It was suggested that replacement of arsenic by P from the soil binding sites was responsible for the enhanced mobility of arsenic and subsequent increased plant uptake. Fayiga and Ma (2006) observed a slight reduction in arsenic uptake (1631 to 1530 mg kg⁻¹) by *P. vittata* in a 5-week pot experiment amended with slow release P-fertilizer. Compost additions were found to facilitate arsenic uptake in a contaminated soil, but not an arsenic spiked soil during a 3-month pot study (Cao et al., 2003). It was suggested that the effect of compost on arsenic uptake was likely due to changes in soil pH and water holding capacity; the soil had a neutral pH and compost treatments may have induced an anaerobic environment in the soil, reducing As(V) to As(III), thereby facilitating uptake by the fern (Cao et al., 2003). In contrast, arsenic adsorption onto organic matter applied in acidic soil may have decreased uptake in the spiked soil (Cao et al., 2003).

During an 8 week study, *P. vittata* grown in rhizopots with As-contaminated CCA soil (105 mg kg⁻¹) reduced water-soluble arsenic and increased soil pH in the rhizosphere soil (Gonzaga et al., 2008). Most of the arsenic accumulation was associated with the rhizosphere soil (67–77%), suggesting that higher plant density may improve remediation of arsenic-contaminated soils by increasing root surface area per unit volume of soil. The optimal planting density for *P. vittata* has not been explored for soil remediation. In a field trial with a planting density of 6 plants m⁻², Gray et al. (2005) reported an annual above-ground biomass production of 1.03 t (dw) ha⁻¹. Kertulis-Tartar et al. (2006) saw improved biomass (1.3 t ha⁻¹ yr⁻¹) using a planting density of 10 plants m⁻². Recently, Shelmerdine et al. (2009) conducted a 9-month container-based

experiment which equated to a planting density of 16 plants m^{-2} with a estimated frond biomass of $2.5 \text{ t ha}^{-1} \text{ yr}^{-1}$. Field trials with increased plant density are required to assess optimal strategies for phytoremediation with *P. vittata*.

CHAPTER 3
A NOVEL PHYTASE FROM *PTERIS VITTATA* RESISTANT TO ARSENATE, HIGH
TEMPERATURE, AND SOIL DEACTIVATION

Plant Phytases

Despite being abundant in the lithosphere, phosphorus (P) is one of the most limiting nutrients affecting agricultural production around the world (Cordell et al., 2009). Plants require P to be in a soluble inorganic form to be taken up, but P is often insoluble in soils due to high sorption (Richardson et al., 2009). Even with soluble inorganic P fertilizer, much of the P is quickly sorbed or transformed into forms unavailable to plants (Sánchez-Calderón et al., 2010). Phosphorus fertilizers, which are mined from non-renewable resources, are often required in great abundance to maintain high crop productivity which also contributes to eutrophication of water bodies (Cordell et al., 2009).

To protect the environment while sustaining agricultural production, improved P nutrition strategies are needed. One such method is to develop plants with increased ability to utilize the large pool of unavailable organic P in soils. Organic P (P_o) accounts for 30-80% of total soil P, predominantly as phytate [myo-inositol 1,2,3,4,5,6-hexakisphosphate] (Richardson et al., 2005). Phytate is a stable compound resistant to biochemical degradation, rendering it unavailable for root uptake (Turner et al., 2002). Within the soil fraction, phytate can make up >50% P_o and >25% total P (Richardson et al., 2005).

Inorganic phosphate (P_i) plays a central role in energy metabolism and regulation in plant cells. During periods of low P_i availability, plants respond through changes in root morphology, secretion of organic acids into the rhizosphere, augmentation of P_i uptake systems, and changes in P metabolism (Vance et al., 2003). Plants can use

phosphatases to release P_i from P_o during seed germination, for internal remobilization, and from external mobilization in the soil (Baldwin et al., 2001; Miller et al., 2001). Phosphatases have broad substrate specificities to various forms of P_o . Those that specifically cleave P_i from phytate are known as phytases (Duff et al., 1994; Brinch-Pedersen et al., 2002). Intracellular phytases and phosphatases are involved in utilization of P_i reserves or other P_i -containing compounds (Duff et al., 1994). Root exudation of phosphatases, especially phytases, could be an effective mechanism to provide additional sources of P_i in soil, but this biochemical strategy has not been widely used by plants. In roots, phytase enzymes can occur in the apoplast but are often localized to the cell wall, epidermal cells, and apical meristem (Duff et al., 1994; Miller et al., 2001). Despite this, most plant root phosphatases (especially agronomically important ones) are unable to hydrolyze sufficient P_i to maintain growth owing to either poor substrate availability in soils due to sorption and precipitation, proteolytic breakdown or limited capacity to effectively exude P_i mobilizing enzymes (Findenegg and Nelemans, 1993; Richardson et al., 2000; George et al., 2004, 2005).

Pteris vittata L. (Chinese brake fern) is native to alkaline sub/tropical soils, which are rich in P_o but deficient in available P (Ramaekers et al., 2010). Furthermore, *P. vittata* (PV) can hyperaccumulate arsenic (As) in the frond biomass to >1% of its dry weight (Ma et al., 2001). Arsenate is the most abundant form of As in soils, and is a chemical and structural analog for P_i . In fact, arsenate is a competitive inhibitor of P_i for uptake by plant P transporters (Meharg and Macnair, 1992). In the presence of arsenate, plant roots will readily assimilate As leading to toxicity development, P-deficiency and enzyme deactivation (Meharg and Hartley-Whitaker, 2002). This is not

the case for PV, which tolerates high concentrations of As in the soil and even higher in the fronds. In addition to its natural adaptation to P-deficient soil, PV phosphatases including phytase may effectively hydrolyze P_i from phytate and other P_o while being uniquely resistant to the deleterious effects of As.

To test this hypothesis, phosphatase and phytase activities from *P. vittata* and *Pteris ensiformis* (PE; a non-hyperaccumulator) were assessed following exposure to As stress and P_i limitation. Furthermore, the role of phytases in P_i acquisition was examined by: 1) quantifying phytase activities in root exudates; 2) growing *P. vittata* on media with phytate as the sole source of P; and 3) evaluating the efficacy of *P. vittata* phytases in soil environments.

Materials and Methods

Hydroponic Plant Culture

Two month old ferns, *P. vittata* (PV) and *P. ensiformis* (PE; a non-hyperaccumulator), were transferred to hydroponic culture in 0.2× strength Hoagland-Arnon nutrient solution (HNS) for three weeks. Plants were rinsed with deionized (DI) water and transferred 500 mL 0.2× modified HNS with 0 or 210 μM P_i (KH_2PO_4) and 0 or 267 μM As ($\text{Na}_2\text{HAsO}_4 \cdot 2\text{H}_2\text{O}$) for 3 d. Treatments are referred to as control (No P), P_i , As, and P_i +As and were replicated four times.

Seedling and Gametophyte Culture

Seeds from *Lactuca sativa*, *Trifolium subterraneum*, and *Allium schoenoprasum* and spores from PE, *Thelypteris kunthii*, and PV were surface sterilized in a 20% bleach solution for 20 minutes followed by three washes in sterile DI water. Spores were suspended in 2 mL sterile DI water. Half strength modified Murashige & Skoog (MS) media was prepared with 0.8% agar without P prior to autoclaving. Phosphate, phytate,

and arsenate solutions were filter sterilized and added to autoclaved MS media to obtain final concentrations of 0.6 mM P as P_i or phytate (P₆; myo-inositol hexaphosphoric acid dodecasodium salt) with 0 or 0.6 mM arsenate. The MS media (pH 6.5) was then poured into sterile petri dishes (100 mm × 13 mm). Seeds and spores (10 µL or 0.05 mg spore) were placed on agar (5 per plate, 4 plates per treatment) under cool/warm fluorescent lamps at 25°C and 60% humidity for 15 and 40 d for seeded plants and ferns, respectively.

Enzyme Collection

Tissues were rinsed in 10 mM Ca(NO₃)₂ and blotted dry, weighed, and mixed (1:2 w/v) with 10 mM acetate buffer (pH 5.0) containing 1 mM EDTA, 1 mM DTT (dithiothreitol), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), and 4% PVPP (polyvinyl polypyrrolidone). Samples were homogenized using a Magic Bullet® blender (Four 15 s pulses), passed through cheesecloth and centrifuged at 10,000 g for 15 min. Supernatants were subjected to gel filtration on Sephadex G-25, pre-equilibrated with 10 mM acetate buffer (pH 5.0). Root exudates were collected from media of 40 d old PV sporophyte, analyzing enzyme activity following gel filtration. Ammonium sulfate fractionation was performed on PV gametophyte extracts, collecting precipitates in 20% intervals from 0 - 80% fractions followed by gel filtration.

Phytase and Phosphatase Assays

Protein content was measured against bovine serum albumin (BSA) standards using the Bradford method (Walker and Kruger, 2002). Enzyme activity was analyzed by incubating ~100 µg protein in 1 mL of 10 mM acetate buffer (pH 5.0) containing either 5 mM phytate or 5 mM pNPP (*p*-nitrophenylphosphate disodium salt; Sigma) at 37°C for phytase and phosphatase, respectively. Reactions were terminated with equal

volumes of 10% (wt/v) trichloroacetic acid after 120 min (phytase) or 25 mM NaOH after 30 min (phosphatase). Specific activities were calculated as the difference between P_i or *p*NP concentration in the extracts with and without incubation, expressed as nmol of P_i or *p*NP released per min per mg protein. Phosphate was measured spectrophotometrically at 880 nm using the molybdenum-blue reaction at a fixed time (20 min) following addition of the color reagent (Carvalho et al., 1998). Phosphatase activity was calculated from the release of *p*NP as determined by measuring absorbance at 405 nm against standard solutions.

Arsenic and Phosphorus Analysis

Pteris vittata tissue was dried at 60°C for 96 h, weighed, and ground through a 2-mm mesh screen. Samples (0.1 g) were subjected to hot block digestion using USEPA Method 3050 (1983) and analyzed for total As using graphite furnace atomic absorption spectroscopy (GFAAS, Varian AA240Z, Walnut Creek, CA). Total P was calculated using the molybdenum blue method previously mentioned. To prevent interference of arsenate when using the molybdenum-blue method, samples were incubated with 300 μ L 5% cysteine at 80°C for 5 min to reduce arsenate to arsenite (Carvalho et al., 1998).

Phytase Arsenic Resistance and Thermostability

Arsenic tolerance of phytase and phosphatase enzymes was analyzed by performing previously described assays in the presence of of arsenate. In addition to 5 mM P_6 or *p*NPP, plant extracts of PV, PE and wheat phytase were incubated with 0, 0.5, 2, 2.5, and 5 mM arsenate. Thermostability of enzyme activities was determined by pre-incubation of enzyme extracts in a water bath at 40, 60, 80, and 100°C for 10 min.

Phytase Stability After Mixing With Soils

The phytase activity in root extracts from PV and PE and wheat phytase were measured after mixing with soils. Briefly, 2.0 g of air dried soil was mixed with DI water and enzyme extracts (or BSA as a negative control) to a 20 mL volume containing 50 μg protein per ml. Samples were placed on a rotary shaker (150 rpm) for 120 min at room temperature. Aliquots of well-mixed soil slurry (250 μL) were removed using a pipette tip with a wide opening and centrifuged at 7,500 g for 5 min, using the supernatant (250 μL) to measure phytase activity. Activities were derived from the difference between plant enzyme mediated P_i release and the amount of P_i in the BSA soil suspensions.

Statistical Analysis

Data are presented as the mean of all replicates with standard error. Significant differences were determined using analysis of variance and treatment means compared by Duncan's multiple range test, at $p \leq 0.05$.

Results

***Pteris vittata* Phytase Showed Arsenic-Resistance and Thermostability**

Partial purification of PV phytase greatly increased its enzyme activities. The PV activities in the crude protein were 2.6 nmol P_i and 8.6 nmol $p\text{NP}$ mg^{-1} protein min^{-1} . Gel filtration tripled specific enzyme activities and ammonium sulfate precipitation increased activities by 9 to 26 fold. The the highest purification was associated with the 20-40% ammonium sulfate fractions (68 nmol P_i and 181 nmol $p\text{NP}$ mg^{-1} protein min^{-1}) which were used to estimate As tolerance and thermostability. Phytase and phosphatase activities were measured by production of P_i and $p\text{NP}$ hydrolyzed by the extracts of PV, PE and a crude wheat phytase in the presence of increasing concentrations of arsenate (0-5 mM). At 5 mM phytate or $p\text{NPP}$ suspensions buffered

at pH 5.0, enzyme activities for PV, PE, and WP were 46.7, 42.7, and 51.8 nmol P_i mg⁻¹ protein min⁻¹ for phytase and 79.5, 149, and 163 nmol pNP mg⁻¹ protein min⁻¹ for phosphatase respectively (Figure 3-1). Phytase activities in PV extracts were unaffected by arsenate up to 2 mM (46.7 to 46.1 nmol P_i mg⁻¹ protein min⁻¹), with a slight decrease (~41.1 nmol P_i mg⁻¹ protein min⁻¹) at concentrations above 2.5 mM, which were not significantly different than the control (p < 0.05; Figure 3-1a). However, phytase activities from PE and wheat extracts exhibited a linear decrease (~50, 43, 34 and 24% decrease) with increasing arsenate (Figure 3-1a). At 5 mM, their activities were ~25% of the control. Phosphatase activities in extracts from PV, PE and wheat were similarly impacted by arsenate (Figure 3-1b). At 5 mM As, their activities were 36-45% of the control.

The thermostability of extracts were tested by incubating samples for 10 min at temperatures ranging from 40°C to 100°C prior to the activity assays. Phytase activities of PV extracts were unaffected by all heat treatments compared to PE and wheat phytase, which lost all activities after incubating at 100°C (Figure 3-2a). Unlike phytase, phosphatase activities from enzyme extracts of all three plants decreased at a similar rate with increasing temperatures (Figure 3-2b).

Phytase and Phosphatase in *P. vittata* and *P. ensiformis* Tissues

After growing in media with P_i, arsenate or both for 72 h, PV and PE showed no toxicity symptoms. Phosphatase and phytase activities were detected in the frond, root, and rhizome extracts of both PV and PE (Table 3-1). Phosphatase activities in all treatments were much greater in PE than PV in all tissues, with the greatest difference in the fronds (85-198 times) and smallest in the roots (1.2-2.0 times). Unlike

phosphatase, phytase activities in all treatments were generally greater in PV, illustrating an inherent difference between the two species.

Neither P_i or As treatment had an impact on activities in the fronds or rhizomes of both PV and PE. However, some treatments reduced enzyme activities in their roots. With no exception, addition of P_i was the most effective in reducing phosphatase and phytase activities in the roots. Strangely enough, addition of As had the same effect as P_i in PV, reducing phytase activity from 19.7 to 6.1 nmol P_i mg⁻¹ protein min⁻¹ (Table 3-1).

***Pteris vittata* Growth on Media Amended with Arsenic and Phytate**

To estimate the ability of PV to utilize phytate as a sole source of P, its growth on modified MS media amended with either 0.6 mM P_i and/or phytate (P_6) with and without 0.6 mM arsenate (P_i +As, P_6 +As, and P_i + P_6 +As) was compared to three angiosperms with known phytase activity (*Lactuca sativa*, *Trifolium subterraneum*, and *Allium schoenoprasum*) and two pteridophytes (*P. ensiformis* and *T. kunthii*). Fresh weights of plants after growing for 15 d for angiosperms and 40 d for ferns are listed in Table 3-2.

Germination rate for seeds were >90% and 100% for fern spores grown on modified MS media amended with 0.6 mM P_i . Though all three angiosperms grew on P_6 -amended media, their biomass production was reduced by 2.1-3.3 times compared to the P_i treatment. The two ferns (PE and *T. kunthii*) were unable to use phytate to grow.

Pteris vittata was the only plant that effectively utilized phytate, and in the presence of 0.6 mM As, survive beyond germination (Table 3-2). Growth on media amended with P_i + P_6 were similar to P_i treatments, verifying that the presence of phytate had no negative effect on growth. Interestingly, P_i +As increased PV biomass 2-fold

(115 to 225 mg) and 1.5-fold in P_i+P_6+As (119 to 151 mg) but was slightly reduced in P_6+As (90 to 66 mg) (Table 3-2).

Phosphorus and As Uptake by *P. vittata* Gametophyte

Total P and As in PV gametophyte grown on MS media with 0.6 mM P_i , P_6 and/or As for 40 d are listed in Table 3-3. Average P concentrations in the P_i treatment were 2,208 mg/kg compared to 1,579 mg/kg in the P_i+As treatment, a significant decrease ($p < 0.05$). It was also similar to that of the P_6 treatment (2,351 mg/kg), indicating that PV gametophyte readily hydrolyzed and accumulated P from phytate. At equal As and P concentrations of 0.6 mM in the P_i+As treatment, the total P and As tissue concentrations were 1,579 and 1,777 mg/kg or 51 and 24 mmoles/kg respectively (Table 3-3). This indicates that PV was more effective in taking up P than As. Compared to the P_i+As treatment, concentrations of P and As in tissue from the P_6+As treatment were both increased, which were 2,672 mg/kg and 2,630 mg/kg ($p \leq 0.05$). However, this did not happen in the absence of As or in the presence of high P (as total P in the P_i+P_6 treatments), which had similar tissue P concentrations. This indicates that phytate (low P) coupled with As promoted up-regulation of P transporters, helping with both P and As uptake.

Phytase Activity in *Pteris vittata* Gametophyte and Root Exudate

Given that *P. vittata* effectively utilized phytate as a sole source of P for growth, we quantified phytase activities from gametophyte and its root exudates in response to P/As stress and phytate. Activities from tissue extracts did not differ significantly between treatments except for the As treatments, which lowered phytase activities by ~5.9 to 2.8 nmol P_i mg^{-1} protein min^{-1} (Figure 3-3a). Exudates collected from phytate-amended media exhibited the highest phytase activity. However, except for P_6 , enzyme

activities from all treatments were not statistically different ($p \leq 0.1$; Figure 3-3b). Compared to phytase activities in the root tissues (5.1 to 20 nmol P_i mg⁻¹ protein min⁻¹; Table 1), those in the root exudates were comparable or higher (9.3 to 19 nmol P_i mg⁻¹ protein min⁻¹), indicating that phytase in the root exudates plays the largest role in P acquisition. Grown in a low available P media (P_6), PV gametophytes increased the amount of exudates. This was indicated by an increase of total protein in exudates from P_6 and P_6+As (2.2 and 2.0 mg protein g⁻¹ tissue) compared to P_i media (1.0, 1.1, and 1.0 mg protein g⁻¹ tissue for P_i , P_i+As and P_i+P_6 respectively). Phosphatase enzymes did not appear to play a significant role in root exudates, as the activity values were only 9 to 18% of those in the PV root extracts (Table 3-1).

***Pteris vittata* Phytase Activity was not Deactivated by Soils**

Root extracts from PV, PE and wheat phytase were mixed with three soils for 2 h. Soil 1 was an acidic (pH 5.6) sandy soil containing 2% OM with a cation exchange capacity (CEC) of 4.2 cmol⁺ kg⁻¹. Soil 2 was a neutral (pH 6.5) silty clay soil with 0.8% OM and a CEC of 16.4 cmol⁺ kg⁻¹. Soil 3 was an acidic (pH = 5.5) clay soil with 0.4% OM and CEC of 24.8 cmol⁺ kg⁻¹. The effect of soils on phytase activity was analyzed by measuring the rate of P_i hydrolysis from phytate in solution following centrifugation. The amount of P_i hydrolyzed represented phytase enzymes that were not sorbed to the soil matrix. For comparative analysis, enzyme samples were incubated without soil (control) and soil samples were mixed with a non-enzymatic protein, bovine serum albumin (BSA), to estimate residual soil P_i released from protein-soil interactions.

In the absence of soil, phytase enzymes from all three plants were similar, ranging from 15 to 19 nmol P_i mg⁻¹ protein min⁻¹ (Figure 3-4). After mixing with soils, PV phytase enzymes retained 66, 50 and 45% of their activity in soil 1, soil 2 and soil 3. In

comparison, PE and wheat phytase retained only ~6% activity (~1 nmol P_i mg⁻¹ protein min⁻¹) in all soils (Figure 3-4).

Discussion

Arsenic Tolerance in *Pteris vittata*

Numerous studies have demonstrated the unique ability of *P. vittata* to acquire, tolerate and accumulate high concentrations of As from soils and culture media (Ma et al., 2001; Tu et al., 2002). The selective factors driving the evolution of As hyperaccumulation in *P. vittata* is unknown, but hypotheses include a role in metal tolerance, protection against herbivores/pathogens, increased antioxidant responses, and allelopathy (Rascio and Navari-Izzo, 2011). As a member of the Pteridaceae, *P. vittata* is an advanced taxa with morphological characteristics placing it in the more recent portion of fern evolution (Smith et al., 2006). During its evolution, As hyperaccumulation and tolerance could be a carryover from marine algae native to As-rich hot springs, which had scant amount of available P_i (Meharg, 2002). Thus, due to the chemical homology of arsenate and P, *P. vittata* must be efficient in scavenging and maintaining P homeostasis while preventing interference from arsenate. Hence, it is no surprise that *P. vittata* commonly inhabits environments depleted of available P_i but with abundant phytate (Jones, 1987; Turner et al., 2006). This unique characteristic was the impetus for our examination of phytase activity in *P. vittata*. The most prevalent form of organic P in many soils is phytate, contributing over 50% of total soil P (Turner et al., 2002). Thus, *P. vittata* would be expected to depend more on phytases to gain P_i from phytate. Because phytase mediated hydrolysis is strongly inhibited by As (Hayes et al., 1999; Päivöke and Simola, 2001), our results suggest *P. vittata* has evolved novel phytase enzymes resistant to As allowing for sufficient acquisition of P_i from phytate.

***Pteris vittata* Phytase showed As-Resistance and Thermostability**

Phytase and phosphatase activities from ammonium sulfate precipitation of PV extracts were detected in all fractions, with the highest concentration in the 20-40% fraction. When PV enzyme extracts were incubated with increasing concentrations of arsenate (up to 5 mM), phytase enzymes, but not phosphatase, retained significant activity. Unlike PV, phytase activity in PE and wheat extracts diminished with increasing concentrations of arsenate (Figure 3-1). It is well established that arsenate interferes with enzyme function including phytases (Zhao et al., 2008). This suggests *P. vittata* had a unique phytase enzyme, which was consistent with its high thermostability. Phytase activities, but not phosphatase of PV enzyme extracts were unaffected by 10 min pretreatments at 100°C while PE and wheat phytase activities were lost (Figure 3-2). *Pteris ensiformis* extracts did retain some activity at 80°C, suggesting a degree of commonality between the two closely related species. Enzyme incubation at pH >7 resulted in loss of activity in PV extracts (data not shown), corroborating previously described optimum pH of 5 (Tu et al., 2010). Metal tolerance and thermostability in phytases and other enzymes have been attributed to glycosylation, hydrogen bonds, disulfide bonds, salt bridges, and presence of co-factors (i.e., chaperones and heat shock proteins) (Wang et al., 2004; Guo et al., 2008). It is possible that the final folded-state of PV phytases were highly stable, conferring arsenate tolerance and thermostability, thereby increasing the ferns' range of potential habitats.

Phytase and Phosphatase Activity in *P. vittata* and *P. ensiformis* Tissues

During periods of P limitation, plants increase their internal phosphatase and phytase production to maintain P_i levels (Sánchez-Calderón et al., 2010). To assess the role of phosphatases in *P. vittata* internal P_i mobilization during P-limitation and

arsenate exposure, their activity in the frond, root, and rhizome tissues were compared to the As sensitive fern PE. When grown in the presence of As, plants often show symptoms of P-deficiency because arsenate competes with P_i uptake and disrupts processes involving phosphorylation and phosphate signaling pathways (Abercrombie et al., 2008). This response was observed for phytase and phosphatase activity in PE root tissues, which were significantly elevated when growing in the absence of P_i or presence of As ($p < 0.05$; Table 3-1). Interestingly, this was not the case for the enzyme activity in PV root extracts. Unlike phytase, phosphatase activity from PV root extracts increased in treatments without P_i while phytase activity increased only in the control (No P) ($p < 0.05$; Table 3-1). The lack of phytase activity response in As-treated PV roots was unexpected. Since arsenate is a phosphate analog, PV roots may not differentiate between them. Instead, the metabolic and regulatory systems may have perceived the toxic metalloid as an abundant supply of P_i , inhibiting the up-regulation of phytase production. After 3 d of growth, different treatments had no effect on enzyme activity in the frond and rhizome tissues for both ferns. Frond and rhizome enzyme activity may have been unaffected because the 3-d incubation period was not long enough to elicit sufficient P-deficiency responses in those tissues. Furthermore, *P. ensiformis* does not translocate As to the rhizome and frond. Alternatively, enzyme activity in both ferns may be associated with acquisition of P_i from soil and not with internal P-homeostasis, which would explain why activity responses were only observed in root tissues (Table 3-1).

***Pteris vittata* Growth on Phytate**

Due to the high enzymatic phytase activity in PV roots, especially under P_i limiting conditions, we investigated whether PV spores could grow on sterile media amended

with P_6 as the sole source of P. Phytate has been shown to be a poor source of P for plants due to both substrate availability and enzyme activity constraints (Hayes et al., 2000; George et al., 2004). This was not the case for *P. vittata*, which grew equally well on P_i or P_6 (Table 3-2) with similar total P (2,208 and 2,351 mg/kg) after 40 d of growth. Most plants lack the ability to access external phytate because their phytases are confined to the endodermal region (Hayes et al., 1999), which was supported by the fact that other plants produced similar biomass in phytate treatment as the control without P (Table 3-2). Even though *T. subterraneum* and *L. sativa* have been shown to increase root phytase activity in P-limiting and other stressful environments (Hayes et al., 1999; Nasri et al., 2010), they were unable to hydrolyze sufficient quantities of phytate in our experiment to sustain growth. The ability of PV to grow using phytate as a sole source of P_i and its lack in two other ferns suggests that phytate utilization is an adaptive trait specifically evolved in only some fern taxa.

As expected, *P. vittata* was the only plant to survive beyond germination in the presence of 0.6 mM arsenate. In the presence of arsenate, PV biomass and total P concentration were affected by the source of P. After 40 d of growth, PV grown on P_i+As agar were ~2 times larger than all other treatments ($p \leq 0.05$; Table 3-2). Despite having the largest biomass, PV tissue from P_i+As agar had the lowest P concentration, which is consistent with previous findings that arsenate stimulates growth and competes with P_i for uptake (Gumaelius et al., 2004). However, arsenate had the opposite effect on gametophyte grown with phytate, reducing biomass below the P_i control while significantly increasing total P ($p \leq 0.05$; Table 3-3). Similar to the lack of phytase response in PV root tissue (Table 1), the presence of arsenate in the growth media may

be perceived as P_i (due to their homology) by PV gametophyte, delaying the necessary transcriptional, physiological, and morphological responses required to facilitate phytate hydrolysis. Although growth was slowed, tissues from the P_6+As media had significantly higher concentrations of P and As compared to P_i+As treatments ($p \leq 0.05$; Table 3-3). With the addition of P_i to the P_6+As treatments, biomass and total P concentrations were in between the results of P_i+As and P_6+As treatments, suggesting that the presence of phytate tempers the growth promoting effect of As. It should be noted that, after 80 d of growth, the initial slow growth of *P. vittata* on P_6+As treatments abated, achieving weights equivalent to the P_i treatments (data now shown).

Phytase Activity in *P. vittata* Gametophyte and Root Exudates

Once it became clear that *P. vittata* could effectively utilize phytate, we assessed the response of enzyme activities in gametophyte and their root exudates following 40 d of growth on modified MS media amended with 0.6 mM P_i , phytate, and arsenate. Phytase activity from tissue grown with phytate exhibited the highest phytase activities compared to P_i+As treatments, which had the lowest (Figure 3-3a). However, compared to the P_i treatment, phytase activities did not differ significantly from other treatments. Similarly, phytase in root exudates from gametophyte grown with phytate exhibited the highest activities although not significantly different than the P_i treatment. Thus, production and exudation of phytase enzymes in PV gametophyte appears to be constitutive, regardless of P_i availability. However, total protein content in exudates of P_6 and P_6+As treatments were double that of P_i treatments, suggesting that PV responds in a low available P environment by increasing total enzyme exudation.

***Pteris vittata* Phytases Were Not Deactivated by Soils**

Pteris vittata grew with a relatively low concentration of phytate (0.6 mM) while maintaining P concentrations equal to the P_i treatment (Table 3-3). Phytase activity in root exudates was enhanced when grown with phytate, the likely mechanism for the P_i acquisition. Studies have shown that while plants have the capacity to exude phytases in roots, sorption and precipitation reactions in soil limit their capacity to directly obtain P_i from soil phytate (Brejnholt et al., 2011). This was not the case for PV enzymes, which retained 45-66% of their phytase activities after mixing with soils compared to >90% reduction in PE and wheat extracts, further illustrating the unique properties of PV phytases (Figure 3-4). As the soil cation exchange capacity increased (Soil 1 < Soil 2 < Soil 3), PV phytases could have been more strongly sorbed, explaining the diminishing trend in increasing phytase activity from supernatant samples. Although other factors like pH would contribute to sorption. Under normal circumstances, sorption of phytase impairs the enzyme's ability to hydrolyze phosphate esters from phytate (George et al., 2005) but PV phytases remained active even when sorbed to soil particles (data not shown), indicating a high affinity for phytate.

Plants in an environment with limited P availability and mobility have evolved tightly controlled mechanisms to maintain P-homeostasis, which include acquisition of P_i from soil, remobilization of stored P_i , as well as optimization of metabolic processes to conserve P_i (Rouached et al., 2010). Native to soils poor in P (Jones, 1987), *P. vittata* has adapted to environments that are depleted of available P_i by utilizing a unique phytase to facilitate phytate hydrolysis, even in the presence of As, which hinders enzymatic processes (Tsai et al., 2009). Furthermore, this unique phytase from *P. vittata* retained activity in soils, which readily sorb and inactivate plant-exuded phytases

(George et al., 2005). This is especially significant because few plants can directly obtain P_i from phytate in soils (Hayes et al., 2000; Richardson et al., 2005). *Pteris vittata* has potentially evolved a phytase that circumvents the limitations of other plant phytases, which are restricted by their capacity to be active following exudation into the soil (Hayes et al., 1999; Richardson et al., 2000).

Table 3-1. Enzyme activities in tissues of *P. vittata* (PV) and *P. ensiformis* (PE)^a

Tissue	Treatment	phosphatase activity nmol pNP mg ⁻¹ protein min ⁻¹		phytase activity nmol P mg ⁻¹ protein min ⁻¹	
		PV	PE	PV	PE
Frond	Control	10.9 ± 2.8 a	1,628 ± 299 a	2.9 ± 1.3 a	3.2 ± 0.9 a
Frond	P _i	14.4 ± 4.2 a	1,218 ± 387 a	2.2 ± 0.4 a	1.2 ± 0.3 a
Frond	As	15.2 ± 4.7 a	1,058 ± 303 a	1.6 ± 0.3 a	3.7 ± 1.1 a
Frond	As+P _i	7.7 ± 1.3 a	1,530 ± 788 a	3.4 ± 1.7 a	1.3 ± 0.7 a
Rhizome	Control	10.8 ± 1.5 a	47.8 ± 9.5 a	1.8 ± 0.3 a	1.3 ± 0.5 a
Rhizome	P _i	9.2 ± 2.0 a	20.3 ± 2.6 a	2.4 ± 0.9 a	1.3 ± 0.3 a
Rhizome	As	7.4 ± 1.2 a	42.0 ± 17 a	2.0 ± 0.5 a	1.1 ± 0.2 a
Rhizome	As+P _i	7.9 ± 1.5 a	20.3 ± 6.4 a	2.3 ± 0.8 a	0.7 ± 0.3 a
Root	Control	136 ± 29 a	158 ± 9.5 a	19.7 ± 4.2 a	7.8 ± 1.9 a
Root	P _i	74.4 ± 22 b	101 ± 18 b	5.1 ± 1.4 b	2.3 ± 0.6 b
Root	As	124 ± 18 a	151 ± 8.0 a	6.1 ± 1.2 b	6.0 ± 2.1 a
Root	As+P _i	66.6 ± 19 b	153 ± 11 a	10.8 ± 2.8 b	5.4 ± 1.0 a

^aValues are the mean of four replicates with standard error and columns with the same letters are not significantly different.

Table 3-2. Plant growth on modified MS media^a

Plant	Treatment and Fresh Weight (mg)							
	Control	As	P _i	P _i +As	P ₆	P ₆ +As	P _i +P ₆	P _i +P ₆ +As
<i>L. Sativa</i> ^b	37 ±3b	4±0.2c	125±17a	5±1c	38±3b	4±0.3c	115±23a	6±1c
<i>A. schoenoprasum</i> ^b	15 ±1b	ng ^d	31±1a	ng	15±2b	ng	32±2a	ng
<i>T. subterraneum</i> ^b	43 ±8b	ng	89±26a	ng	43±12b	ng	96±19a	ng
<i>P. ensiformis</i> ^c	2 ±0.2b	ng	95±1a	ng	2±0.1b	ng	96±1a	ng
<i>T. kunthii</i> ^c	2 ±0.3b	ng	83±1a	ng	2±0.1b	ng	81±4a	ng
<i>P. vittata</i> ^c	5 ±1d	7 ±1 d	115±5bc	225±27a	90±4cd	66 ±4 c	119±5 bc	151±5 b

^aValues are the mean of 20 replicates with standard error and rows with the same letters are not significantly different. ^bAverage biomass from one seed after 15 d. ^cBiomass from 0.05 mg spore after 40 d. ^dng = no germination

Table 3-3. Concentration of P and As (mg/kg) in *P. vittata* gametophyte^a

Treatment	Phosphorus	Arsenic
P _i	2208 ±222 ab	nd ^b
P _i +As	1579 ±307 c	1777 ±175 b
P _i +P ₆	2012 ±117 bc	nd
P ₆	2351 ±201 ab	nd
P ₆ +As	2672 ±181 a	2630 ±229 a
P _i +P ₆ +As	2138 ±93 abc	2206 ±340 a

^aValues are the mean of six replicates, bars representing standard error and columns with the same letters are not significantly different. ^bnd = none detected (≤ 5 ng/kg)

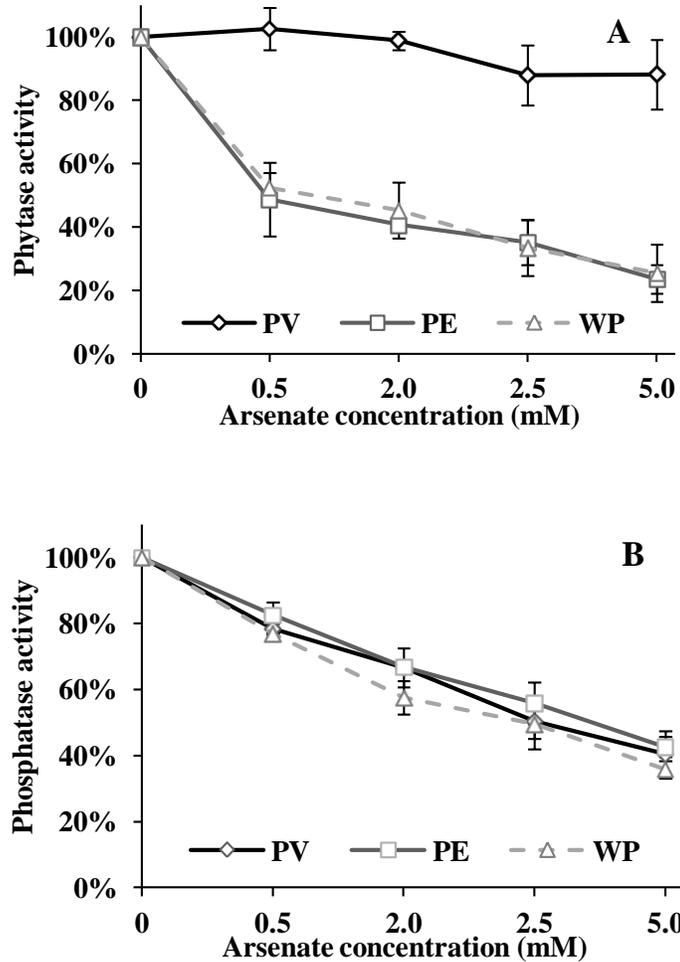


Figure 3-1. *Pteris vittata* phytase was resistant to arsenate. Phytase (A) and phosphatase (B) activities from the extracts of *P. vittata* (PV), *P. ensiformis* (PE) and purified wheat phytase (WP) were determined by incubating samples in 5 mM phytate or pNPP suspensions buffered at pH 5.0 with increasing concentrations of arsenate. Specific activity values for PV, PE, and WP were 46.7, 42.7, and 51.8 nmol P_i mg⁻¹ protein min⁻¹ for phytase and 79.5, 149, and 163 nmol pNP mg⁻¹ protein min⁻¹ for phosphatase respectively. Data are the means of ten replicates with bars representing standard error.

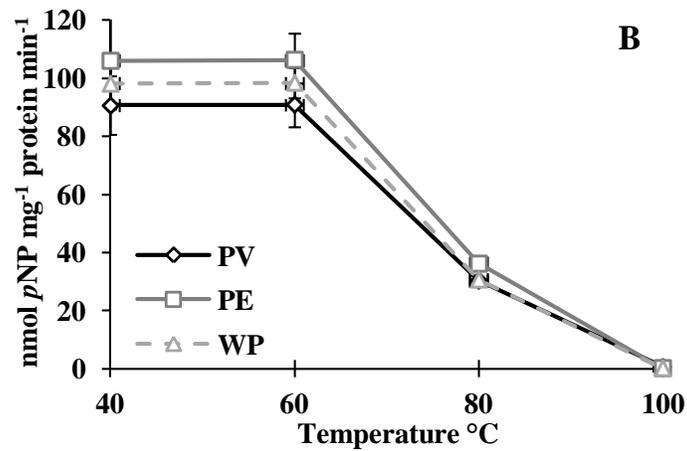
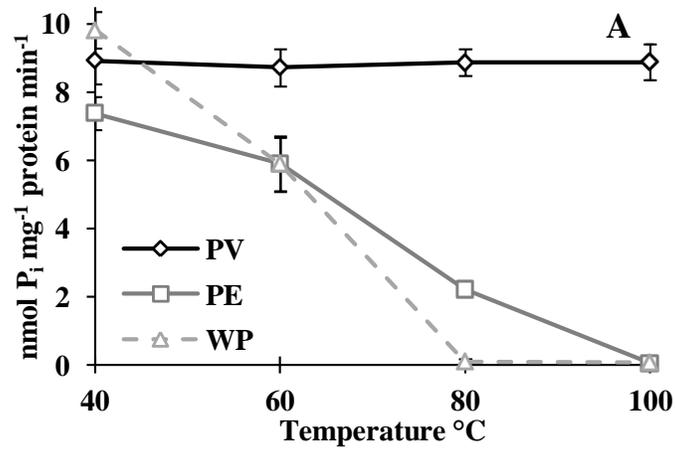


Figure 3-2. *Pteris vittata* phytase was resistant to heat shock. Phytase (A) and phosphatase (B) activities from extracts of *P. vittata* (PV), *P. ensiformis* (PE) and wheat phytase were determined by incubating 5 mM phytate or *p*NPP suspensions buffered at pH 5.0 following 10 min pretreatments in a water bath held at 40, 60, 80, or 100 °C. Data are the means of ten replicates with bars representing standard error.

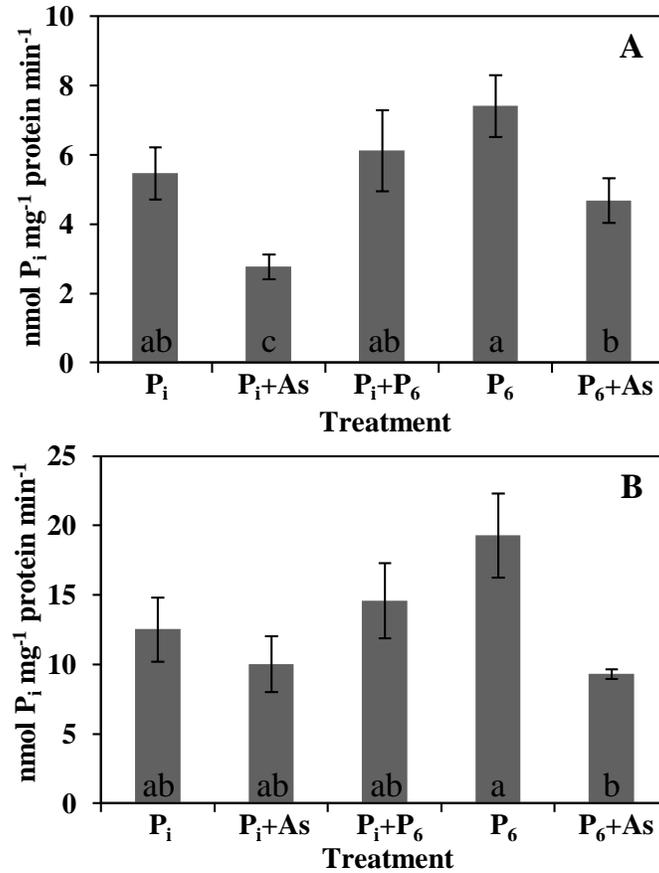


Figure 3-3. Presence of phytate increased phytase activity in *P. vittata*. Phytase activities determined from gametophyte (A) and root exudates (B) determined from *P. vittata* grown with phosphate (P_i), phytate (P_6), and arsenate (As). Data represent the mean of eight replicates with standard error and bars with the same letters are not significantly different.

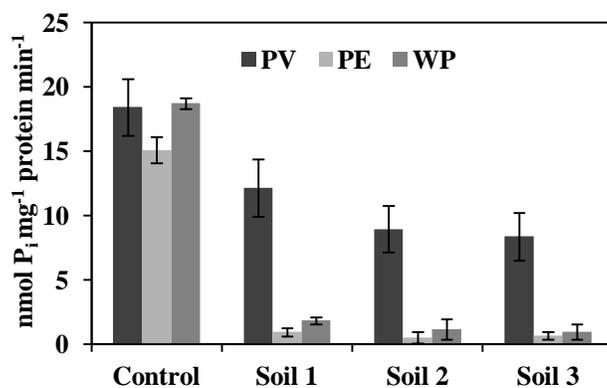


Figure 3-4. *Pteris vittata* phytase activity was resistant to soil inactivation. Phytase specific activities (nmol P_i mg⁻¹ protein min⁻¹) in the supernatant of soil suspensions after mixed with enzyme extracts of *P. vittata* (PV), *P. ensiformis* (PE), and wheat phytase (WP). Enzyme extracts were added to soil suspensions, mixed for 2 h, and centrifuged prior to activity measurement. Data are the means of four replicates with bars representing standard error.

CHAPTER 4
IMPROVED HUSBANDRY AND PHOSPHATE ROCK AMENDMENTS
SIGNIFICANTLY IMPROVE SOIL ARSENIC PHYTOREMEDIATION BY *PTERIS*
VITTATA: A TWO YEAR STUDY

Phytoremediation Using *Pteris vittata*

Due to its toxicity and carcinogenicity, arsenic is ranked by the Agency for Toxic Substances & Disease Registry as the #1 contaminant in the environment (ATSDR, 2007a). For nearly five decades (1930 to 1980), the application of arsenical pesticides (e.g., PbHAsO_4 , and CaHAsO_4) amounted to soil As additions of $\sim 1,300$ metric tons yr^{-1} (Brooks, 2012). The USEPA regional screening level for soil As under residential use averages 0.39 mg kg^{-1} while the Florida direct exposure Soil Cleanup Target Level (SCTL) is 2.1 mg kg^{-1} (Teaf et al., 2010). Despite being a known issue, As accumulation in soils is a continual problem. Natural leaching and inappropriate disposal of As-treated products pose a threat to public health and the environment. By 2002, more than 90% of all outdoor wooden structures in the U.S. were treated with copper chrome arsenate (CCA) pesticide (Gray and Houlihan, 2002). With high concentrations of As ($\sim 1,200 \text{ mg kg}^{-1}$), CCA-treated wood has a long life-span (20-50 years) and acts as a source of As contamination in the vicinity (Stook et al., 2004). Even though CCA wood was banned for residential use in 2004, $\sim 6.1 \times 10^6$ kg of As is used annually for wood treatments in the U.S. (Brooks, 2012). Normally, soil As concentrations exceeding the limit results in regulatory actions at industrial or hazardous waste sites, but no such protocols exist for residential and public spaces suggesting the presence of a widespread regulatory health crisis (Belluck et al., 2003).

Many engineering technologies have been developed for remediation of As contaminated soils, but they are costly and invasive. Methods of removal either disturb

the environment (excavation) or do not remove the As (solubilization and stabilization), allowing potential for future exposure. Alternatively, the use of phytoremediation preserves the topsoil while reducing hazardous contaminants. This technique requires no special equipment or high operating costs and can be aesthetically pleasing, garnering more public acceptance. Arsenic hyperaccumulator *Pteris vittata* L. (Chinese brake fern) (Ma et al., 2001) can accumulate up to 22,630 mg arsenic kg⁻¹ (dry weight) in the aboveground biomass, indicating its capacity for high tolerance and detoxification of As. Numerous studies have demonstrated the unique ability of *P. vittata* to tolerate and accumulate high concentrations of As, but are based on short growing periods (~12 weeks) in conditions unrepresentative of soil environments (i.e., hydroponic, small pots under glasshouse conditions) (Salido et al., 2003; Caille et al., 2004; Cao and Ma, 2004; Kertulis-Tartar et al., 2006; Baldwin and Butcher, 2007; Gonzaga et al., 2008; Shelmerdine et al., 2009). To achieve the As-SCTL in light to moderately contaminated soils, phytoremediation can require years to decades to achieve. Thus, a long-term study to elucidate conditions conducive to maximizing As uptake and biomass production is required to evaluate *P. vittata*'s full potential for phytoremediation.

Arsenate, the most prevalent form of As in soil, uses phosphate transporters in higher plants, acting as a competitive inhibitor (Meharg and Macnair, 1992). Due to shared homology of arsenate and phosphate, plant roots will readily assimilate arsenate, leading to P-deficiency (Meharg and Hartley-Whitaker, 2002). During P starvation, many plants respond by increasing root length, density of root hairs and by exuding organic compounds to mobilize different types of P-associated compounds (Raghothama and Karthikeyan, 2005). *Pteris vittata* is native to soils which are

characterized by low available P in addition to tolerating high concentrations of arsenic (Jones, 1987). These properties make *P. vittata* unique in its ability to scavenge for P, even in the presence of high concentrations of As. Arsenic concentrations in fronds and roots of *P. vittata* were shown to increase with decreasing P_i in nutrient solution (Lou et al., 2010).

To maximize the remediative capacity of *P. vittata*, soil should be supplied with a source of P with minimal availability. Phosphate rock (PR), which is the raw material used to manufacture phosphatic fertilizers could provide a long-term source of P with limited plant availability. Phosphate rock is typically not suitable as a direct substitute for soluble P fertilizers because the rate of dissolution is not adequate to meet plants demands. In a study comparing PR and mono ammonium phosphate amendments in 16 *Brassica* species, PR treatments reduced plant biomass >2.5 times and P concentration ~1.5 times in all of the cultivars (Aziz et al., 2011). There are factors that influence rate of PR dissolution like soil characteristics and plant interactions. In a PR study with white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*), dry matter yields at pH 5.3 were equivalent to mono calcium phosphate treatments, but decreased 24% at pH 5.6 and 28% at pH 6.4 (Rajan et al., 1991). Some plants influence the rate of PR dissolution by altering the rhizosphere pH, uptake of Ca, and through production of chelating organic acids (citric, malic and 2-ketogluconic acid) which complex Ca and deplete P in the soil solution (Ramaekers et al., 2010). In a PR amendment study with white lupin (*Lupinus albus*), initial limited P availability stimulated root growth and exudation allowing for increased root-induced dissolution of PR (Hinsinger and Gilkes, 1995).

In *P. vittata*, P limitation has been shown to promote root growth (Santos et al., 2008) and increase As uptake which positively influences the plant's biomass (Tu and Ma, 2003). These unique responses by *P. vittata* could be taken advantage of to improve long-term phytoremediation. Use of a slow release P fertilizer (13 g plant⁻¹) in a two-year field experiment by Kertulis-Tartar et al. (2006) led to relatively small *P. vittata* biomass in the first year (12.1 g plant⁻¹) with a slight reduction (11.7 g plant⁻¹) in the 2nd. In addition to maintaining low available P, appropriate clipping techniques need to be determined to maximize plant re-growth. In a sixteen month pot-study with three harvests by Gonzaga et al. (2008), clipping of *P. vittata* fronds at the rhizome base hindered re-growth, leading to biomass declines of 74 and 40% in the 2nd and 3rd harvests. We hypothesize that *P. vittata* can remove As on a long-term basis, over multiple harvests by maintaining low available P to increase P scavenging responses which will enhance remediation efficiency. The objectives were to: 1) demonstrate the feasibility of using *P. vittata* to remediate As-contaminated soils over a long period, 2) study the effect of PR amendments on P/As uptake, and 3) determine if proper husbandry practices improve re-growth of biomass between harvests.

Materials and Methods

Soil Collection

Three soils were collected from As contaminated areas in central Florida. Two soils (A_o and B_t horizons; Arenic Albaqualfs) were collected from abandoned cattle dipping vats (DVA and DVB), contaminated with an arsenical tickicide, and a third soil (A horizon; Grossarenic Paleudult) from an abandoned wood treatment facility which used copper chromate arsenate (CCA). Soils were air-dried, sieved through a 2 mm mesh screen and analyzed for pH (1:2 soil to water), organic matter content (Walkley-

Black method), cation exchange capacity (ammonium acetate method) and particle size (pipette method) (Tan, 2005). Soil samples were subjected to $\text{HNO}_3/\text{H}_2\text{O}_2$ digestion (USEPA Method 3051) on a hot block (Environmental Express, Ventura, CA). The digested samples were analyzed for total As concentration using graphite furnace atomic absorption spectroscopy (GFAAS, Perkin Elmer SIMMA 6000, Perkin-Elmer Corp., Norwalk, CT) and total P was measured spectrophotometrically (UVI1800U, Shimadzu Corp., Columbia, MD) at 880 nm using the molybdenum-blue reaction. Due to arsenate interference with the molybdenum reaction, samples were first incubated with cysteine at 80°C for 5 min to reduce arsenate (AsV) to arsenite (AsIII). Water-soluble P and exchangeable fractions of As were analyzed using extracts of deionized water (1:2 soil to solution ratio) and 0.05 M $(\text{NH}_4)_2\text{SO}_4/(\text{NH}_4)\text{H}_2\text{PO}_4$ (1:4 soil to solution ratio) respectively. The digests were used to determine total Fe, Al, Ca, and Mg by ICP-AES. Selected physico-chemical properties of the soils are shown in Table 4-1.

Experimental Setup

Raised beds were constructed (0.36 m² to a 35 cm depth) and filled with soil (four beds per soil) mixed with 15 g⁻¹ kg⁻¹ phosphate rock [PR, $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$ (CaCO_3)_x, <1 mm; PCS Phosphate, White Springs, Florida] and without PR as a control. The beds were watered to field capacity which was maintained for two weeks and had a final soil depth of 30 cm. In December 2009, three month old *P. vittata* (3-4 fronds ~15 cm in length) purchased from Milestone Agriculture (Apopka, Florida) were washed clean of potting mix and transplanted 15 cm apart (9 per bed) in hand dug holes ~5 cm deep. Containers (20 L) with PR amended soil without plants were maintained as a negative control. At time of transplant, P-free granulated fertilizer (N:P:K ratio of 10:0:10; 28 g⁻¹ bed; 7 g⁻¹ container, Rite Green; Sunniland Corporation, Sanford Florida) was surface

applied to PR amended soils while a granulated fertilizer with P (N:P:K ratio of 6:4:6; 35 g⁻¹ bed) was used on control soils, which was repeated bimonthly. Overhead and drip irrigation were employed to maintain soil moisture (60-80% field capacity) which was measured with a Kelway HB-2 Acidity and Moisture tester (Kel Instruments, Wyckoff, New Jersey). One application of hydrated lime was spread (28 g⁻¹ quadrant; 7 g⁻¹ container) to the surface of the dipping vat soils (A and B).

Plant Harvest

Four harvests were conducted in six month intervals (July 2010, January 2011, July 2011, and January 2012). Frond biomass was collected by cutting mature fronds ~20 cm above the rhizome, ensuring at a few leaflets remained and leaving young fiddleheads intact to expedite re-growth. Samples were oven dried at 60°C for 96 h, weighed, and ground through a 2-mm mesh screen in a Wiley Mill (Thomas Scientific, Swedesboro, NJ). Frond (0.1 g) samples were subjected to HNO₃/H₂O₂ digestion and analyzed for As and P as previously described.

Soil and Root Sampling

At planting and each harvest, 30 cm soil cores were taken with an auger (3 cm diameter) approximately 7.5 cm from the base of the ferns. Samples were taken before subsequent fertilizer applications to minimize influence of soluble P in controls. Two cores were taken from each bed, separated by depth (top 0-15 cm and bottom 15-30 cm) and composited. Samples were immediately sieved through a 2 mm screen to separate root tissue which was then weighed. Soil samples dried at 60°C for 48 h and analyzed for elemental analysis as previously described.

Statistical Analysis

Data are presented as the mean of all replicates and error bars (where shown) represent one standard error either side of the mean. Significant differences were determined using analysis of variance and treatment means compared by Duncan's multiple range test, at $p \leq 0.05$.

Results and Discussion

Soil Characteristics

Select physico-chemical properties of soils at time of collection and following the two year experiment are listed in Tables 4-1 and 4-2. The textural classes of the CCA, DVA and DVB soils were loamy sand, sand, and sandy loam respectively. Dipping vat soils (A and B) were acidic (~pH 5.2) and lightly contaminated (26 to 30 mg kg⁻¹ As). The calcareous CCA soil was slightly alkaline (pH 7.2) and moderately contaminated with As at 129 mg kg⁻¹. These concentrations substantially exceed the SCTL of 2.1 mg kg⁻¹ for Florida, indicating they are a potential health risk and require remediation.

The fraction of As in the soil that is more available for plant uptake needs to be considered when employing phytoremediation. Plant available As depends on adsorbing soil constituents (i.e. Fe, Al, Ca), pH, organic matter, and clay minerals (Zhang and Selim, 2008). Iron and Al oxides and hydroxides have particularly high affinity to As, and adsorption increases in presence of Ca (Smith et al., 2002). The CCA soil had the highest total exchangeable As (9.5 mg kg⁻¹) and DVA/B had 3.2 and 4.0 mg kg⁻¹ respectively. However, in the CCA soil, exchangeable As accounted for 7% of total As compared to ~13% in DVA and DVB soils. The smaller fraction of exchangeable As in CCA soil might be associated with the relatively high concentrations of available Ca (1541 mg kg⁻¹) along with amorphous Fe (2004 mg kg⁻¹) and Al (854 mg kg⁻¹), which

more strongly bond As, making them less available. In the DVA/B soils, exchangeable As was likely controlled by amorphous Al (~512 mg kg⁻¹) as the available Ca (~194 mg kg⁻¹) and amorphous Fe (~56 mg kg⁻¹) were low, explaining the higher percentage of exchangeable As relative to CCA soil.

Phosphate rock amendments contained 9% P, 24% Ca, 3% K and 2% Mg, and did not significantly alter soil pH, soluble P, or available Ca, K and Mg. However, control soils were slightly more acidic after two years, possibly due to buffering effects of PR (Table 4-2). Initial total P concentrations were 382, 166 and 500 mg kg⁻¹ in CCA, DVA and DVB soils respectively. Initial water soluble P concentrations were <0.4 mg kg⁻¹ in all soils and did not increase with addition of PR amendments, despite total P concentrations increasing to ~2300 mg kg⁻¹ (data not shown). Soil-related factors that increase dissolution of PR are low pH, high cation exchange and high P sorption (Robinson et al., 1994). The soluble P concentration remained stable in PR amended plant-less soils (data not shown), suggesting that soil properties had little effect on PR dissolution. This was not entirely unexpected because the PR was very coarse (size fraction: 0.05-2.0 mm) and soils were not highly acidic with relatively low cation exchange (Table 4-1). When PR is used to supply P to field crops, it is often ground to a fine powder or partially acidulated to expedite dissolution (Robinson et al., 1994). Soluble P in control soils, which were supplied with P fertilizer, ranged between 4 to 10 mg kg⁻¹ throughout the two year experiment, a ~20 fold increase over the PR amended soils (data not shown). Minimizing soluble P in soils is advantageous when nutrient leaching is a concern, which is an added benefit of using PR in lieu of soluble P fertilizer.

Harvest Scheme Improved Re-growth of *P. vittata*

Hyperaccumulation based phytoremediation is influenced by rate and amount of biomass production. *Pteris vittata* is a perennial fern requiring repeated frond removal to facilitate the remediation process. After six months of growth, regardless of treatment, harvested frond biomass averaged 17 g plant⁻¹, increasing to 19, 28, and 34 g plant⁻¹ in the 12, 18, and 24 month harvests, respectively (Figure 4-1). A six-month harvest interval was used because it coincides with peak frond maturity which minimizes As loss via frond senescence (Kertulis-Tartar et al., 2006) and spore dispersal (Lombi et al., 2002). Furthermore, at harvest, fronds were clipped ~15 cm from the rhizome base and fiddleheads were left intact to promote faster re-growth. Overall, frond clippings yielded an average 100 g (or 50 g year⁻¹) of biomass over four harvests, with an increase of 26% in between each harvest (Figure 4-1). In similar long-term phytoremediation experiments with multiple harvests, yearly average biomass yields were 12, 6, 32, 40, and 8 g plant⁻¹ (Caille et al., 2004; Li et al., 2005; Kertulis-Tartar et al., 2006; Gonzaga et al., 2008; Shelmerdine et al., 2009). In each experiment, re-growth was slowed, reducing harvestable biomass in subsequent harvests. For example, in separate pot studies, initial harvests of 13 and 29 g plant⁻¹ were reduced to 4 and 8 g plant⁻¹ in the 2nd harvest, an average reduction of 73% (Caille et al., 2004; Gonzaga et al., 2008). These declines were attributed to cutting fronds at the rhizome, which severely hinders the plant due to the lack of photosynthesis, forcing the plant to rely on carbon stores in the roots and rhizome for re-growth (Wade and Westerfield, 2009). Furthermore, through the use of containers,

plant and root environments are restricted due to small reservoirs for water and short substrate columns that adversely affect drainage (Fonteno, 1993).

The results in this study represent the first report of increasing frond biomass of *P. vittata* over subsequent harvests. Average increase in frond biomass between harvests exhibited a linear increase ($R^2 = 0.95$) which is likely attributed to increased soil volume (compared to containers), modified harvesting practices, and a consistent fertilizing regime.

Phosphate Rock Amendments Increased *P. vittata* Biomass

Pteris vittata, which is native to soils with limited P-availability, responded to PR amendments by increasing frond and root biomass relative to controls. In four harvests over two years, frond biomass of *P. vittata* in PR amended soils averaged 115 g plant^{-1} compared to 82 g plant^{-1} in control soils, a 40% increase (Figure 4-1). Similarly, root density of two-year old *P. vittata* plants (fresh weight) in PR amended soils averaged 64 g kg^{-1} soil compared to 42 g kg^{-1} soil in control treatments, a 52% increase. In addition to having larger root biomass, *P. vittata* roots in PR amended soils had extensive root hair and adventitious root growth which were absent in control roots (Figure 4-2). This increase in root mass maximizes interactions at the root-soil interface, allowing for more nutrient acquisition, including PR mineralization (Péret et al., 2011). Similar observations have been made in *Lupinus albus*, where limiting P availability increased root biomass and proteoid root development facilitating dissolution of PR in the rhizosphere (Hinsinger and Gilkes, 1995). This reaction is very species specific however, as many plants are not capable of mineralizing PR at a rate that provides enough P to meet the plant demands (Rajan and Watkinson, 1992). In a study with 15 wheat cultivars, average shoot biomass in PR treatments was reduced ~1.5 times

compared to ammonium phosphate treatments (Yaseen and Malhi, 2009). The observed increase in growth suggests that *P. vittata* may be uniquely adapted to P-limiting environments, especially due to its novel ability to hyperaccumulate and tolerate As.

Phosphate Rock Improved Arsenic Uptake in *P. vittata*

Fronde arsenic concentrations ranged from 764 - 3480 mg kg⁻¹ in the first harvest to 264 - 1813 mg kg⁻¹ in the fourth harvest. The addition of PR amendments increased average frond As concentrations ~78% in the first year and ~43% in the second in all soils (Table 4-3). In CCA soil amended with PR, frond As concentrations averaged 3150 mg kg⁻¹ in the first year and 2290 mg kg⁻¹ in the second. Comparatively, CCA control fronds averaged 1500 mg kg⁻¹ As in the first year and 1900 mg kg⁻¹ in the second. The increased *P. vittata* biomass (Figure 4-1) and As uptake in PR amendments doubled As accumulation from CCA soil, increasing from 172 in controls to 345 mg plant⁻¹ over four harvests (Table 4-3). This trend also extended to DVA and DVB soils, with PR amendments increasing As uptake in *P. vittata* to ~93 mg plant⁻¹ from ~40 mg plant⁻¹ in controls.

The increased As uptake in PR amended soils can be partly attributed to the limited soluble P (<0.4 mg kg⁻¹) maintained throughout the two year study. Arsenic and P exert antagonistic effects on each other during plant uptake and transport (Meharg and Hartley-Whitaker, 2002). Thus, even though soluble P fertilizers increase As availability, root uptake is negatively impacted, resulting in lower frond As accumulation (Table 4-3). The use of PR circumvents this problem by supplying the plant with a source of P without elevating soluble P concentrations in the soil. This encourages greater root exploration to increase PR mineralization, which has the added benefit of

increasing As uptake. Furthermore, As is known to stimulate biomass growth in *P. vittata*. In a study by Gumaelius et al. (2004), addition of 75 mg kg⁻¹ As to growth media improved total *P. vittata* biomass by 20% while 375 mg kg⁻¹ As increased growth by an additional 55%. Plants in fertilized control soils lacked these growth promoting benefits (Figure 4-1) due to increased P availability which decreased plant affinity for As relative to PR amended plants (Table 4-3).

The approximate two-fold increase in As uptake from harvested *P. vittata* biomass in PR amended soils is best illustrated by comparing bioconcentration ratios. The bioconcentration ratio (BC), which is the ratio of frond to soil As concentration; showed plants in PR amendments were more efficient in extracting As in all soils (Table 4-3). The sustained high BC rates demonstrated by *P. vittata* in PR amendments are critical to facilitating more rapid As removal. Average BC ratio for total soil As increased from 21 in the control to 40 in PR amendments and from 169 to 327 for available soil As. Even though exchangeable As concentrations were ~53% higher in control soils, BC rates were half of PR. The effect of PR amendments on BC was significant, which is impressive considering that BC ratios in our controls were very high. For comparison, in similar long-term phytoremediation studies using *P. vittata*, BC rates for total As were reported to be 3, 6, 18 and 10 (Caille et al., 2004; Cao and Ma, 2004; Kertulis-Tartar et al., 2006; Gonzaga et al., 2008). The lower BC's are likely attributed to poor re-growth from severe clipping and absence of sufficient fertilization schemes.

***Pteris vittata* P Acquisition**

During periods of the low P availability, plants respond morphologically to facilitate acquisition of P from previously unavailable sources (Vance et al., 2003). Since *P. vittata* is native to calcareous soils (Jones, 1987), they may share similarities with other

acidifuge plants, known to effectively mobilize P from previously unavailable sources (Ström et al., 1994). In the PR treatments, *P. vittata* responded to low soluble P by increasing root biomass which would enhance P uptake. Total P concentration in fronds grown in PR treated soils averaged 1,975 mg kg⁻¹ compared to 2,260 mg kg⁻¹ in the controls (Table 4-5). Since PR is highly insoluble and the control plants were supplied with a continuous supply of P, it was not surprising that frond biomass from PR treatments had slightly lower concentrations of P. However, considering the increased frond biomass in PR treatments, total P uptake over four harvests increased from 181 mg plant⁻¹ in controls to 224 mg plant⁻¹, a 23% increase in the PR amended soil (figure 4-3). The P-limiting environment in the PR treatments induced greater root biomass in *P. vittata* containing more adventitious roots and root hairs (Figure 4-2) which are more metabolically efficient in acquiring P due to the large absorptive surface area relative to the root volume (Ramaekers et al., 2010). Furthermore, *P. vittata* roots increase exudation of dissolved organic carbon content including oxalic and malic acid in P limiting conditions (Lou et al., 2010). In the PR amended soil, these exudates would facilitate P acquisition by increasing dissolution of PR. Frond tissue in PR amended soils averaged 2480, 1680, and 1720 mg kg⁻¹ P in CCA, DVA and DVB soils respectively. Frond biomass from CCA soils had the highest P concentrations despite the alkaline soil conditions, suggesting that mineralization of PR was root-mediated. Similar results have been observed in *L. albus*, whose larger root biomass and proteoid root development was shown to dissolve PR in an alkaline soil (Hinsinger and Gilkes, 1995). Furthermore, soluble P in plant-less PR amended soils did not increase over time (data not shown), indicating PR solubilization was not influenced by soil properties.

Soil Arsenic Removal

Regardless of treatment, *P. vittata* significantly lowered As concentrations ($p < 0.05$) in all three soils, exhibiting a linear decline over two years ($R^2 > 0.97$; Figure 4-4). Phosphate rock amendments improved As removal, decreasing concentrations in all soils ~41% compared to ~27% in controls. The largest decline after two years was observed in the CCA soil, which began at 130 mg kg^{-1} , lowering to 98 mg kg^{-1} in control and 88 mg kg^{-1} in PR treatments. A similar trend was observed in DVA and DVB soils, with average As reductions of 28% in control and 46% in PR amendments (Figure 4-4B and C). Compared to controls, PR amendments removed 7% (10 mg kg^{-1}), 12% (3 mg kg^{-1}), and 24% (7.1 mg kg^{-1}) more As from CCA, DVA and DVB soils respectively.

Following two years of growth with *P. vittata*, depletion of exchangeable As in CCA soil was reduced from 9.5 to 4.3 mg kg^{-1} in PR amendments and to 6.4 mg kg^{-1} in control treatments (Table 4-2). In DVA and DVB soils, exchangeable As was reduced 18 and 36% in PR amendments, respectively while increasing ~11% in control fractions. Higher concentrations of exchangeable As in control soils can be attributed to the 21-fold increase (0.19 to 4.0 mg kg^{-1}) in soluble P from fertilizers which displace As due to competition at soil sorption sites (Smith et al., 2002). In our experiment, the exchangeable As concentrations did not correlate with of As uptake by *P. vittata*. After two years, control soils contained 34% more exchangeable As than PR amended soils, but As uptake by *P. vittata* remained more efficient in PR amended soils (Table 4-4).

Depth of As removal is another important factor to consider in successful phytoremediation. Regardless of soil or treatment, approximately 16% more As was removed from the top 15 cm of soil than the bottom 15-30 cm (Figure 4-5). The root densities were similarly structured, with slightly less biomass associated with the 15-30

cm fraction (data not shown). This shows that *P. vittata* can readily access the top 30 cm of soil, further exemplifying its ability to remediate As.

In both treatments, *P. vittata* removed equal or more As in the 2nd year compared to the 1st, showing that remediation efficiency increased over time. This observation is unique compared to other phytoremediation experiments where As removal declined after the first harvest, increasing estimated time length for remediation (Caille et al., 2004; Gonzaga et al., 2008; Kertulis-Tartar et al., 2006; Shelmerdine et al., 2009). The combination maintaining low soluble P with PR, increased soil volume, and improved husbandry practices were shown to drastically improve rate and efficiency of soil As remediation using *P. vittata* (Table 4-6). Based on the rate of As removal, we estimate that the SCTL of 2.1 mg kg⁻¹ could be achieved in approximately 6, 5, and 4 years for CCA, DVA, and DVB soils respectively. Based on As removal from similar experiments, we have shown that *P. vittata* can remediate moderately contaminated soils 5-10 times faster than previously reported (Table 4-6).

Conclusion

The majority of outdoor wooden structures in the U.S. were treated with arsenical pesticide which readily leaches into soil. Due to the abundance and relatively small areas affected, phytoremediation using *P. vittata* is ideal to clean and protect public health. In this study, we show that maintaining low soluble P concentrations in natural soil environments with improved husbandry practices dramatically improves the remediation capacity of *P. vittata*. Furthermore, we show that *P. vittata* can accumulate As more efficiently over time, suggesting it is a viable option to achieve SCTL's. *Pteris vittata* provides a plausible, sustainable, and affordable solution to the pervasive soil As

contamination in the U.S. and around the world, especially for residential areas, where problems need to be addressed.

Table 4-1. Selected physiochemical properties of soils used in this study

Soil characteristic	CCA	DVA	DVB
pH	7.2	5.3	5.1
Total As (mg kg ⁻¹)	129.4	25.5	29.9
Exchangeable As (mg kg ⁻¹) ^a	9.5	4.0	5.5
Water soluble P (mg kg ⁻¹)	0.38	0.09	0.09
Amorphous Al (mg kg ⁻¹) ^b	854	543	481
Amorphous Fe (mg kg ⁻¹)	2004	83	29
Available Ca (mg kg ⁻¹) ^c	1541	132	256
Available Mg (mg kg ⁻¹)	115	18	54
Available K (mg kg ⁻¹)	27	12	23
Organic matter (%)	1.1	2.2	0.4
CEC (cmol ⁺ kg ⁻¹)	7.8	3.3	12.4
Sand (%)	86.3	95.5	80.7
Silt (%)	9.9	2.7	6.6
Clay (%)	3.8	1.8	12.7
Textural class	Loamy sand	Sand	Sandy loam

^a Ammonium phosphate, 0.05 mM

^b Oxalic acid + ammonium oxalate, 0.2 M

^c Mehlich III

Table 4-2. Physicochemical properties of soils amended with phosphate rock (PR) or control treatments following two years of growth with *P. vittata*

Soil characteristic	CCA		DVA		DVB	
	PR	Control	PR	Control	PR	Control
pH	7.3 ±0.1	7.0 ±0.1	5.8 ±0.1	5.5 ±0.2	5.8 ±0.2	5.0 ±0.1
Total As (mg kg ⁻¹)	88.3 ±3.2	98.2 ±3.8	15.2 ±0.5	18.2 ±0.6	14.5 ±1.0	21.6 ±0.9
Exchangeable As (mg kg ⁻¹) ^a	4.3 ±0.2	6.4 ±0.8	3.3 ±0.2	4.4 ±0.1	3.5 ±0.6	6.2 ±0.3
Water soluble P (mg kg ⁻¹)	0.38 ±0.01	4.2 ±0.2	0.15 ±0.01	4.0 ±0.3	0.15 ±0.01	4.0 ±0.4
Amorphous Al (mg kg ⁻¹) ^b	870 ±26	947 ±34	484 ±16	358 ±48	281 ±15	400 ±12
Amorphous Fe (mg kg ⁻¹)	1958 ±29	2165 ±54	63 ±1.3	53 ±14	28 ±1	31 ±3
Available Ca (mg kg ⁻¹) ^c	1680 ±96	1878 ±153	192 ±8	239 ±65	334 ±80	274 ±77
Available Mg (mg kg ⁻¹)	235 ±9	298 ±6	73 ±7	92 ±6	156 ±37	131 ±4
Available K (mg kg ⁻¹)	83 ±17	127 ±41	37 ±8	30 ±5	37 ±6	151 ±15

^a Ammonium phosphate, 0.05 mM

^b Oxalic acid + ammonium oxalate, 0.2 M

^c Mehlich III

Table 4-3. Arsenic concentration and uptake in *P. vittata* frond biomass

Soil	Treatment	Frond As concentration, mg kg ⁻¹				Frond As uptake, mg plant ⁻¹			
		Harvest 1	Harvest 2	Harvest 3	Harvest 4	Harvest 1	Harvest 2	Harvest 3	Harvest 4
CCA	PR	3480 ±328	2826 ±203	2762 ±112	1813 ±65	70 ±11	82 ±8	107 ±9	86 ±6
	Control	1489 ±360	1222 ±58	2764 ±199	1665 ±99	21 ±6	26 ±2	71 ±9	54 ±10
DVA	PR	1387 ±192	909 ±14	767 ±110	524 ±40	32 ±6	17 ±1	23 ±3	18 ±1
	Control	771 ±13	385 ±36	843 ±110	523 ±26	11 ±2	6 ±1	21 ±4	17 ±3
DVB	PR	1798 ±261	869 ±64	724 ±80	681 ±97	33 ±5	17 ±1	22 ±4	23 ±3
	Control	764 ±43	439 ±164	251 ±38	264 ±20	10 ±1	5 ±2	4 ±1	6 ±1

Table 4-4. Bioconcentration factor^a

Soil	Treatment	Exchangeable Soil As ^b				Total Soil As			
		Harvest 1	Harvest 2	Harvest 3	Harvest 4	Harvest 1	Harvest 2	Harvest 3	Harvest 4
CCA	PR	518 ±49	380 ±27	607 ±31	421 ±10	29 ±3	25 ±2	27 ±2	21 ±1
	Control	242 ±59	140 ±7	441 ±32	260 ±16	12 ±3	10 ±1	26 ±2	17 ±1
DVA	PR	392 ±54	331 ±5	210 ±20	158 ±8	61 ±8	48 ±1	45 ±5	35 ±2
	Control	203 ±4	121 ±11	165 ±19	118 ±6	31 ±1	16 ±2	42 ±5	27 ±1
DVB	PR	281 ±41	376 ±28	149 ±11	194 ±18	70 ±9	38 ±3	31 ±2	50 ±9
	Control	175 ±10	94 ±35	52 ±9	43 ±3	27 ±2	17 ±6	12 ±2	12 ±1

^a Concentration ratio of arsenic in fronds to soil

^b Ammonium phosphate, 0.05 mM

Table 4-5. Frond P concentration, mg kg⁻¹

Soil	Treatment	Harvest 1	Harvest 2	Harvest 3	Harvest 4
CCA	PR	2569 ±188	2519 ±110	2774 ±60	2066 ±74
	Control	3052 ±343	2735 ±397	3293 ±237	2424 ±118
DVA	PR	1901 ±23	1825 ±48	1524 ±41	1557 ±35
	Control	1743 ±45	2009 ±291	2134 ±73	2764 ±83
DVB	PR	2133 ±58	1778 ±75	1523 ±16	1530 ±42
	Control	1579 ±21	1688 ±21	1701 ±28	1829 ±66

Table 4-6. Multi-harvest phytoremediation studies with *P. vittata* grown in moderately^a contaminated soils

Reference	Fronde biomass ^b g year ⁻¹	As uptake mg plant ⁻¹ year ⁻¹	BC	Soil As removed ^c kg ha ⁻¹ year ⁻¹	Time ^d to remediate, years
Gonzaga et al. (2008)	40	32	10	14	~17-30
Kertulis-Tartar et al. (2006)	12	40	18	18	~18-28
Caille et al. (2004)	24	18	3	8	~25-63
Shelmerdine et al. (2009)	6	1	<1	<1	>100
Li et al. (2005)	6	15	18	7	~67-70
This study	76	172	26	76	~6-7

^aSoil As concentration range was 100 to 360 mg kg⁻¹

^bAverage harvested biomass normalized to one year

^c Assuming 15 cm plant spacing on a soil containing 125 mg kg⁻¹ As

^dTime estimates are based on the highest and average plant As uptake values reported and assumes a final soil As concentration < 2 mg kg⁻¹

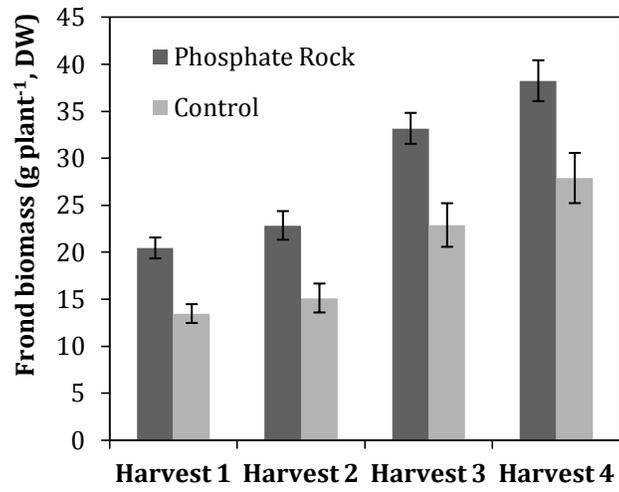


Figure 4-1. Harvested frond biomass from *P. vittata* (g plant⁻¹, dry weight) increased at each six-month harvest in CCA, DVA and DVB soils with phosphate rock amendments significantly improving biomass over the control ($p \leq 0.05$). Values are the mean of 4 replicates with standard error.



Figure 4-2. Two year old *P. vittata* roots growing in phosphate rock amended CCA soils contained abundant adventitious roots and root hairs. Photo courtesy of Jason Lessl.

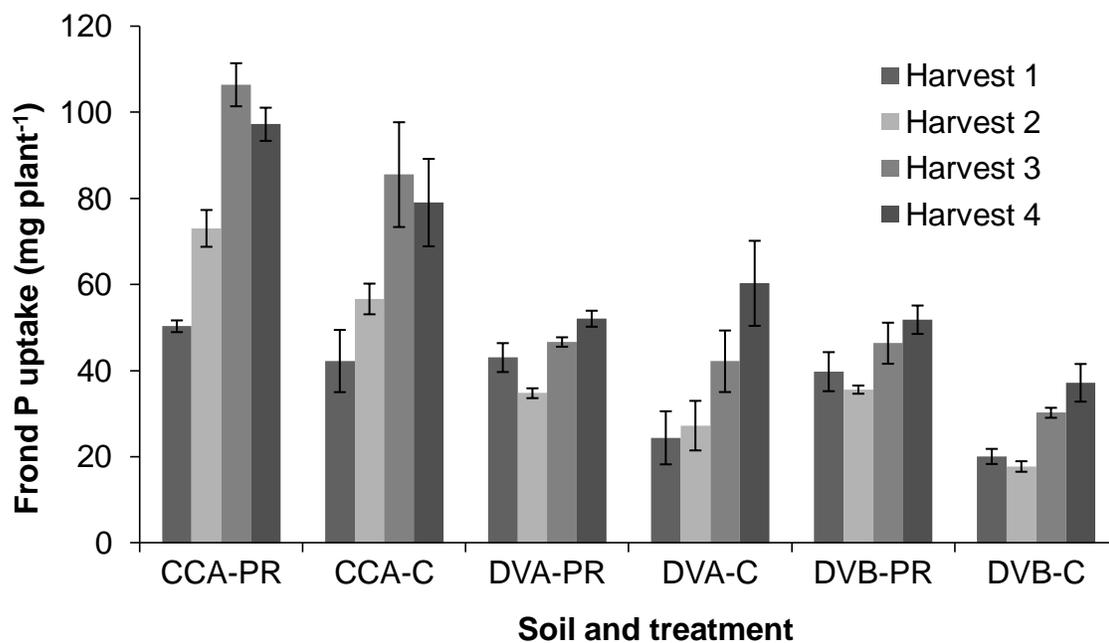


Figure 4-3. Total P uptake in *P. vittata* frond biomass collected at each six-month harvest from CCA, DVA and DVB soils with phosphate rock (PR) and control (C) amendments from July 2009 to Jan 2012. Values are the mean of 4 replicates with standard error.

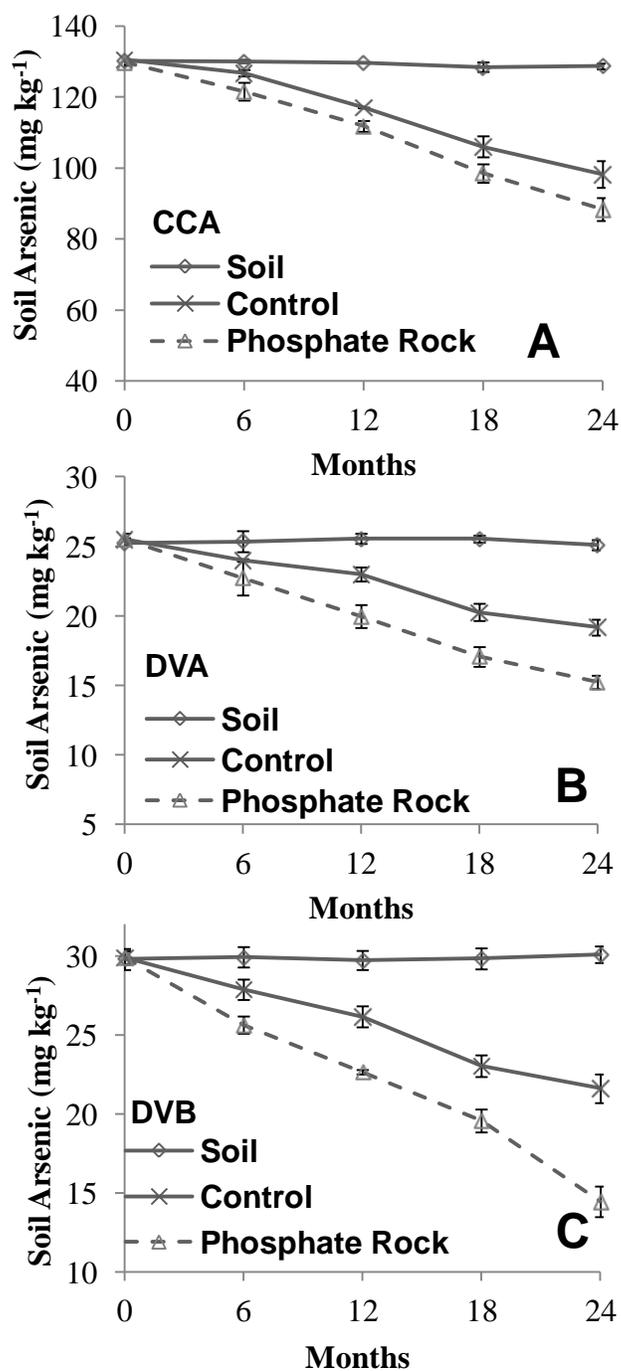


Figure 4-4. Soil As concentrations (mg kg⁻¹) declined at a linear rate in CCA (A), DVA (B) and DVB (C) soils over two years. Phosphate rock amendments significantly improved As removal in all soils compared to controls ($p \leq 0.05$). The soil line represents the plant-less control. Values are the mean of 4 replicates, bars representing one standard error.

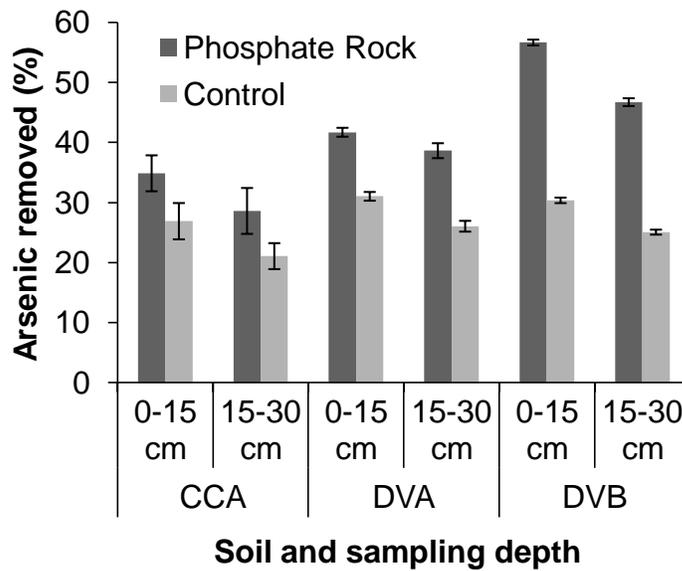


Figure 4-5. Soil As removed from the top 0-15 and bottom 15-30 cm fractions of CCA, DVA and DVB soils with phosphate rock and control amendments after two years of *P. vittata* growth. Values are the mean of 4 replicates, bars representing one standard error.

CHAPTER 5
ARSENIC DISTRIBUTION IN THE SOIL AND FROND OF *PTERIS VITTATA* L.
DURING PHYTOEXTRACTION HARVESTED OVER TWO YEARS

Arsenic Soil Distribution

Human exposure to arsenic increases mortality through acute toxicity and multiple internal organ cancers (liver, kidney, lung, and bladder) (ATSDR, 2009). ATSDR ranks arsenic number one on the 2001 CERCLA Priority List of Hazardous Substances (ATSDR, 2007a). The soil arsenic chronic Reference Dose Media Evaluation Guide (RMEG) is 20 mg kg⁻¹ for non-carcinogenic effects and 0.5 mg kg⁻¹ for the Cancer Risk Evaluation Guide (CREG) (MPCA, 1999). One of the most pervasive sources of soil As-contamination comes from arsenical insecticides in wood treatments. Until 2002, more than 90% of all outdoor wooden structures in the United States were made with As-treated lumber which averages 1200 mg kg⁻¹ As, accounting for an estimated 550 million pounds of arsenic from 1964 to 2001 (Gray and Houlihan, 2002). Arsenic continually leaches from treated wood, acting as a large reservoir for environmental contamination (Belluck et al., 2003).

Although a number of techniques exist to remove arsenic from soils, most sites remain contaminated due to high economic and environmental costs. Owing to the large number of potentially contaminated areas (i.e., residential decks, fencing, playgrounds), methods of environmental restoration using plant-based technology offers a viable alternative. Phytoremediation is a cost effective and environmentally friendly remediation method for contaminated soils that is especially well-suited for As-remediation associated from treated lumber.

Due to its high rate of As-accumulation, fast growth, and high production of biomass, *Pteris vittata* L. (Chinese brake Fern) is ideal for phytoremediation (Ma et al.

2001). The capacity to remove high concentrations of arsenic from soil offers an easy, non-invasive, and cost-effective remediation method compared to traditional clean-up techniques (U.S. Environmental Protection Agency, 2002). Successful phytoremediation depends on several factors including the extent of soil contamination, As-availability, and the plants' capacity to mobilize, absorb, and accumulate arsenic into shoots. Understanding the mechanisms and processes that govern As-uptake will help improve the utility of *P. vittata* as a viable clean-up option.

Arsenic is slowly mobile in soils and exists predominantly in its most stable form, As(V), a deprotonated oxyanion, including the arsenate anion, AsO_3^{4-} . Arsenic sorption largely depends on the amount of amorphous Al and Fe oxyhydroxides which exist as colloidal precipitates, surface coatings and along edges of clay minerals (Bissen and Frimmel, 2003). The continual physical, chemical and biological processes in soil effect As-redistribution among solid-phase components. Using the sequential extraction procedure developed by Wenzel et al. (2001), arsenic soil distribution can be operationally defined as being soluble, exchangeable, amorphous hydrous oxide-bound, crystalline hydrous-oxide bound, and residual. The soluble and exchangeable fractions represent the most environmentally important forms of As due to their increased bioavailability. Furthermore, these fractions are a good indicator of bioavailability for plant uptake since plants preferentially take up their nutrients from the soil solution (Linehan et al., 1985) (McBride, 1994).

In a phytoremediation experiment by Fitz et al., (2003), soil As concentrations associated with the bioavailable fraction did not decrease during the 41 d experiment despite substantial As removal by *P. vittata*. This was attributed to the large buffering

capacity of the soil, with As from amorphous and crystalline-bound fractions replenishing the bioavailable fractions. This was also observed by Gonzaga et al. (2006) during a 56 d phytoremediation experiment with *P. vittata* who found most As removed from soil was associated with the the amorphous-bound fraction. The capacity of the soil to replenish the soluble and exchangeable forms of metals depend on the diffusion rates (Hinsinger et al., 2005). Fractionation provides an understanding of the relative mobility and bioavailability of metals in soils (Fitz and Wenzel, 2002; Gonzaga et al., 2006). This is because plant metal uptake or metal toxicity is related to those fractions (Gulz et al., 2005). Soluble and exchangeable forms of any nutrient or metal are considered to be the most available to plants (Jungk, 2001). All these factors influence the ability of *P. vittata* to access As from soils, but little is understood about the As redistribution over several years.

Monitoring changes of bioavailable As fractions and its redistribution from less available pools during long periods of phytoextraction is essential to evaluate the efficiency of phytoremediation (Fitz and Wenzel, 2002; Wenzel et al., 2003). In order to understand how different fractions change As lability during remediation over a long-period, we used an operationally-defined sequential fractionation method to evaluate the effect of As distribution in soils by *P. vittata* over several growing seasons.

Materials and Methods

Soil Collection

Three soils were collected from As contaminated areas in central Florida. Two soils (A₀ and B_t horizons; Arenic Albaqualfs) were collected from abandoned cattle dipping vats (DVA and DVB), contaminated with an arsenical tickicide, and a third soil (A horizon; Grossarenic Paleudult) from an abandoned wood treatment facility which

used copper chromate arsenate (CCA). Soils were air-dried, sieved through a 2 mm mesh screen and analyzed for pH (1:2 soil to water), organic matter content (Walkley-Black method), cation exchange capacity (ammonium acetate method) and particle size (pipette method) (Tan, 2005). Plant and soil samples were subjected to $\text{HNO}_3/\text{H}_2\text{O}_2$ digestion (USEPA Method 3051) on a hot block (Environmental Express, Ventura, CA). The digested samples were analyzed for total As concentration using graphite furnace atomic absorption spectroscopy (GFAAS, Perkin Elmer SIMMA 6000, Perkin-Elmer Corp., Norwalk, CT). The digests were also used to determine total Fe, Al, Ca, and Mg by ICP-AES. The improved sequential extraction procedure developed by Wenzel et al. (2001) was followed to fractionate arsenic into five operationally-defined fractions, including soluble (S), exchangeable (E), amorphous hydrous-oxide bound (A), crystalline hydrous-oxide bound (C), and residual (R).

Experimental Setup

Raised beds were constructed (0.36 m^2 to a 35 cm depth) and filled with soil (four beds per soil) mixed with $15 \text{ g}^{-1} \text{ kg}^{-1}$ phosphate rock [PR, $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2 (\text{CaCO}_3)_x$, <1 mm; PCS Phosphate, White Springs, Florida]. In December 2009, three month old *P. vittata* (3-4 fronds ~15 cm in length) purchased from Milestone Agriculture (Apopka, Florida) transplanted 15 cm apart (9 per bed). Containers (20 L) with PR amended soil without plants were maintained as a control. Granulated fertilizer (N:P:K ratio of 10:0:10; 28 g^{-1} bed; 7 g^{-1} container, Rite Green; Sunniland Corporation, Sanford Florida) was applied bimonthly. A roof was constructed over the beds using clear corrugated plastic roofing material with a light transmission rating of 93%. Overhead and drip irrigation were employed to maintain soil moisture (60-80% field capacity) which was measured with a Kelway HB-2 Acidity and Moisture tester (Kel Instruments, Wyckoff,

New Jersey). An application of lime was spread (28 g^{-1} quadrant; 7 g^{-1} container) to the surface of the dipping vat soils (A and B).

Soil Sampling and Plant Harvest

Four harvests were conducted in six month intervals (July 2010, January 2011, July 2011, and January 2012). Fronds were collected by cutting stems ~20 cm above the rhizome and were immediately separated by age. Young and mature fronds were differentiated by the development of sori (green/absent or brown) while senescent tissues were characterized by a browning of the leaflets. Fronds were further separated by stem (rachis and stipe) and leaflets. At planting and each harvest, 30 cm soil cores were taken with an auger (3 cm diameter) approximately 7.5 cm from the base of ferns. Samples were oven dried at 60°C for 96 h, weighed, and sieved through a 2-mm mesh screen. Soil and plant digests were subjected to $\text{HNO}_3/\text{H}_2\text{O}_2$ digestion for As analysis. Soil and mature frond digests were used for elemental analysis.

Statistical Analysis

Data are presented as the mean of all replicates and error bars (where shown) represent one standard error either side of the mean. Significant differences were determined using analysis of variance and treatment means compared by Duncan's multiple range test, at $p \leq 0.05$.

Results and Discussion

Soil Characteristics

Select physico-chemical properties of soils at time of planting are listed in Table 1. The calcareous CCA soil was slightly alkaline (pH 7.2) and moderately contaminated with As at 129 mg kg^{-1} . Dipping vat soils (A and B) were acidic (~pH 5.2) and lightly

contaminated with As (26 to 30 mg kg⁻¹). These As-concentrations exceed the RMEG and CREG, indicating they are a potential health risk and require remediation.

Phosphate rock amendments were very coarse (size fraction: 0.05-2.0 mm) and contained 9% P, 24% Ca, 3% K and 2% Mg which did not significantly alter soil pH, soluble P, or available Ca. Normally, PR is ground to a fine powder or partially acidulated to expedite dissolution (Robinson et al., 1994). However, to more accurately assess the effect *P. vittata* has on As-distribution, it was important to minimize soil P_i concentrations as it displaces As in the exchangeable fraction. Water soluble P concentrations were <0.4 mg kg⁻¹ in all soils and did not increase with PR amendments (Table 5-1). Furthermore, low soluble P has the advantage of lowering nutrient leaching, which is an added benefit of using PR in lieu of soluble P fertilizer.

Biomass and Arsenic Accumulation in *Pteris vittata*

Pteris vittata is a perennial fern, so for phytoremediation, numerous frond harvests need to be employed to expedite the clean-up process. A six-month interval was used because it coincided with peak frond maturity. After four harvests in two years, *P. vittata* grown in CCA, DVA, and DVB soils produced 4.54, 3.45, and 3.49 kg of frond biomass (dry weight) with average As concentrations of 2720, 900, and 1020 mg kg⁻¹, respectively. Arsenic concentrations in the frond declined in subsequent harvests, from 3480 to 1813 mg kg⁻¹ in CCA, 1387 to 524 in DVA, and 1800 to 681 in DVB soils. Each plant yielded an average 60 g of frond biomass per year, increasing 18% between six month harvests. In similar studies with *P. vittata* using multiple harvests, yearly average biomass yields were 12, 6, 32, 40, and 8 g plant⁻¹ (Caille et al., 2004; Li et al., 2005; Kertulis-Tartar et al., 2006; Gonzaga et al., 2008; Shelmerdine et al., 2009). These declines were attributed to cutting fronds at the rhizome, which severely hinders the

plant due to the lack of photosynthesis (Wade and Westerfield, 2009). Our results showed that *P. vittata* proliferated with raised beds, PR amendments and the use of a regimented fertilizing schedule. Furthermore, plant re-growth between harvests was promoted by leaving fiddleheads and a few leaflets from mature fronds.

FronD As distribution was highest in *P. vittata* leaflets followed by spores and then stems. Tissue As concentrations correlated with total soil As levels (Table 5-1), with fronds from CCA soil containing ~4 times more As than from DVA and DVB soils (Table 5-2). This also extended to spores, which contained ~1170 mg kg⁻¹ As compared to ~146 mg kg⁻¹ from plants in dipping vat soils. Arsenic concentrations declined in leaflets and stems at maturity. However, mature tissues accounted for ~56% of the harvested biomass compared to ~20% in young leaflets, equating to substantially more As uptake. The As-decline between young and mature tissues is likely attributed to a diluting effect due to plant growth. Of the leaflets, the senescent tissue had the smallest As concentrations while senescent stem concentrations were similar to mature. The continued As-decline in senescent fronds can be attributed to As leaching and volatilization as they dry. Tu et al. (2003) found that during air-drying of *P. vittata* fronds, As concentrations dropped ~15%. In a field study with *P. vittata*, As concentrations were 49 and 25% less in the senescent fronds over two harvests (Kertulis-Tartar et al., 2006).

FronD elemental concentrations fell into normal ranges for mature plants and were similar to a previous report on nutrient uptake by *P. vittata* (Tu and Ma, 2005), indicating that soil type or phosphate rock amendments had no apparent negative impact on overall plant health (Table 5-3). Except for P, As and Mn, elemental analysis of fronds

showed little difference between all three soils. The higher P and As in fronds from CCA soil is due to the higher As concentrations, which have been shown to correlate with each other in tissues. In a hydroponic study, the addition of As up to 30 mg L⁻¹ induced higher P uptake by *P. vittata* (Tu and Ma, 2005). Fronds from the dipping vats contained more Mn because of the lower soil pH (Table 5-1), which increases mobility and uptake of Mn.

Soil As Fractionation

Arsenic sorption depends on soil constituents (i.e. Fe, Al, Ca), pH, organic matter, and clay minerals (Zhang and Selim, 2008). Iron and Al oxides and hydroxides have particularly high affinity to As which increases in presence of Ca (Smith et al., 2002). To differentiate between As fractions, a five-step sequential extraction procedure was used. The first extraction step, 0.05M (NH₄)₂SO₄, represents the most soluble (labile) As. In the second extraction, 0.05M NH₄H₂PO₄ was used to assess exchangeable As, in that it can be specifically replaced by phosphate. Although not as easily released as the first fraction, the exchangeable fraction is also considered labile. The next three steps target As bound to amorphous and crystalline hydrous oxides of Fe and Al, along with the residual fraction, which are all considered non-labile.

Of the three soils, CCA had the highest amorphous Al, Fe and available Ca (Table 5-1). This was reflected in the As distribution of the CCA soil before planting, where 87% of the As (113.2 mg kg⁻¹) was associated with the amorphous and crystalline fractions compared to 5% (6.8 mg kg⁻¹) in the soluble and exchangeable fractions (Figure 5-1A). Comparatively, the DVA (Figure 5-1B) and DVB (Figure 5-1C) had a higher proportion of soluble and exchangeable As at 13 and 18% (3.3 and 5.4 mg kg⁻¹) with ~75% (19.3 and 21.9 mg kg⁻¹) associated with amorphous and crystalline

fractions, respectively. The higher bioavailability of As in the dipping vat soils is likely due to low concentrations of amorphous Fe and available Ca compared to the CCA soil (Table 5-1). The residual fractions of all three soils were similarly proportioned at ~9.1%. Overall, the As fractions in our soils were consistent with data reported by Wenzel et al. (2001) who conducted As-fractionations in twenty different As-contaminated soils (96-2183 mg kg⁻¹). Arsenic in their soils were primarily associated with the amorphous (~42%) and crystalline (~29%) fractions (Wenzel et al., 2001) which was similar to our soils at ~50% and ~30%, respectively.

Following two years of phytoremediation with *P. vittata*, As concentrations were significantly reduced in CCA (130 to 88 mg kg⁻¹), DVA (26 to 15 mg kg⁻¹) and DVB (30 to 14 mg kg⁻¹) soils ($p \leq 0.05$). Even though As uptake by *P. vittata* arises from the available fractions, after two years, a small decline was observed in the soluble with little change or slight increase in exchangeable fractions in the three soils (Figure 5-2). The lack of depletion from the exchangeable fraction can be attributed to As replenishment from the amorphous and crystalline fractions. In the CCA soil, amorphously bound As was reduced from 75.1 to 44.4 mg kg⁻¹ while crystalline bound As was reduced from 38.1 to 30.5 mg kg⁻¹ As (Figure 5-2A). A similar trend was observed in the dipping vat soils, with the greatest reduction of As associated with the amorphously bound As after two years followed by the crystalline fraction (Figure 5-2B and C). In all three soils, ~22% of the total soil As reduction was associated with the amorphous fraction followed by ~13% in the crystalline. There was little change in the residual fraction of CCA soil (9.7 to 9.1 mg kg⁻¹) and slight declines in the dipping vat

soils, accounting for 2.5 and 3.6% reductions in DVA (2.9 to 0.9 mg kg⁻¹) and DVB (2.5 to 1.8 mg kg⁻¹) soils respectively.

Predicting Arsenic Uptake in *P. vittata* Using Sequential Extraction Data

Regression analysis was used to assess potential correlation between frond and soil As concentrations. We compared the As in sequentially extracted fractions of soil during two years of phytoextraction to develop a model predicting As accumulation by *P. vittata*. For most plants, a linear correlation exists between total As concentrations and plant growth inhibition (Sheppard, 1992). Further investigation by Gulz et al. (2005) found that the As solubility, P availability and P demand should be collectively considered to predict As uptake in common plants. However, these models may not apply to *P. vittata*, due to its unique ability to hyperaccumulate and tolerate high concentrations of As. In a nine month pot study with *P. vittata* by Shelmerdine et al. (2009), a model was developed from soluble As data to predict the length of remediation needed to clean-up a variety of As contaminated soils. While the model was found to correlate well with frond As concentrations in the study ($R^2 = 0.71$), it predicted that after 30 years, a soil contaminated with ~24 mg kg⁻¹ would be reduced to ~15 mg kg⁻¹ (Shelmerdine et al., 2009). In our study, a soil containing 30 mg kg⁻¹ As (DVB) was reduced to 14 mg kg⁻¹ after two years of remediation with *P. vittata*. This highlights one of the benefits of a long-term study, which could allow for more realistic predictions of remediation length.

FronD bioconcentration, which is the ratio of As in fronds to soil, was most useful in predicting As uptake. The use of bioconcentration rates also normalizes rate of As uptake, allowing for comparisons of soils with wide ranges contamination. The pH was also considered because it affects the lability of As is in soil. Considering individual As-

fractions for model development, the best to worst in predicting frond As uptake were: amorphous > total > residual > crystalline > exchangeable > soluble. Despite plant mediated As uptake originating from the available pools, the soluble ($R^2 = 0.46$) and exchangeable ($R^2 = 0.09$) fractions did not accurately predict frond As accumulation. Normally, concentrations of soluble nutrients are a good indicator of bioavailability for plant uptake in soil since plants preferentially take up their nutrients from the soil solution (Neumann, 2007). However, the supply of elements such as P and As at the root-soil interface is limited by diffusion. If the rate of plant facilitated As uptake exceeds rate of soil As-redistribution to the available fractions, concentrations will remain low and static while total As declines. This suggests that for more accurate predictions of As uptake, the pool responsible for replenishing the available fractions should be used. In our study, the greatest decline in As in all three soils was associated with the amorphously-bound fractions (Figure 5-2). Regression analysis indicated that bioconcentration rates based on the amorphously bound As had good correlation ($R^2 = 0.77$) between frond As concentrations throughout the two year study. This model was further improved ($R^2 = 0.94$) when using the natural logarithm of the ratio between available (S+E) and amorphously bound fractions (Figure 5-3). This model accounts for the relative difference between pools of As which are available for plant uptake and the source of primary replenishment, which was shown to decrease over time (Table 5-4). When the As in the amorphous fraction approaches equilibrium with the available, the rate of diffusion will slow, which accounts for the declining frond As concentrations in subsequent harvests.

Conclusions

It is important to be able to predict the time frame required to remediate contaminated soil. Inclusion of the amorphously bound fraction of As will aid in accessing the length of remediation required in achieving soil-As cleanup levels. Phosphate rock amendments maintained low soluble P without negatively impacting plant health. Our results show that soil with moderate to low concentrations of Arsenic can be efficiently remediated using *P. vittata*. Furthermore, by sequentially extracting As from soils, a reasonable time frame of clean-up can be predicted based on the ratio of available to amorphously bound As concentrations.

Table 5-1. Select characteristics of soils used in this study

Soil characteristic	CCA	DVA	DVB
Total As (mg kg ⁻¹)	129.4	25.5	29.9
Total Al (mg kg ⁻¹)	3661	1710	7455
Amorphous Al (mg kg ⁻¹) ^a	780	417	470
Total Fe (mg kg ⁻¹)	2227	324	1298
Amorphous Fe (mg kg ⁻¹)	1309	46	60
Total Ca (mg kg ⁻¹)	7300	356	700
Available Ca (mg kg ⁻¹) ^b	1540	129	244
Total P (mg kg ⁻¹)	382	166	516
Water-soluble P (mg kg ⁻¹)	0.35	0.18	0.33
pH	7.2	5.3	5.1
Organic matter (%)	1.1	2.2	0.4
CEC (cmol ⁺ kg ⁻¹)	7.8	3.3	12.4
Sand (%)	86.3	95.5	80.7
Silt (%)	9.9	2.7	6.6
Clay (%)	3.8	1.8	12.7
Textural class	Loamy sand	Sand	Sandy loam

^a Oxalic acid + ammonium oxalate, 0.2 M

^b Mehlich III

Table 5-2. Arsenic distribution in harvested *P. vittata* fronds

Tissue	Arsenic mg kg ⁻¹			Fron
	CCA	DVA	DVB	weight (%)
Y. leaflet	3472	1488	1735	16.7
M. leaflet	3246	1057	1297	47.3
S. leaflet	3053	611	864	18.1
Y. stem	478	70	112	2.9
M. stem	227	40	43	6.3
S. stem	201	55	56	6.5
Spore	1116	149	143	2.3

Table 5-3. Elemental analysis of harvested
P. vittata fronds

Element (mg kg ⁻¹)	CCA	DVA	DVB
As	2720	900	1020
P	2266	1806	1784
K	22314	20984	21475
Ca	5153	5132	5251
Mg	3217	3600	3621
Al	193	171	228
Fe	123	116	151
Cu	77	66	67
Mn	29	112	55

Table 5-4. The ratio of amorphous:available arsenic in soil over two years

Sample	CCA	DVA	DVB
1	22.4	6.0	5.3
2	17.5	3.3	3.1
3	17.3	2.6	2.8
4	13.2	2.4	1.9

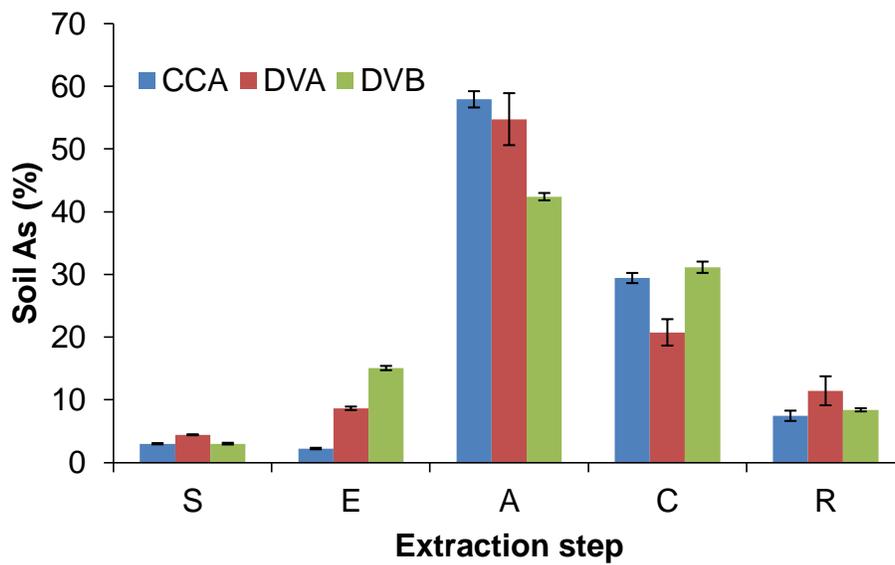


Figure 5-1. Relative distribution of arsenic in the soluble (S), exchangeable (E), amorphous (A), crystalline (C) and residual (R) fractions at time of planting.

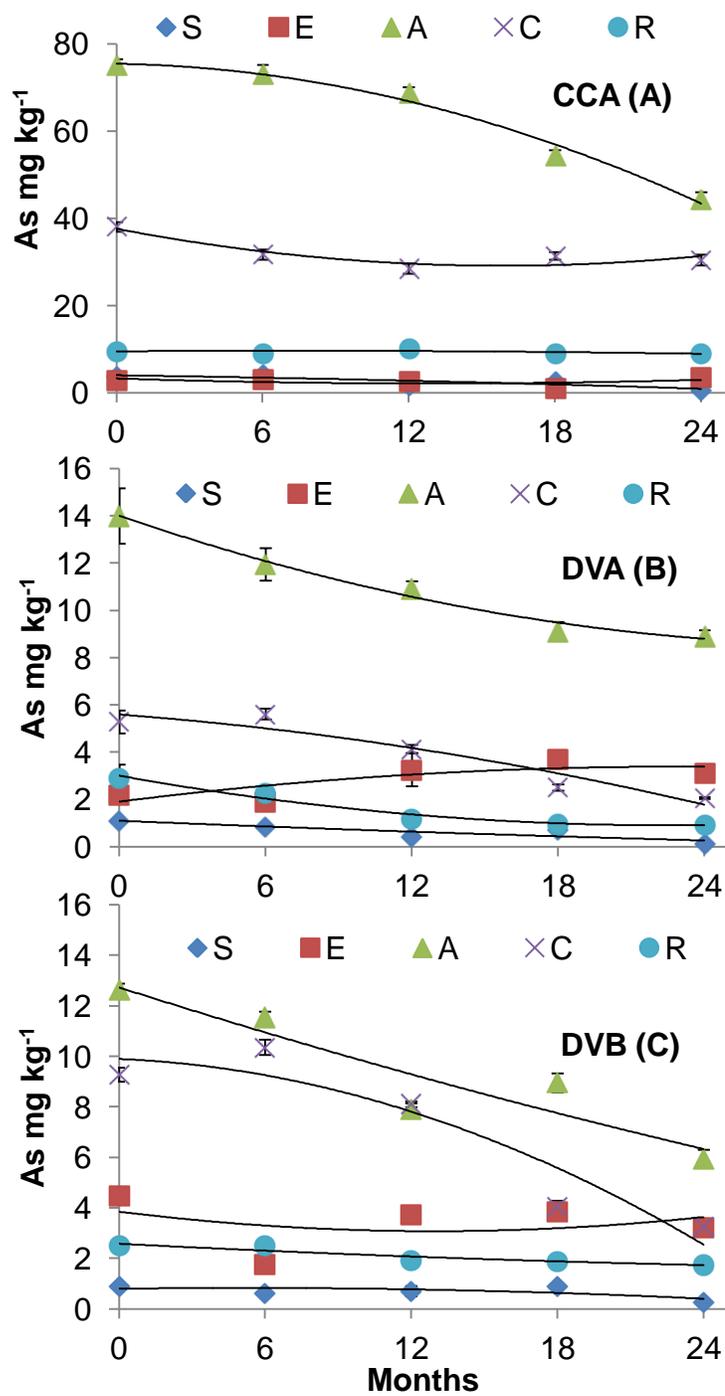


Figure 5-2. Arsenic in the soluble (S), exchangeable (E), amorphous (A), crystalline (C) and residual (R) fractions of CCA (A), DVA (B), and DVB (C) soils during two years of growth with *P. vittata*.

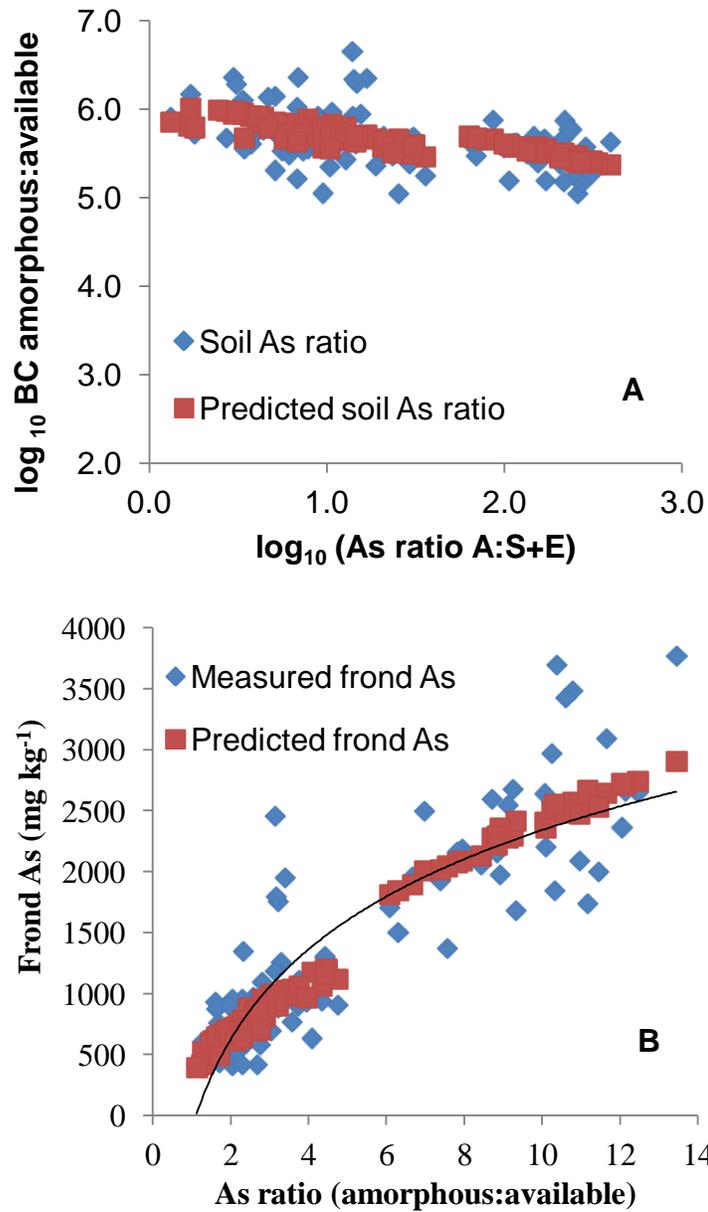


Figure 5-3. Predictive model based on the natural logarithm of measured bioconcentration (BC) ratios between amorphous and available soil As fractions (A) to predict As uptake (B) in *P. vittata*.

CHAPTER 6
ROLE OF ARSENIC HYPERACCUMULATION IN *PTERIS VITTATA*

Role of Metal Accumulation in Plants

Environmental stress plays a significant role in the evolution of biological systems, from gene to entire ecosystems. Many areas of the world are contaminated by metals, either naturally or by anthropogenic activity. All heavy metals, and metalloids such as arsenic and selenium are toxic to plants when concentrations exceed trace quantities (Macnair, 1997). The properties that make some metal ions essential for life can also be toxic in elevated concentrations. The formation of hydroxyl radicals or high-affinity binding to S, N, and O-containing functional groups in biological molecules can cause inactivation and/or damage. Furthermore, there are elements which can interfere with essential elements of the same group, including Cd (for Zn), As (for P) and Se (for S), making them extremely toxic. (Clemens, 2006). The physiological range for essential metals between deficiency and toxicity is narrow, requiring tightly controlled mechanisms to adapt to changes in micronutrient availability (Nelson, 1999).

Some plants can grow on soil containing metal concentrations that would normally inhibit growth. They possess naturally selected higher levels of tolerance, which is typically specific for certain metals (Schat and Vooijs, 1997). Metal tolerance in natural plant populations is largely due to selective pressure from growing on soils enriched with metals. Plants have a range of cellular mechanisms that might be involved in the detoxification and thus tolerance to heavy metal stress. Most plant tolerance is achieved by avoiding the build-up of metal concentrations. Comparatively, there also exist plants with ubiquitous basal tolerance (e.g. *Pteris vittata*).

Heavy metal phytotoxicity results from alterations of numerous physiological processes by inactivating enzymes, blocking functional groups, displacing or substituting for essential elements or disrupting membrane integrity (Rascio and Navari-Izzo, 2011). A common consequence of heavy metal poisoning is the enhanced production of reactive oxygen species (ROS) due to interference with electron transport activities (Pagliano et al., 2006). Increased ROS induces oxidative stress leading to lipid peroxidation, macromolecule deterioration, membrane dismantling, ion leakage, and DNA-strand cleavage (Quartacci et al., 2001). Plants responses include a series of defense mechanisms that control uptake, accumulation and translocation of metals and detoxify them by excluding the free ionic forms from the cytoplasm. A common plant response is to hinder entrance of metals into the root by binding them to exuded organic acids or to anionic groups of cell walls (Rascio et al., 2008). If heavy metals enter the plant, tolerant plants will often store them in root cells, where they can be detoxified by complexation with amino acids, organic acids or metal-binding peptides (Rascio and Navari-Izzo, 2011). This restricts translocation to the above-ground organs, protecting the metabolically active photosynthetic cells from damage. Another defense mechanism used by tolerant plants is enhancement of the antioxidant system to counteract oxidative stress (Sgherri et al., 2003). Typically, metal tolerant species rely on strategies which restrict metal translocation. However, many hyper-tolerant (hyperaccumulators) species exhibit the opposite behavior as far as metal uptake and distribution in the plant (Hall, 2002).

Genetic analysis of intra- and inter-specific crosses determined that tolerance and hyperaccumulation are independent characters. For example, tolerance of Cu, Cd,

or Zn in different *Silene vulgaris* accessions from normal soil and metalliferous sites are controlled by one or two major genes (pleiotropic) with additional modifiers controlling the level of tolerance (Schat, 1999). The selective factors driving the evolution of hyperaccumulation are unknown and difficult to identify. The different hypotheses in the literature include: increased metal tolerance, protection against herbivores or pathogens, inadvertent uptake, drought tolerance, and allelopathy (Rascio and Navari-izzo, 2011). There is wide variability between populations of hyperaccumulator species in their capacity to tolerate and accumulate metals. For example, numerous arsenic hyperaccumulators have been identified in the Pteridaceae family, including *Pteris vittata*, *P. cretica*, *P. longifolia* and *P. umbrosa* and *Pityrogramma calomelanos* (Meharg, 2002). Though the number of ferns tested for arsenic hyperaccumulation is small, the Pteridaceae contain over 400 species, which means less than 1.25% can accumulate arsenic. Ferns are among the most primitive plants and morphological characteristics place the Pteridaceae in the latter portion of fern evolution. During the evolution of land plants, those that evolved in arsenic rich environments would have required mechanisms for coping with this element, with hyperaccumulation being one strategy. Arsenic tolerance could be a carryover from marine algae that contain arsenate in high amounts due to the scarcity of phosphate in seawater. Another theory suggests the evolution of early terrestrial life began around arsenic-rich hot springs (Meharg and Hartley-Whitaker, 2002). Tolerance mechanisms may have been lost as plants spread out into non-arsenic contaminated environments, with members of the Pteridaceae retaining these mechanisms, either as evolutionary baggage or because this trait conferred them with some advantage, arsenic related or not (Meharg, 2002).

Alternatively, the hyperaccumulation trait could have evolved later in response to selective pressure, or perhaps the ferns were confined to arsenic-rich habitats throughout their evolutionary development (Meharg 2002). It is also possible that arsenic had little to do with arsenic hyperaccumulation. The physiological mechanisms may have evolved for other reasons, conferring arsenic hyperaccumulation to the ferns as a side-effect. This is the case for arsenic resistance in angiosperms where resistance is a consequence of suppressed high-affinity phosphate transport (Lou et al., 2010), as arsenate and phosphate are chemical analogues. Arsenic hyperaccumulation may therefore be a consequence of unusual phosphorus metabolism in ferns, although there is little evidence to suggest this.

The most interesting aspects of metal tolerance pertains to the plants that hyperaccumulate toxic elements (e.g. As, Pb, Cd), especially since the reasons for this unusual behavior remain elusive. Why do some plants do it, what functions does hyperaccumulation perform and what are the benefits and the adaptive values of metal hyperaccumulation? Clemens (2006) hypothesizes three possible explanations: (i) the detoxification pathways for non-essential metals play an important role in essential metal homeostasis; (ii) molecular determinants of toxic metal tolerance serve additional, as yet unknown, essential functions; (iii) toxic metals are actually essential elements and the detoxification pathways are part of the homeostatic network for these elements. In marine ecosystems, Cd has been observed to behave like a micronutrient. Its distribution is similar to phosphate, in that concentrations remain low in surface water due to uptake by photosynthetically active algae and concentration increases with depth (Butler, 1998). The marine diatom *Thalassiosira weissflogii* shows improved growth

under conditions of low Zn availability and low CO₂ when Cd ions are added to the medium. The algal cells were found to use Cd(II) as a co-factor for a carbonic anhydrase that is expressed only under these conditions to replace the regular Zn-requiring enzyme (Lane et al., 2005). In plants, accessions of the Zn/Cd hyperaccumulator *T. caerulescens* grow better in nutrient solution containing low concentrations of Cd than in nutrient solution without Cd (Clemens, 2006).

In addition to translocation, the accumulation of toxic metals in plants remains an interesting puzzle. A variety of hypotheses have been proposed to explain the role of toxic metal accumulation in aboveground biomass, including: metal tolerance, metal disposal, improved stress responses, interference with neighboring plants, and defense against herbivory. According to the tolerance/disposal hypothesis, the hyperaccumulation pattern allows plants to displace metals away from the roots and eliminating them from the plant body by shedding the high-metal tissues. Heavy metals may increase plant drought resistance, with a water-conserving role in the cell walls or acting as osmolytes inside the cells. The interference hypothesis (or elemental allelopathy), suggests that perennial hyperaccumulator plants interfere with neighboring plants through enrichment of metal in the surface soil. This would be an additional benefit to the tolerance/disposal hypothesis. The high-metal leaf litter would potentially prevent the establishment of less metal tolerant species. High Ni levels in the surface soil under the canopy of hyperaccumulator *S. acuminata* has been observed when compared to surface soil in non-hyperaccumulator species (Boyd, 2001).

Another hypothesis suggests that the toxic metal concentrations in aboveground biomass function as a self-defense strategy against natural enemies, such as

herbivores and pathogens. Recent studies confirm the defensive function of Ni (Jhee et al., 2006), Cd (Jiang et al., 2005), Zn (Behmer et al., 2005), As (Rathinasabapathi et al., 2007) and Se (Galeas et al., 2008) in plant defense. In spite of important progress made in recent years by the numerous studies accomplished, the complexity of hyperaccumulation is far from being understood. Metal tolerant and metal hyperaccumulator plants, which are widespread on metal soils in both tropical and temperate zones of all the continents, belong to several unrelated families. This shows that tolerance has evolved more than once, further complicating the questions pertaining to evolutionary fitness. More elements and tolerant species require examination in order to validate any one particular hypothesis of the defensive effects of heavy metals.

In this study, we observed some of the beneficial roles that arsenic hyperaccumulation potentially play in *P. vittata*. Specifically, we looked at how arsenic effects *P. vittata* 1) growth in low nutrient environments; 2) defense against herbivory; and 3) enrichment of arsenic at the soil surface.

Materials and Methods

Fern and Gametophyte Setup

Topsoil was air-dried, sieved through a 2 mm mesh screen and analyzed for pH (1:2 soil to water), organic matter content (Walkley-Black method) and particle size (pipette method) (Tan, 2005). Soils were separated into separate buckets and mixed with suspensions of sodium arsenate ($\text{Na}_2\text{HAsO}_4 \cdot 2\text{H}_2\text{O}$) to achieve final concentrations of 25, 50 and 100 mg kg⁻¹ As. Samples were subjected to $\text{HNO}_3/\text{H}_2\text{O}_2$ digestion (USEPA Method 3051) on a hot block (Environmental Express, Ventura, CA). The digested samples were analyzed for total As concentration using graphite furnace

atomic absorption spectroscopy (GFAAS, Perkin Elmer SIMMA 6000, Perkin-Elmer Corp., Norwalk, CT). Three month old *P. vittata* (3-4 fronds ~15 cm in length) purchased from Milestone Agriculture (Apopka, Florida) were transplanted 15 cm apart (6 per bed). Growth was observed for one year with no fertilizer applications. Drought stress was simulated in the first month by allowing soil moisture levels to decline until visible wilt symptoms were observed, at which time, soil moisture was brought back to field capacity where it was maintained for the duration of the experiment.

Spores from *P. vittata* were collected from plants growing in arsenic contaminated soils with varying concentrations (25-130 mg kg⁻¹ As) and surface sterilized in a 20% bleach solution for 20 minutes followed by three washes in sterile DI water. Spores were suspended in 2 mL sterile DI water. Half strength modified Murashige & Skoog (MS) media was prepared with 0.8% agar without P prior to autoclaving. Phosphate, phytate, and arsenate solutions were filter sterilized and added to autoclaved MS media to obtain final concentrations of 0.6 mM P as P_i (KH₂PO₄) or phytate (P₆; myo-inositol hexaphosphoric acid dodecasodium salt) with 0 or 0.6 mM arsenate. The MS media (pH 6.5) was then poured into sterile petri dishes (100 mm × 13 mm). Spores (10 µL or 0.05 mg spore) were placed on agar (10 per plate, 4 plates per treatment) under cool/warm fluorescent lamps at 25°C and 60% humidity for 30 d.

Herbivory Setup

During the summer of 2009, caterpillars were observed eating *P. vittata* leaflets growing in a campus greenhouse. The caterpillars were captured and sent to the Florida department of Agriculture and Consumer Sciences who identified them as *Spodoptera latifascia* (ID# E2009-7753-1). To gauge the role of arsenic accumulation in herbivory prevention, five month old *P. vittata* ferns were transferred to hydroponic

culture in 0.2× strength Hoagland-Arnon nutrient solution (HNS) containing 0 or 267 μM As. The caterpillars identified as *S. latifascia* were transferred onto ferns to observe their herbivory behavior. Frass and leaflet samples were taken after one week to analyze As concentration by digestion analysis as previously described.

Allelopathy Experiment

Pteris vittata ferns were observed growing naturally at an apartment complex in Gainesville Florida built in 1980. The ferns were isolated to areas underneath stairwells which were constructed from arsenic treated wood. According to the management, the ferns, which were not planted, had been left unattended for at least 6 years. Samples were taken from 25 different stairwells containing actively growing *P. vittata*. Soil cores (20 cm deep) were collected at the base of *P. vittata* plants and a distance of 50 cm directly away. Soil cores were separated by top (0-2 cm) and bottom (18-20 cm) to analyze total arsenic as previously described.

Statistical Analysis

Data are presented as the mean of all replicates with standard error. Significant differences were determined using analysis of variance and treatment means compared by Duncan's multiple range test, at $p \leq 0.05$.

Results and Discussion

The Effect of Arsenic on *P. vittata* Growth

The soil was 89% sand, 6% silt and 5% clay with a pH of 7.5. At the beginning of the experiment, average fresh weight of transplanted *P. vittata* plants was 31 g. There was no observable difference between treatments during the first three weeks of growth (Figure 6-1). During the fourth week, the ferns were subjected to drought stress, which was characterized by a wilting of the fronds. *Pteris vittata* is sensitive to drought, which

was reflected by the wilting tissues immediately senescing. One week following the drought stress, and one month into the experiment, the ferns had lost all photosynthetic tissues. At this point, new frond growth is required in order for plants to survive. New frond growth was observed one week after the drought stress in plants growing in the As treated soils while one plant in the control soil began producing frond growth. For the next four months, plants in the arsenic treated soil continued to proliferate while the remaining plant in the control soil remained stunted (Figure 6-1). After six months, average frond biomass (dry weight) was 2, 18, 36, and 32 g plant⁻¹ with arsenic concentrations of 70, 3700, 8700 and 9600 mg kg⁻¹ in soil with 0, 25, 50 and 100 mg kg⁻¹ As, respectively.

Despite the toxicity associated with arsenic, *P. vittata* is able to survive high concentrations (Ma et al., 2001). Many heavy metals stimulate the formation of free radicals and reactive oxygen species (ROS), leading to uncontrolled oxidation and radical chain reactions, which stress the plant (Zaman and Pardini, 1996). Drought stress has been shown to induce the same responses in plants (Mittler, 2002). In *P. vittata*, the presence of As has been shown to stimulate the production of anti-oxidants like superoxide dismutase, catalase, and ascorbate peroxidase (Srivastava et al., 2005). Thus, in the presence of arsenic, an increase in anti-oxidant enzymes may improve survivability of *P. vittata* during drought stress.

To gauge the effect of arsenic on spore germination and growth, spores were germinated on MS media. Although the presence of arsenic in the media significantly improved biomass, pre-exposure to arsenic had little impact on rate of biomass growth over a 30 day period (Table 6-1). Spores containing no arsenic prior to germination

generally exhibited the highest biomass, indicating that pre-exposure of arsenic may actually slow growth, even though presence of arsenic in the media improves growth. Phytate was used because it requires a plant produced enzymatic reaction to cleave phosphate for uptake by the growing gametophyte. Pre-exposure to arsenic did not inhibit the activity of this enzyme, as growth was not impeded on media amended with phytate.

Arsenic as a Defense against Herbivory

The *S. latifascia* observed eating the *P. vittata* plants in our greenhouse was the first report of herbivory by the caterpillar (Figure 6-2). The plants did not however, contain arsenic, as they were growing in un-contaminated potting mix. Upon moving the caterpillars to *P. vittata* growing in arsenic amended media, they continued to eat, unperturbed by arsenic in the tissues. However, upon analysis of the frond tissue, arsenic concentrations were relatively low at $\sim 200 \text{ mg kg}^{-1} \text{ As}$ (fresh weight). The arsenic concentrations in the collected frass reflected this, with approximately $10 \text{ mg kg}^{-1} \text{ As}$ (dry weight). The caterpillars consumed approximately $\sim 10 \text{ g}$ of tissue on both control and arsenic contaminated plants, with roughly all of the arsenic passing through their system. We were unable to rear a second generation of the caterpillars and therefore could not test the effect of higher arsenic concentrations in the frond. It should be noted that the caterpillars were only found on plants free of arsenic in the greenhouse, as there were several experimental plants they could have eaten, but did not.

Allelopathy

Arsenic readily leaches from arsenic treated wood, contaminated the soil around it. This was the the case pertaining to the soil underneath the decking of an apartment

complex built with arsenic treated wood. *Pteris vittata* were found to grow naturally underneath the stairwells, likely because the elevated arsenic concentrations are toxic to most other plants, providing them with a unique niche. To maintain such a niche, it has been theorized that metal hyperaccumulation could be used to enrich toxic metals at the soil surface to deter competitive plants. At the apartment complex, all soils sampled around structures built with arsenic treated structures site were extensively contaminated with arsenic, ranging from 20 to 100 mg kg⁻¹ As. Soil samples taken at the base of plants was slightly elevated compared to concentrations just outside the canopy (50 cm from plant) (Figure 6-3). The average soil arsenic concentration at the base of plants was 44 mg kg⁻¹ compared to 33 mg kg⁻¹ in areas with no *P. vittata*. At a depth of 20 cm, arsenic concentrations were significantly lower, averaging 10 mg kg⁻¹ underneath *P. vittata* and 13 mg kg⁻¹ at a 50 cm distance away (Figure 6-3). The higher concentration at the surface is likely due to arsenic leaching from wood, not necessarily plant mediated. A point by point comparison of surface soils with and without *P. vittata* showed that the difference in arsenic concentrations was not significantly different. This suggests that *P. vittata* may be enriching surface soil with arsenic, but contamination at this site was not homogenous enough to accurately assess this theory.

Table 6-1. *Pteris vittata* spore growth

Spore As mg kg ⁻¹	Treatment and Fresh Weight (g)				
	P	P+As	P ₆	P+P ₆	P ₆ +As
0	0.34	0.46	0.23	0.44	0.25
88	0.26	0.28	0.14	0.25	0.15
138	0.18	0.25	0.17	0.30	0.14
177	0.19	0.37	0.12	0.15	0.16
186	0.25	0.28	0.16	0.31	0.22
282	0.18	0.26	0.23	0.25	0.11
819	0.19	0.28	0.20	0.34	0.10
996	0.22	0.25	0.22	0.18	0.10
1717	0.20	0.24	0.18	0.28	0.13



Figure 6-1. *Pteris vittata* growing in soil spiked with 0, 25, 50 and 100 mg kg⁻¹ arsenic. Plants after transplant (top) and after six months (bottom) of growth. Photos courtesy of Jason Lessl.



Figure 6-2. *Spodoptera latifascia* caterpillar (top) and adult moth (bottom). Photos courtesy of Lyle Buss.

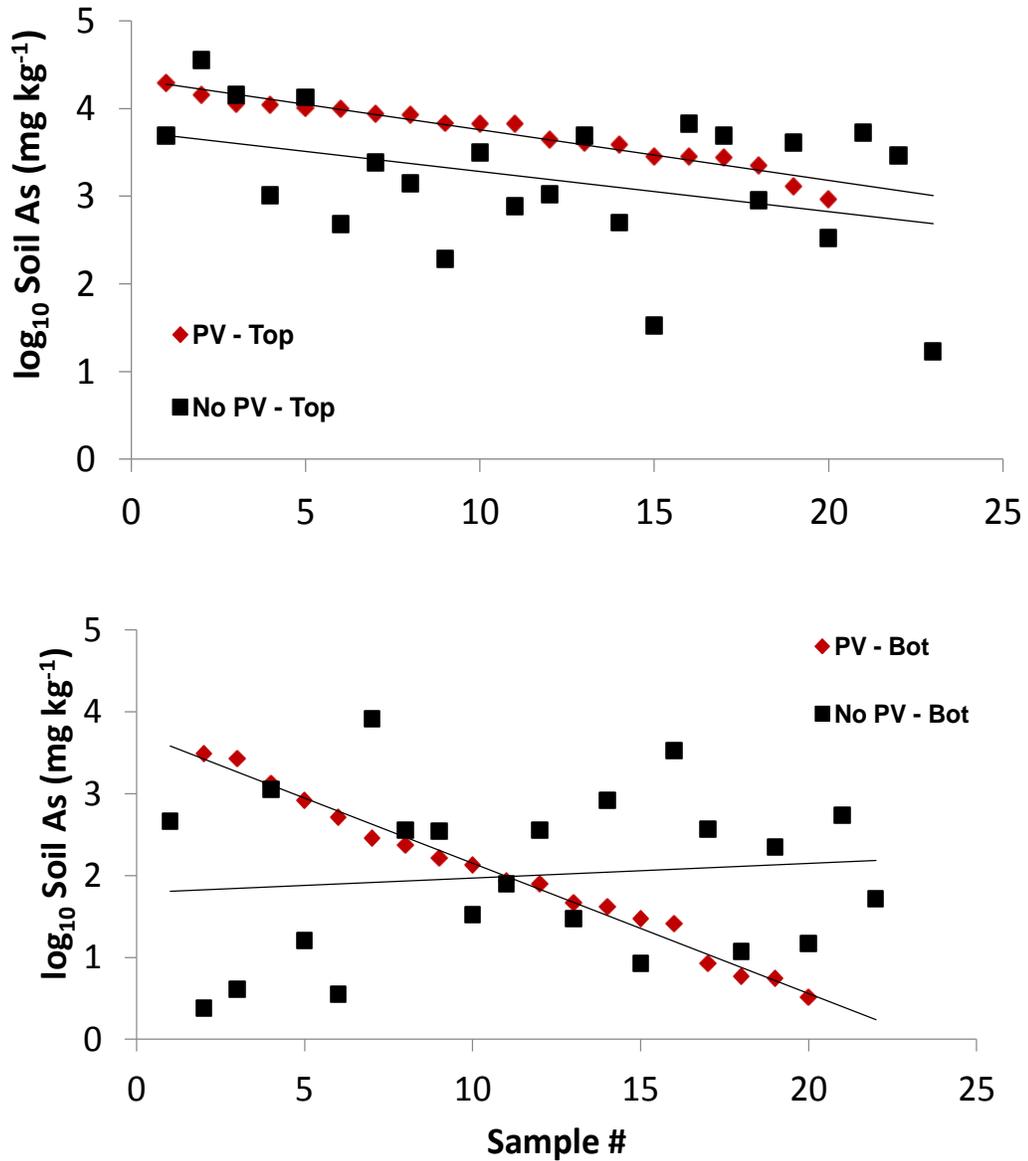


Figure 6-3. The natural logarithm of soil arsenic concentrations at the surface soil (top) and at a 20 cm depth taken at the base of *P. vittata* (PV) and at a distance 50 cm away from plants.

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BIOGRAPHICAL SKETCH

Jason T. Lessl, was born in Milwaukee Wisconsin to Tom and Ruth Lessl. After his high school education, he attended the University of Georgia for his bachelor's degree in biology and a master's degree in plant pathology. The title of his master's thesis was "The role of bacterial motility in watermelon blossom colonization and seed infestation by *acidovorax avenae* subsp. *citrulli*, causal agent of bacterial fruit blotch." Awarded an alumni graduate fellowship at the University of Florida, he joined the department of Soil and Water Science in 2008 to pursue a PhD studying the arsenic hyperaccumulator *Pteris vittata*.