PHYTOREMEDIATION OF ARSENIC-CONTAMINATED SOIL AND GROUNDWATER

By

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This research is dedicated to my dear parents, Chief and Chief (Mrs) Adelegan who nurtured me and taught me to read right from a very early age.

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PHYTOREMEDIATION OF ARSENIC-CONTAMINATED SOIL AND GROUNDWATER

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Pteris vittata was effective in taking up arsenic in the presence of various metals and transporting it to the fronds. Arsenic uptake, bioconcentration factors and transfer factors reduced with increasing metal concentration in the soil. Greater concentrations of metals resulted in greater catalase activity in the plant. Most of the arsenic in the plant was present as arsenite (the reduced form) indicating little impact of the metals on plant arsenic reduction. Fractionation analysis before and after plant arsenic uptake in the control soil with no metals spiked showed that Ca-As was the dominant fraction, with Ca-As > Fe-As > Al-As > WE-As. Actual concentrations of Al-As and Fe-As were significantly ($P<0.01$) greater in all metal-spiked soils than in the control at 8 weeks. Phosphate rock significantly increased arsenic uptake and reduced lead uptake by *P. vittata* in a multi-metal-arsenic-polluted soil. Arsenic uptake by the fern was positively correlated with both total and available arsenic in the soil. *Pteris vittata* had significantly higher aboveground and total biomass than *P. cretica*. *Pteris vittata* also had higher As

uptake than *P. cretica* except in the golf-course soil. GSH availability was not implicated as a detoxification mechanism for arsenic in these ferns. The ability of *P. vittata* to accumulate As and tolerate metal toxicity better than *P. cretica* makes it a better candidate for phytoremediation of arsenic contaminated soils. *Pteris vittata* was also more efficient than *P. cretica* in hydroponic conditions with significantly greater arsenic accumulation and translocation. Arsenic reduction from AsV to AsIII in the fronds of *P. vittata* did not occur appreciably after 1-day exposure. *P. vittata* accumulated arsenic in the fronds while *N. exaltata* accumulated it in the roots. Pre-exposure to arsenic increased the ability of *P. vittata* to accumulate arsenic from solution containing higher arsenic concentration. The nitrate-fed ferns (*P.vittata*) significantly took up more arsenic than did ferns (*P.vittata*) fed with the other nutrients. Adding calcium carbonate resulted in greater reduction of solution arsenic. This is very good for the phytofiltration of arsenic-contaminated water in areas where limestone is present in the rock strata.

CHAPTER 1
INTRODUCTION

The presence of arsenic in the environment may be due to both natural and anthropogenic sources. Arsenic minerals are natural sources of arsenic in the environment. Natural activities such as volcanic action, erosion of rocks, and forest fires introduce arsenic into the environment (EPA, 2001). Anthropogenic sources include arsenic sources added to the soil plant system as insecticides, herbicides, pesticides, livestock dips and wood preservatives. Indiscriminate use of arsenical pesticides during the early to mid-1900s led to an extensive contamination of soils worldwide (Smith et al., 1998). Mining and smelting processes contribute to arsenic contamination because arsenic is a natural component of lead, zinc, copper and gold ores.

Arsenicals can cause surface soil contamination of 600 ppm or more (Adriano, 1986). In the United States, surface soils contain an average of 7.2 ppm (range <0.1 to 97 ppm) (Shacklette and Boerngen, 1984). Arsenic use in 1992 was 23,900 metric tons; of which 67% was for production of the wood treatment chemical chromated-copper-arsenate (CCA). Agricultural use was 23% of the total in 1992 but declined after cancellation of approval for use of arsenic chemicals as cotton leaf desiccants (EPA, 1995).

Epidemiological studies show a direct relationship between environmental exposure of humans to inorganic arsenic and cancer of the skin and lungs (National Academy of Sciences, 1977). Various technologies are in place to clean up arsenic or to reduce exposure to arsenic from contact with (or ingestion of) arsenic-contaminated soil

and water. Technologies for remediation of arsenic contaminated soils include excavation, immobilization, vitrification, soil washing/flushing and phytoremediation. Treatment technologies applicable for arsenic-contaminated water include precipitation, membrane filtration, adsorption, ion exchange, permeable reactive barriers and biological treatment.

Phytoremediation has been defined as the use of green plants to remove pollutants from the environment or render them harmless (Cunningham and Berti, 1993). There are various aspects of phytoremediation: phytoextraction, phytodegradation, rhizofiltration, phytostabilization and phytovolatilization. Phytoextraction involves using hyperaccumulating plants to remove the contaminant from the contaminated media and concentrate it in their aboveground plant tissues, which is periodically harvested. The metal-enriched plant residue can be disposed of as hazardous material and if economically feasible, used for metal recovery (Salt et al., 1998).

Hyperaccumulating plants are plants that are able to take up metals above established background concentrations and more than other species from the same soil (Kabata-Pendias and Pendias, 2001). The key to using hyperaccumulators in phytoremediation lies in the rate of biomass production, coupled with the concentration of the element transferred to the plant matter (Reeves and Baker, 2000). Hyperaccumulating plants species represent perhaps the ultimate in plant tolerance to extremely hostile edaphic environments. Many have been reported for Cu, Co, Zn, Ni, Se, and Pb (Harris and Oparka, 1994). The fern *Pteris vittata* reported to be the first arsenic hyperaccumulator (Ma et al., 2001) was discovered in an abandoned wood-treatment site in Central Florida. Several other fern species in the Pteridales have been

identified to be As hyperaccumulators (Zhao et al., 2002). Some of these ferns are also being screened for their potential for phytoremediation of arsenic-contaminated soil and water.

The main objective of this research was to evaluate and enhance the efficiency of *P. vittata* to remediate arsenic-contaminated soil and water.

Specific aims are as follows.

- **Specific aim 1.** Determine the effects of other heavy metals on arsenic uptake and accumulation by *P. vittata* in an arsenic contaminated soil.
- **Specific aim 2.** Look at the possibility of using phosphate rock to immobilize metals and increase arsenic uptake in arsenic contaminated soil impacted with various metals.
- **Specific aim 3.** Compare arsenic uptake by *P. vittata* and *P. cretica* in different arsenic contaminated soils.
- **Specific aim 4.** Evaluate the effect of different arsenic sources and doses on arsenic uptake of *P. vittata* and *P. cretica* in water.
- **Specific aim 5.** Determine effect of nutrition and arsenic pre-exposure on arsenic uptake of *P. vittata* in water.

CHAPTER 2
LITERATURE REVIEW

2.1 Arsenic in the Soil

Arsenic is a crystalline metalloid with three allotropic forms that are yellow, black and gray. It also exists in several forms and oxidation states (–3, 0, 3, & 5). Arsenate (V) is the stable oxidation state in aerobic conditions. In strongly reducing conditions, elemental As, As(III) and arsine (III) can exist. Arsenite (III) exists in moderately reducing conditions and is one of the most toxic arsenic compounds. Methanogenic bacteria reduce As (V) to As (III) and methylate it to methylarsinic acid. Arsenic trioxide (white arsenic) As_2O_3, constitutes 97% of arsenic produced that enters end product manufactured. Arsenic trioxide is the raw material for arsenical pesticides including lead arsenate, calcium arsenate, sodium arsenite, and organic arsenicals. These compounds are used in insecticides, herbicides, fungicides, algicides, sheepdips, wood preservatives, and dyestuffs; and for eradication of tapeworm in sheep and cattle. As (III) exists in most natural water as $As(OH)_3$ (pKa = 9.2) and is more mobile than As(V) because it is less strongly absorbed on most mineral surfaces than the negatively charged As(V) oxyanions (H_3AsO_4; pKa = 2.22, 6.98, 11.53). Iron (III) oxy species have a high affinity for As (V) (Waychunas et al. 1993).

Arsenic and P are chemically similar. Both form insoluble compounds with Al and Fe in soils. Al-As and Fe-As are the dominant forms of arsenic in most soils, though arsenic shows less affinity for Al oxides than does phosphates. As (III) seems to be adsorbed on iron (III) surfaces (Sun and Doner, 1996). Activated alumina has a 2-fold

higher affinity for As (V) than for As (III) at pH 7 (Ghosh and Yuan, 1987). Kaolinite and montmorillonite have higher affinities for As (V) than for As (III) (Frost and Griffin, 1977). Abiotic oxidation of As (III) is enhanced in the presence of the clay minerals kaolinite and illite, a process that results in strongly-bound As(V) species (Manning and Goldberg, 1997). Arsenic mobility and phytotoxicity are greater in sandy than in clayey soils.

The total concentration of arsenic in the soil does not reflect the amount available for plant uptake because of the adsorption of arsenic in the soil. Therefore different methods are used to determine availability of arsenic in the soil. Various extractants that could simulate release of arsenic under different conditions in the soil have been reported to correlate significantly with plant arsenic uptake.

Sequential extraction has been widely used to assess metal availability and mobility in soils. Different reagents are used to separate metals into operationally-defined chemical associations. Among all the methods, the one developed by Tessier et al. (1988) is the most widely used to assess metal geochemistry in soils. However, for soil arsenic, due to its chemical similarity to phosphate, the method used for P fractionation has been used for arsenic fractionation (Onken and Adriano, 1997). Soil arsenic is operationally separated into four fractions: water-soluble plus exchangeable arsenic (WE-As), Al-bound arsenic (Al-As), Fe- bound arsenic (Fe-As), and Ca-bound arsenic (Ca-As), using NH_4NO_3, NH_4F, NaOH and H_2SO_4. Based on sequential extraction, information about the chemical binding form, retention, and partitioning of metals in soils can be estimated. Even though sequential extraction suffers from the lack of specificity

during chemical fractionation and resorption of dissolved metals by soils during the extraction, it is still a useful tool to evaluate metal biochemistry in soils.

In arsenic contaminated soils, soil pH is one of the major factors determining arsenic availability (Bech et al., 1997). Akins and Lewis (1976) examined the effects of pH (4-8) on arsenic sorption by soils using a sequential fractionation procedure. They found that, at low pH (pH=4), Fe-As is the most abundant form followed by Al-As, whereas at high pH (pH=6-8), Ca-As is more predominant. This is similar to behaviors of P in soils; that is, Fe-P and Al-P are predominant in acid soils, whereas Ca-P dominates in alkaline and calcareous soils (Adriano, 1986). In addition to soil pH, arsenic partitioning and retention in soils is also influenced by the presence of organic matter, Fe, Mn, and Al oxides, and clay minerals (Balasoiu et al, 2001). However, the capacity of a soil to retain arsenic mainly depends on the content of extractable hydrous oxides of Fe and Al in the soil (Livesey and Huang, 1981; Jacobs et al., 1970).

Efficiency of phytoremediation depends on soil characteristics and contaminant. Adsorption of arsenic in soils is influenced by a number of factors: types and amount of soil colloids (clay minerals; Fe, Al and Mn oxides; and organic matter); pH; presence of organic and inorganic ligands; and soil texture (Ross, 1994). The difference in the rate of sorption of arsenite on A and B-horizons of five West Virginia soils was explained by differences in pH, iron oxide and organic matter content (Elkhatib, 1984). There is a strong association between As and Fe (mainly goethite) in soils for both natural and added As. Hydroxy aluminium on the external surfaces of micaceous minerals significantly retains As (Kabata-Pendias and Pendias, 2001). Arsenic mobility and

phytotoxicity are greater in sandy soils than in clayey soils because hydrous Fe and Al oxides vary directly with clay content of the soil.

2.2 Arsenic in Water

Arsenic concentrations in groundwater are of increasing environmental concern because of the risk arsenic poses to plants, animals, and human health (Mueller et al., 2001). The EPA is in the process of setting the new arsenic standard for drinking water at 10 ppb (μg/L) to protect humans against the effects of long-term, chronic exposure to arsenic in drinking water. Roughly 5% (or 3,000) of community water systems serving 11 million people will have to take corrective action to lower the current levels of arsenic in their drinking water (EPA, 2001).

Higher levels of arsenic are found in groundwater sources than in surface-water sources. In parts of the southern San Joaquin Valley, California; and parts of Arizona and the middle Rio Grande Basin, New Mexico; oxic, alkaline groundwater contains high arsenic concentrations that may result from desorption from iron oxide. In the southwest, high arsenic concentrations are associated with iron-rich groundwater (which is consistent with dissolution of iron oxide as a source of arsenic). High pH groundwater in felsic volcanics contains high concentrations of arsenic in parts of the Willamette Basin, Oregon. In the upper Midwest, glaciated quaternary sediments appear to be associated with high arsenic concentrations in groundwater (Ryker and Welch, 2001). Arsenic in groundwater is mainly inorganic with arsenate comprising about 50% of the total (Abedin et al., 2002).

Many countries around the world (including Taiwan, Argentina, India, Bangladesh, Mexico, Hungary, and Chile) have reported extensive arsenic contamination of their

groundwater supplies (Nikolaidis et al., 2004; Smedley et al., 2002). In the United States, arsenic-contaminated groundwater has been reported in New England (Peters et al., 1999), the Mid-west (Welch et al., 2000), Oklahoma (Schlottmann and Breit, 1992), Nevada (Welch and Lico, 1998) and California (Wilkie and Hering, 1998).

Groundwater in Bangladesh is currently contaminated by up to 2mg/L As with reports of widespread arsenic-related health effects on millions of people (Abedin et al., 2002). Use of this contaminated water for irrigation of crops has led to elevated concentrations of arsenic in agricultural soils.

Out of the groundwater investigated in the quarternary loess aquifers in northern La Pampa Province of central Argentina, 95% exceeded the World Health Organisation (WHO) guideline value of 10 μg/L As. Groundwater arsenic correlated positively with pH, alkalinity, (HCO_3), F, and V. Weaker correlations were observed with B, Mo, U, and Be. High concentration of As in the aquifer resulted from desorption of arsenic from Fe and Mn oxides; weathering of primary silicate minerals, and apatite; high pH and alkalinity from silicate and carbonate reactions (Smedley et al., 2002).

In Michigan, (USA) 12% of wells sampled had arsenic concentrations exceeding the USEPA contaminant level; and most of the arsenic detected was arsenite (AsIII). In shallow groundwater (<15 m), low arsenic concentrations are likely due to formation of insoluble ferrosoferrichydroxide complex while in deep groundwater (>15 m). Concentrations of arsenic are probably controlled by reductive dissolution of arsenic-rich iron hydroxide/oxyhydroxide and dissolution of arsenic-sulfide minerals (Kim et al., 2002).

2.3 *Pteris vittata*

Brake fern (*P. vittata*) is native to South Africa, Madagascar, Asia, Japan, Malaysia, New Guinea, and Australia. The fern is usually found in sunny situations; shuns shade; and requires free drainage but appreciates watering during the dry season. It grows on limestone and requires neutral to alkaline conditions. Brake fern was found growing on a site in Central Florida contaminated with chromated copper arsenate (Ma et al., 2001). This fern species accumulated up to 5,000 mg kg^{-1}As in its aboveground biomass under normal conditions in a soil with an average of 184 mg kg^{-1}As. It produces a large amount of aboveground biomass, which makes it ideal for phytoremediation purposes.

Calcium is probably of special importance to *P. vittata* because it's lime-loving (Jones, 1987). Phosphate rocks have been shown to release Ca ions thereby increasing the soil pH (Hammond et al., 1986; Ma and Rao, 1999; and Rajan et al., 1996). Phosphate rock could be useful at arsenic-contaminated sites impacted with metals such as lead because it supplies both Ca and P (two essential nutrients for plant growth) and increases soil pH (which aids the immobilization of lead thereby reducing phytotoxicity of lead to the arsenic hyperaccumulator). Ma and Rao (1999) showed that phosphate rock effectively immobilized aqueous lead from Pb-contaminated soils although its effectiveness was affected by soil pH and extent of Pb contamination.

2.4 Detoxification Mechanisms in Plants

Hyperaccumulating plants possess efficient mechanisms for detoxifying accumulated metal. These mechanisms include chelation, compartmentalization, biotransformation and cellular repair (Salt et al., 1998).

Heavy metals are generally transported and deposited in the vacuole as metal chelates. Baker et al. (2000) explained that the solution concentration of free metal ions taken up by plants into their tissues is reduced greatly when they are chelated by specific high-affinity ligands (like oxygen-donor ligands, sulfur-donor ligands, and nitrogen-donor ligands).

They gave examples of oxygen-donor ligands as organic acids, in particular carboxylic acid anions, which are abundant in the cells of terrestrial plants and form complexes with divalent and trivalent metal ions of reasonably high stability. Carboxylates (such as malate, aconitate, malonate, oxalate, tartrate, citrate, and isocitrate) are commonly the major charge-balancing anion present in the cell vacuoles of photosynthetic tissues. Several of these carboxylates have been associated with high metal concentrations in plants (Ma et al., 1997; Gabbrielli et al., 1997; Homer et al., 1995). Malate was proposed as a carrier to transport Zn^{2+} ions into the vacuole in *T. caerulescens* (Mathys, 1977).

Sulfur-donor ligands (like metallothioneins and phytochelatins) form highly stable complexes with heavy metals because sulfur is a better electron donor than oxygen. Metallothioneins are gene-encoded low-molecular-weight, cysteine-rich peptides found in fungi and mammals recently shown to be induced by Cu in plants (Robinson et al., 1993). In fungi and mammals, metallothioneins are involved in metal detoxification (Tohayama et al., 1995) but their role in plants is not yet well understood.

Recent studies show the existence of a group of organic solute transporters, belonging to the ATP binding cassette (ABC) transporters superfamily, that is directly energized by MgATP (Rea et al. 1998). These pumps are competent in transporting a

broad range of substances including sugars, peptides, alkaloids, and inorganic anions. Belonging to the ABC family, the multidrug resistance–associated proteins (MRPs) identified in plants are thought to participate in transporting exogenous and endogenous amphipathic anions and glutathionated compounds from the cytoplasm to the vacuole. They function in herbicide detoxification, cell pigmentation, storage of anti-microbial compounds, and alleviation of oxidative damage. Plant MRPs are also suspected to play a role in channel regulation and transporting heavy-metal chelates. Glutathione S-conjugate (GS-X) and metabolite (M) transport is achieved by specific ABC transporters.

Phytochelatins are low-molecular-weight, cysteine-rich peptides that are especially produced by plants when exposed to heavy metals and are known to bind cadmium and copper in plants (Rauser, 1995). The PC-metal complexes are less toxic than free metal ions to cellular plant metabolism (Prasad, 1999). They have been shown to be essential for cadmium detoxification in *A. thaliana* (Howden et al., 1995a and 1995b) and are believed to bind Pb and Hg by competing with Cd (Abrahamson et al. 1992). The GSH-mediated transfer of Cd2+, Pb2+, Cu2+ and Hg2+ to PCs was demonstrated by Mehra et al. (1996). Phytochelatin synthesis has been induced on exposure to arsenate in a number of plant species (Sneller et al. 1999, Schmoger et al. 2000, Zhao et al. 2003). Intact PCs-As complexes have also been isolated from plant tissues (Schmoger et al. 2000) suggesting that phytochelatins are also involved in arsenic detoxification in plants. Though phytochelatin (PC) synthesis was induced on exposure to arsenate in *P. vittata*, only PC2 was detected in the plant. The molar ratio of PC-SH to As suggested that only a small proportion (1-3%) of the As in *P. vittata* can be complexed with PCs (Zhao et al., 2003).

Resistance to arsenic in arsenic non-hyperaccumulating plants has been shown to involve a decreased uptake of arsenate due to suppression of the high affinity phosphate uptake system (Meharg and Mcnair, 1991). This is because the mechanisms of arsenic uptake are similar to those of phosphorus resulting in competitive uptake. Arsenic detoxification might also include methylation and biotransformation by microorganisms. Some bacteria enzymatically reduce arsenate to arsenite by ArsC and the arsenite is then pumped out by the membrane protein Ars B (Cai and Ma, 2003).

2.5 Phosphate Rocks

Effectiveness of phosphate rock as a direct-application fertilizer is directly related to the degree of substitution of carbonate for phosphate in the apatite structure. The greater the carbonate substitution, the greater the solubility of the phosphate rock and the greater the agronomic benefit of the rock for direct application (Khasawneh and Doll, 1978). Solubility of a phosphate fertilizer directly influences the quality of plant available phosphorus that will be released from the fertilizer at any given time after its application. Plant uptake of P has previously been shown to be related to a fundamental crystal property, the unit cell a (A) dimension of carbonate apatite (Anderson et al., 1985). Increasing carbonate decreases the unit cell a- dimension and increases its solubility and reactivity (Rajan et al., 1996). Phosphate rocks have been shown to release Ca ions thereby increasing soil pH (Ma and Rao, 1999; Hammond et al., 1986; and Rajan et al., 1996).

Addition of phosphate rock supplies calcium and phosphate to the soil (Hellums, 1989; Rajan et al., 1996), so it is expected to aid plant arsenic uptake and accumulation in contaminated soils. Both calcium and phosphate are essential plant nutrients that are beneficial to plant growth. It has been observed that phosphate rock immobilizes Pb in

contaminated soils (Ma et al., 1995). Phosphate rock was the only treatment that reduced gastrointestinal available Pb in both gastric and intestinal solutions, by 23 and 92%, respectively. Phosphate rock decreases risk from exposure to Pb via the soil-ingestion pathway (Basta et al., 2001).

Phosphate rocks for direct soil application are a potential alternative to water-soluble fertilizers. They are less likely to cause eutrophication of surface waters and are cost-effective compared to water-soluble P sources (Robinson, 1992). High P concentration could also increase As availability because P competes for As fixation sites because of their chemical similarities (Adriano, 1986). Increased P status may increase plant tolerance as arsenate toxicity is due to the competition of arsenate with phosphate in ATP (Ullrich-Eberius et al., 1989). Increasing cell phosphate levels will lead to decreased formation of the arsenate ATP analogue and therefore to increased arsenic tolerance (Meharg et al., 1994). This explains the positive correlation observed between plant P and As accumulation in the brake fern (Komar, 1999). The phosphate uptake system by which arsenic is taken up (Meharg & Macnair, 1994) is induced under a low-phosphate status like that of some angiosperms and fungi.

CHAPTER 3
EFFECTS OF HEAVY METALS ON GROWTH AND ARSENIC ACCUMULATION OF ARSENIC HYPERACCUMULATOR *Pteris vittata* L.

3.1 Introduction

Arsenic contamination has become of major concern to government and industry as more arsenic-related human health problems have surfaced worldwide (EPA, 2001). Phytoremediation, a process that uses plants for environmental restoration, has been proposed as a cost-effective alternative technology to remediate arsenic-contaminated soils (Lasat, 2002). Phytoremediation preserves the topsoil and reduces the amount of hazardous materials generated during cleanup (Ensley, 2000). The rate of plant biomass production and elemental concentrations in the aboveground biomass is the key to using hyperaccumulators in phytoremediation (Reeves and Baker, 2000). Hyperaccumulators are plants and/or genotypes that accumulate metals above certain concentrations in their aboveground biomass (Kabata-Pendias and Pendias, 2001). More than 400 hyperaccumulators have been identified for Cu, Co, Zn, Ni, Se, and Pb (Harris and Oparka, 1994).

Arsenic accumulation has been reported in grasses growing on mine waste in the United Kingdom (Porter and Petersen, 1975). The first arsenic hyperaccumulator (*Pteris vittata)* was discovered growing on a site contaminated with chromated copper arsenate (CCA), a commonly used wood preservative in Central Florida (Komar et al., 1998; Ma et al., 2001). The fern efficiently accumulates As (up to 2.3% in its fronds) and produces a large amount of aboveground biomass (up to 1.7 m in height), which makes it feasible for

phytoremediation purposes. This fern has also been reported growing on arsenic-contaminated soils in Thailand (Francesconi et al., 2002).

Though *P. vittata* is effective in hyperaccumulating arsenic from both CCA contaminated soil and non-contaminated soils (Ma et al., 2001), its ability to survive in soil contaminated with heavy metals other than Cu and Cr is unknown (Komar, 1999). The impacts of these metals on plant biomass production and arsenic hyperaccumulation by *P. vittata* are also unknown. To effectively remediate arsenic contaminated soils, *P. vittata* must be tolerant of other metals and effective in hyperaccumulating arsenic in the presence of other co-contaminants.

Previous research on arsenic hyperaccumulation by *P. vittata* (Ma et al., 2001; Zhang et al., 2002) showed that arsenic exists in the plant predominantly as inorganic species with little detectable organic arsenic. In addition, up to 80% of the As in the fronds (stems and leaves) is present as arsenite. Reduction of arsenate (As-V, AsO_4^{3-}) to arsenite (As-III, AsO_3^{3-}) is thought to be one of the detoxification mechanisms in arsenic hyperaccumulation by the fern. Thus, it is important to determine whether the presence of other heavy metals affects the ability of *P. vittata* to reduce arsenic.

Heavy metals are known to cause oxidative stress in plants, which in turn induces antioxidant enzymes to catalyze the reduction of the superoxide radical (Sairam et al, 1998; Teisseire and Vernet, 2001). In our study, catalase activity (an anti-oxidant enzyme) was used as a biomarker for the response of the fern to heavy metal stress from the experimental treatments. In this chapter, we attempted to determine the impacts of heavy metals on plant biomass and antioxidant activity of *P. vittata* and examine the effects of heavy metals on arsenic uptake, distribution, and speciation by *P. vittata*. The information obtained from this

study should be useful for application of *P. vittata* to arsenic-contaminated sites contaminated with other heavy metals.

3.2 Materials and Method

3.2.1 Soil Characterization

The soil used in this experiment was collected from an abandoned wood preservation site in Central Florida. The site is contaminated with chromated copper arsenate (CCA) and is where the hyperaccumulating ability of *P. vittata* was first recognized (Komar et al., 1998; Ma et al., 2001). The soil is classified as an Arredondo–urban land complex (loamy, siliceous, hyperthermic Grossarenic Paleudult). The soil was air-dried and analyzed for total Pb, Cd, Cr, Cu, As, Ni, and Zn concentrations, and water-soluble arsenic. Soil pH was measured using a 1:2 soil to water ratio. Cation exchange capacity (CEC) was determined by an ammonium acetate method (Thomas, 1982). Organic matter content was measured by the Walkley Black method (Nelson and Sommers, 1982) and particle size by the pipette method (Day, 1965). Selected physical-chemical properties of the soil are listed in Table 3-1.

3.2.2 Experiment Setup

Air-dried arsenic-contaminated soil was separately amended with nitrate salts of Pb, Cd, Ni, or Zn as solutions at two levels: 50 and 200 mg kg^{-1}. Each of these treatments was replicated four times. Metal-spiked soil (1.5 kg) was thoroughly mixed with 1.5 g of Osmocote time-release fertilizer (18-6-12) and 1.5 g/kg of peat moss, and placed in 15 cm diameter (2.5 L) plastic pots. After one-week equilibration, one healthy fern was planted in each pot. The plants used in this study were propagated from spores in our laboratory using a method described by Jones (1987). The plants were grown for approximately four months and had 5-6 fronds before transplanting. Efforts were made to ensure visual uniformity across all plants. Plant biomass and metal concentrations were determined before being

transplanted (Table 3-1). The plants were grown in the greenhouse where the average temperature ranged from 14 (night) to 30°C (day), with an average photosynthetically photoactive radiation of 825 μmol m-2 s^{-1}. Plants were harvested eight weeks after transplanting.

3.2.3 Catalase Assay

Catalase activity was determined in the fern fronds a day before harvesting using the method of Fu and Huang (2001). The enzyme was extracted at 4^{o}C from 1g of fresh leaves using a mortar and a pestle with 50 mM sodium phosphate buffer (pH 7.0). The extract was then centrifuged at 14,000 g for 10 min at 4^{o}C. Crude extract obtained was desalted by using a sephadex G-25 column. Catalase activity was assayed by measuring the decline in absorbance at 240 nm for 1 min in a 3-mL reaction mixture containing 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 0.1 mL extract. Protein was quantified by the dye-binding micro-assay (Bradford, 1976).

3.2.4 Arsenic Speciation in Fern Fronds

Speciation of arsenic in the fern fronds was determined immediately after harvesting by extracting plant samples ultrasonically in 1:1 methanol/ water twice following the method of Zhang et al. (2002). Arsenate and arsenite were separated using an arsenic speciation cartridge (Metal Soft Center, Highland Park, NJ), which retains arsenate (Meng et al., 2001). The samples were then analyzed for total arsenic and arsenite.

3.2.5 Soil and Plant Analysis

The harvested plants were separated into aboveground (fronds) and belowground biomass, oven-dried at 65^{o}C for 3 days and ground into powder. Soil samples were taken at five and eight weeks, air-dried and analyzed for soil pH, and water soluble and total As, Cd, Pb, Zn, and Ni content. Soil pH was measured using a pH meter and 1:2 soil: solution ratio,

while the water-soluble metals were determined in a suspension at 2:10 soil: solution ratio after shaking for one hour. Soil and plant samples were digested with nitric acid using the Hot Block Digestion System (Environmental Express, Mt. Pleasant, SC; EPA Method 3050A). Total As, Ni and Cd concentrations were determined with a graphite furnace atomic absorption spectrophotometer (Perkin Elmer SIMMA 6000, Perkin-Elmer Corp, Norwalk, CT) while Pb and Zn contents were analyzed on a flame atomic absorption spectrophotometer (Varian 220 FS with SIPS, Varian, Walnut Creek, CA).

3.2.6 Fractionation of Arsenic

Arsenic fractionation procedure used by Onken and Adriano (1997) was used in this experiment. The procedure was briefly summarized below using 2 g of soil and 40 ml extraction solution. Fractions of WE-As, Al–As, Fe-As, and Ca-As were obtained by determining supernatants extracted using 1 M NH_4Cl (shaken for 30 min), 0.5 M NH_4F (0.5 h), 0.1 M NaOH (17 h), and 0.5 M H_2SO_4 (17 h), respectively. Between each fraction, 25 ml of saturated sodium chloride was added to the solid residue, resuspended, centrifuged, and supernatant discarded. This washing step was repeated for all fractions. For each step, the suspensions, after shaken for specific time, were centrifuged at 2000 rpm.

3.2.7 Statistical Methods

The experiment is a two-factored experiment (four metal types at two levels) with four replications arranged in a completely randomized design. Treatment effects were determined by analysis of variance according to the general linear model procedure of the Statistical Analysis System (SAS Institute, 1987). Differences among treatment means were separated by the Duncans multiple range test. Significance was tested at the 0.05 probability level.

3.3 Results and Discussion

This experiment was conducted to determine the impacts of heavy metals, Cd, Ni, Zn and Pb on plant growth and arsenic accumulation of *P. vittata*. Basic properties of the arsenic contaminated soil and background metal concentrations in *P. vittata* are presented in Table 3-1. The arsenic-contaminated soil collected from a former CCA wood-treating facility was used as the control. The total arsenic in the soil was 131 mg kg^{-1}, which was much greater than Cr and Cu (40.6 and 8.30 mg kg^{-1}). Concentrations of Cd, Ni, Zn and Pb were relatively low (<8.1 mg kg^{-1}). The soil was deficient in Zn as Zn sufficiency range for most plants is between 25 to 150 mg kg^{-1} (Kiekens, 1993). All metal concentrations are within the range for typical Florida soils except for arsenic (Chen et al., 1999). Hence, only arsenic could be harmful to plant growth because of its high concentration in the soil.

In addition to soil characterization, background metal concentrations in *P. vittata* were also determined prior to transfer (Table 3-1). These were comparable to most plants with two exceptions (Kabata-Pendias and Pendias, 2001). Plant Ni content (11.5-14.9 mg kg^{-1}) was greater than those reported for other plants and plant arsenic concentration (226 mg kg^{-1}) was substantially greater than those in most plants. For example, average Ni concentrations in grasses and crops ranged from 1.2 to 2.7 mg kg^{-1}. Similar to Ni, most plants have relatively low As concentrations, typically <1.5 mg kg^{-1} (Kabata-Pendias and Pendias, 2001). The fact that arsenic concentration in *P. vittata* was 150 times greater than those of typical plants is consistent with its hyperaccumulator characteristics (Ma et al., 2001). In addition to background elemental concentrations, fern biomass was also determined. The average dry aboveground biomass per plant was 0.65 g, while below ground biomass was 1.18 g. The relatively large root biomass of *P. vittata* may facilitate its arsenic uptake from the soil.

Table 3-1. Selected soil properties and background metal concentrations in *P. vittata* before transplanting (mg/kg)

	Soil	*P.vittata*	
Properties		Above ground	Below ground
Total As	131±3.7 *	226±1.6	18.1±0.2
Total Cd	0.08± 0.01	0.03±0.01	0.12±0.03
Total Ni	7.40± 0.6	11.5±2.8	14.9±1.6
Total Pb	8.10±0.6	5.12±1.1	33.5±0.8
Total Zn	0.81±0.01	48.6±0.9	46.1±0.8
Total Cr	40.6±1.6	3.81±1.3	19.5±2.8
Total Cu	8.30±1.5	10.4±0.9	10.4±1.4
Water-soluble As	0.2±0.02		
Soil pH	7.60±0.01		
CEC (cmol/kg)**	4.4±0.01	Plant biomass	(g)
Organic matter (g/kg)	11.0± 1.2	0.65	1.18
Sand (%)	88.2±2.2		
Silt (%)	9.1±0.2		
Clay (%)	2.7±0.01		

* Mean ± standard error **CEC-Cation exchange capacity

3.3.1 Plant Biomass

Plant biomass (dry wt.) can be used as an indicator for the overall health of *P. vittata* growing in the presence of heavy metals. After 8 weeks of growth, the fern biomass increased from 1.8 g to 5.9-19 g (Figure 3-1) in the presence of heavy metals. Mean separation using Duncan multiple range test shows that the aboveground biomass of Cd-50 (i.e. Cd was spiked at 50 mg kg^{-1}) was significantly higher than the others. While the belowground biomass of Pb-200, Cd-50, Zn-200 and Pb-50 were significantly higher than the other treatments with Pb-200 > Cd-50 >Zn-200 >Pb-50. The total biomass of the treatments were in order of Cd-50> Pb-200> Zn-200> Pb-50 > Cd-200> Zn-50> control> Ni-50> Ni-200, with Cd-50 and Pb-200 being significantly higher than other treatments.

Table 3-2. Plant biomass (g) of *P. vittata* after growing for 8 weeks in an arsenic-contaminated soil spiked with metals at 50 or 200 mg kg^{-1}

Treatment	Aboveground	Belowground
Control	5.7bc*	2.7c
Cd-2000	5.4bc	4.9bc
Cd-50	7.7a	11.2a

Ni-200	3.9c	2c
Ni-50	3.8c	2.7c
Zn-200	6.2ab	8.2ab
Zn-50	4.5bc	5.6bc
Pb-200	5.7bc	12.2a
Pb-50	5.1bc	7.9ab

*Mean of four replicates and means with the same letter are not significantly different.

The results indicate that *P. vittata* was tolerant of metals at the amended levels, which are relatively high as they were added as soluble nitrate form (Ma and Rao, 1997).

The fact that only Ni-50 and Ni-200 treatments produced significantly lower total biomass than the control indicates that Ni adversely impacted *P.vittata* growth. The stimulation of plant growth by Cd, Zn and Pb may result from added N nutrition since all metals were added as nitrate salts. The low solubility of spiked metals in soils could also have limited their adverse impact on plant growth. In terms of the quantity of nitrate added to the soil with heavy metals, Ni-200 treatments was the highest, followed by Cd-200, Ni-50, and Cd-50. Thus, impacts of nitrate alone could not explain the changes in fern biomass. Visual observations confirmed that there were no toxicity symptoms in the fern growing in the soil spiked with Cd-50, which had the most vigorous growth. There were, however, toxicity symptoms (burning at leaf edges) in Cd-200, Zn and Pb (-50 and -200) treatments

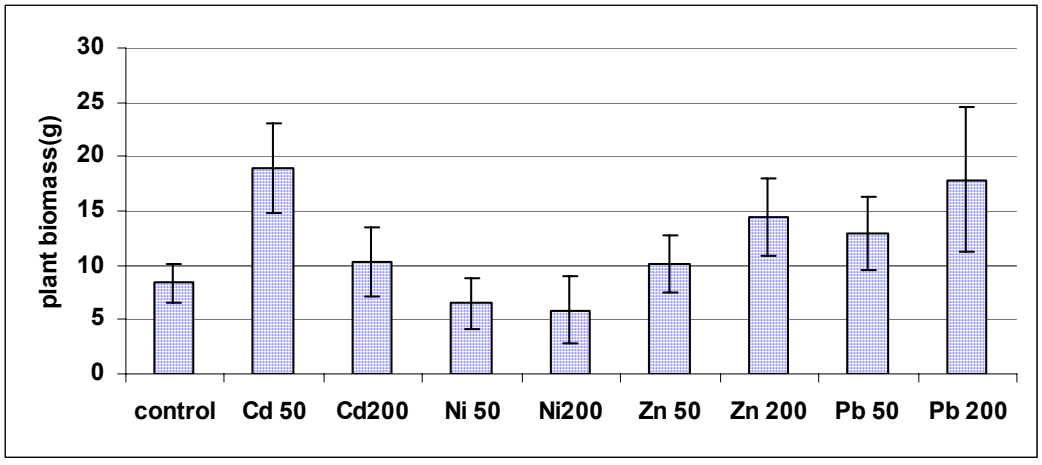


Figure 3-1. Plant biomass of *P. vittata* grown for 8 weeks in CCA soil spiked with various metals. Bars are SE of means.

The fact that total plant biomass in *P. vittata* was greater grown in metal-spiked soils than those of the control with the exception of Ni, and the fact that its biomass increased by as much as 12 times compared to its original mass after 8 wks of growth (Figure 3-1) suggests that *P. vittata* was tolerant to these metals.

3.3.2 Antioxidant Enzyme Activity

Catalase is an antioxidant enzyme produced by plants under conditions of oxidative stress such as the presence of heavy metals (Sairam et al., 1998). The function of catalase is to degrade H_2O_2, which is a potential source of the highly reactive hydroxyl radical and singlet oxygen. The singlet ion can initiate lipid and organic peroxidation (MacRae and Ferguson 1985). Thus, greater catalase activity generally indicates greater stress for a given plant. However, it is important to point out that under extreme conditions of stress, a plant may be too weak to produce enough antioxidant enzymes to protect it.

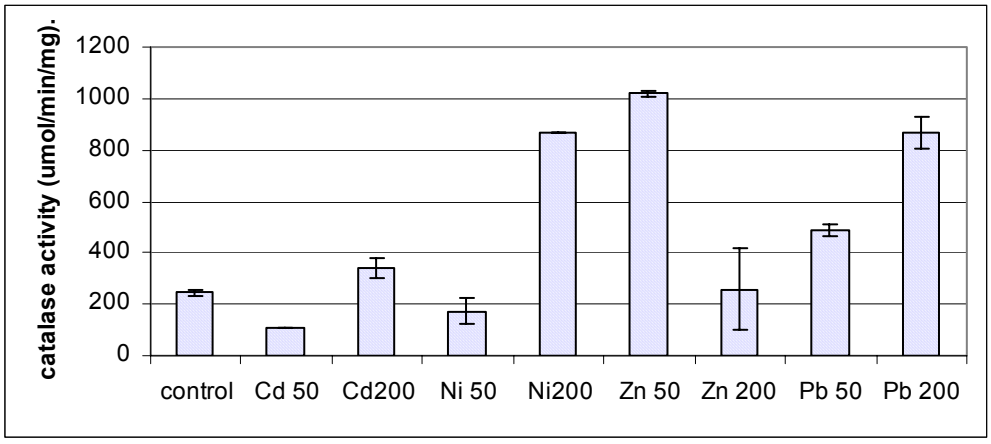


Figure 3-2. Extractable catalase activity in the fronds of *P.vittata* grown for 8 weeks in CCA soil spiked with various metals at 50 or 200mg/kg. Bars are SE of means.

As such, low activity of antioxidant enzyme may not always indicate low stress. Catalase activity in the plant increased with increasing plant concentrations of Cd, Ni, and Pb (Figure 3-2). However, Zn-200 produced lower catalase activity compared to Zn-50, which had the highest catalase activity. This is possible since, among the four metals, Zn is the only

plant nutrient, though Ni is also referred to as an essential plant nutrient. The highest activity of catalase was recorded in the ferns growing in Zn-50 spiked soil. Necrosis in young fronds was observed on two ferns (replicates) growing in the same soil. Interestingly, Zn toxicity in *Phaseolus vulgaris* increased the levels of H_2O_2 (increase of catalase activity) in roots and lipid peroxidation in primary leaves (Weckx and Clijsters, 1997) while Zn deficiency has also been reported to cause photo-oxidation of thylakoid constituents and impairment of detoxifying mechanisms (Cakmak and Marschner, 1993). It is not very clear what is responsible for the high catalase activity of the ferns grown in Zn-50. It doesn't appear to be zinc toxicity since the critical toxicity levels of zinc in crop plants was given as ranging from 100-300 μg Zn g^{-1} dry wt (Marschner, 1995), which were greater than those in the fern. However, at the present time, the critical toxicity level of zinc in the fern is unknown. Also Zn deficiency is expected to be seen in the control plants if this was the case.

The fact that plants grown in Ni-50 had a lower catalase activity than the control was not an indication of low stress. On the contrary, the plants used for the assay were already damaged (2nd lowest biomass among all metals; Figure 3-1). The decrease in catalase activity reflected the poor health of the plant, probably due to lipid peroxidation and plant damage. Assimilation of toxic amounts of Ni in the plant (Tables 3-3 and 3-4) may have caused oxidative damage and inhibited plant growth (Salem et al., 1998). Fu and Huang (2001) showed that decreased catalase activity induced by severe drought stress in tall fescue and Kentucky blue grass was due to lipid peroxidation.

Metal stress has been reported to affect photosynthesis, chlorophyll fluorescence, stomatal resistance and biomass (Mendelssohn et al., 2001; Monni et al., 2001; Xiong, 1997). However, stimulating effects of metal ions on catalase activity in different plant species have

also been reported (Teisseire and Vernet, 2000; Knorzer et al., 1996). Both cases have been observed in our study. Though Pb-200 treated plants had 2nd highest total biomass, Pb seemed to induce plant stress as catalase activity was among the highest, 251% of the control. Ferns grown in soils amended with Cd-50 had the lowest catalase activity (57% of the control), which is consistent with the fact that this produced the highest total plant biomass. So both plant biomass and catalase activity should be used to measure plant health.

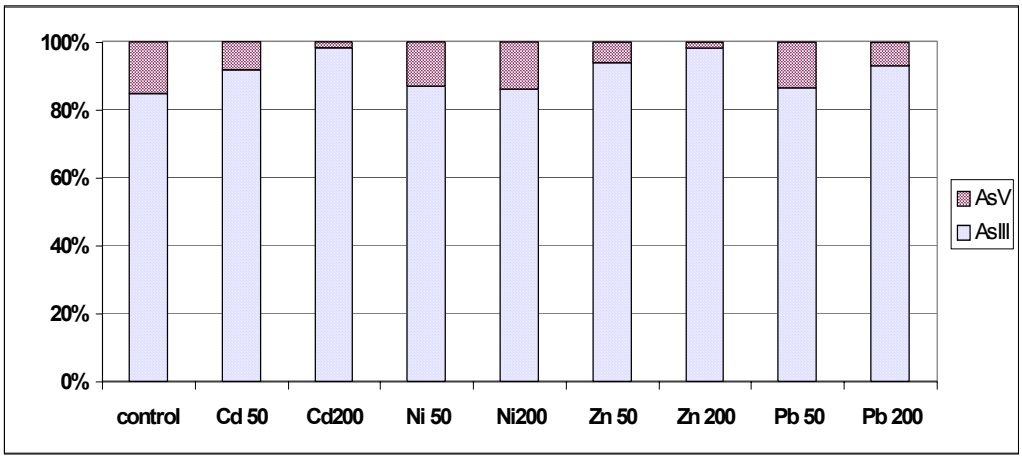


Figure 3-3. Speciation of arsenic in the fronds of *P. vittata* grown for 8 weeks in CCA soil spiked with various metals at 50 or 200 mg/kg.

3.3.3 Arsenic Speciation in the Plant

Once taken up by *P. vittata*, arsenic is translocated from the roots and stored in the fronds, mostly in the reduced form; that is, arsenite (As-III; Ma et al. 2001). Reduction of arsenate to arsenite is considered a detoxification mechanism for the plant (Zhang et al. 2002). Thus, under conditions of physiological stress (i.e., heavy metals) plant arsenic reduction may be impacted. Consistent with previous results (Ma et al., 2001; Francesconi et al. 2002), >80% of the arsenic in *P. vittata* was present as the reduced species, arsenite (Figure 3-3). The addition of other metals seemed to enhance plant As reduction. The percentage of As (III) in the fern fronds increased with increasing metal concentration except in the Ni-amended soils.

As discussed earlier, Ni had negatively impacted fern growth. All the ferns growing in metal-amended soils except Ni and Pb-50 had a greater percentage of As (III) in the fronds than those in the control.

3.3.4 Impact on Arsenic Accumulation

Arsenic concentrations in frond biomass were not significantly affected in soils amended with 50 ppm metals except in Cd-treated soils (Table 3-3). Arsenic uptake in soils amended with 200 ppm metals was, however, significantly affected except in Pb-amended soils. Arsenic uptake decreased significantly with increase in metal concentration for each metal except in lead treated soils. The total amount of arsenic removed from soil (concentration x biomass) described as 'arsenic accumulation' can be used to determine the efficiency of a plant in concentrating arsenic from soil into its biomass. The control showed significantly higher arsenic accumulation by *P. vittata* than the metal amended soils, with Cd and Ni treatments having the lowest values (Fig.3-4).

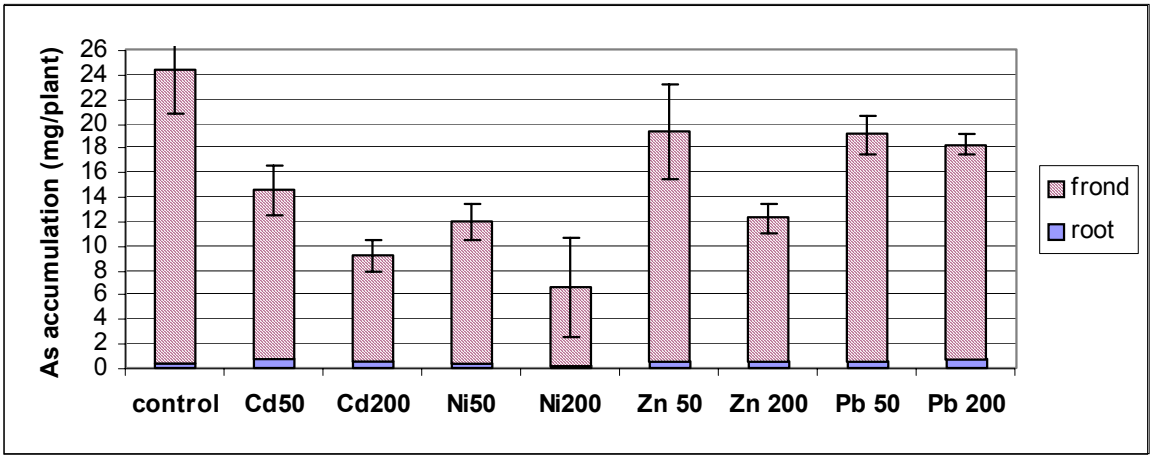


Figure 3-4. Arsenic accumulation in *P. vittata* grown for 8 weeks in CCA soil spiked with various metals. Bars are SE of means

Though effective in taking up arsenic, *P. vittata* had a limited capability to take up other metals. Ferns grown in Ni-200 soils had 73 mg kg^{-1} Ni in the fronds (Table 3-3) and

309 mg kg^{-1} Ni in the roots (Table 3-4). These high concentrations of Ni in the fern had negatively impacted fern growth, with Ni-treated soil producing the lowest fern biomass among all treatments (Figure 3-1). Relatively large amounts of Cd taken up by the fern (48.3 and 186 mg kg^{-1}) also adversely impacted fern growth and arsenic accumulation.

Table 3-3. Total arsenic and metal concentrations (mg kg^{-1}) in fronds of *P. vittata* after growing for 8 weeks in CCA soil spiked with metals at 50 or 200 mg kg^{-1}

	As	Cd	Ni	Zn	Pb
control	4200a*	0.03c	1.4c	49.7bc	38.1d
Cd-50	1824bc	8.38b	0.7c	42.5c	42.5d
Cd-200	1617c	48.3a	7.4c	49.2bc	67.5d
Ni-50	3412a	<0.02c	26.7b	54.6bc	65.0d
Ni-200	1538c	<0.02c	72.6a	59.4b	85.0c
Zn-50	4100a	0.05c	2.5c	83.3a	90.0c
Zn-200	1913bc	<0.02c	1.1c	83.9a	94.4bc
Pb-50	3612a	<0.02c	1.3c	50.9bc	118ab
Pb-200	3075ab	0.01c	1.7c	51.7bc	110a

*Mean of four replicates and means with the same letter are not significantly different.

Table 3-4. Total arsenic and metal concentrations (mg kg^{-1}) in the roots of *P. vittata* after growing for 8 weeks in CCA spiked with metals at 50 or 200 mg kg^{-1}

Treatment	As	Cd	Ni	Zn	Pb
Control	130a*	0.11c	5.40c	69.1c	69.4d
Cd-50	66.0a	39.5b	4.80c	51.9c	68.7d
Cd-200	92.0a	186a	3.50c	66.1c	90.0cd
Ni-50	136a	0.16c	73.6b	59.2c	95.6cd
Ni-200	121a	0.08c	309a	73.2c	106cd
Zn-50	93.0a	0.11c	10.0c	178b	123c
Zn-200	74.0a	0.05c	5.20c	228a	118c
Pb-50	66.0a	0.05c	6.30c	68.7c	179b
Pb-200	68.0a	0.06c	5.80c	60.2c	306a

*Mean of four replicates and means with the same letter are not significantly different.

Pteris vittata also accumulated up to 118 mg kg^{-1} Pb and 83 mg kg^{-1} Zn in its fronds (Table 3-3) with 305 mg kg^{-1} Pb and 228 mg kg^{-1} Zn in the roots (Table 3-4). The concentration of nickel in alfalfa plants were reported to range from 308 to 437 mg kg^{-1} in soils amended with 50 mg kg^{-1} of Cd, Cu, Ni and Zn.

Cadmium in the shoots of alfalfa varied from 100 to 200 mg kg^{-1} while Zn varied from 100 to 150 mg kg^{-1} (Peralta-Videa et al., 2002). Ye et al. (1997) reported metal concentrations in four populations of *Typha latifolia* varied with plant parts with the leaves having low levels (Zn= 22-122, Pb= 4.7 - 40 and Cd=0.2 -0.8 mg kg^{-1} dry wt.) and in the roots (Zn= 46-946, Pb= 25-3628, and Cd=1.0-17 mg kg^{-1} dry wt.). Some plant species grown in the mine tailings showed shoot Cd concentrations varying from 0.4 to 12.5 and Pb from 3.4 to 38.3 mg kg^{-1}, while the root varied from 0.1 to 6.2 mg kg^{-1} Cd, and 8.1 to 920 mg kg^{-1}Pb (Stoltz and Greger, 2002).

Transfer factor (TF), defined as the arsenic concentration ratio between aboveground and belowground biomass, is used to measure the effectiveness of a plant in translocating arsenic from root to shoot. The presence of heavy metals at 50 mg kg^{-1} did not affect the plant's capability to transport arsenic from roots to its aboveground biomass, with Pb-50 having the highest TF. Ferns growing in Zn-50, and Pb-amended soils had significantly higher TF than the control. The presence of metals at 200 mg kg^{-1}reduced arsenic transfer from root to frond (Table 3-5). Arsenic transfer factors ranging from 15.8 to 46.1 indicate *P. vittata* was able to effectively translocate arsenic in the presence of other metals.

Bioconcentration factor (BF), defined as the concentration ratio of arsenic in the plant to that in the soil, is used to measure the effectiveness of a plant in concentrating arsenic into its biomass. The metal-amended soils had significantly lower BFs than the control, showing the metals adversely impacted the efficiency of *P. vittata* to concentrate arsenic into its biomass. These BFs were reduced by 70% in Cd- and Ni-200 spiked soils; 62% in Zn-200 and 47% in Pb-spiked soils. Bioconcentration factors reduced with increasing Zn and Ni concentration, but not Cd and Pb (Table 3-5). Since the efficiency of phytoremediation is

determined by the ability of the fern to concentrate the contaminant in its biomass, the presence of these metals will slow down this process as reflected by lower TFs and BFs.

Table 3-5. Arsenic bioconcentration and transfer factors of *P. vittata* after growing for 8 weeks in an arsenic-contaminated soil spiked with metals at 50 or 200 mg kg^{-1}

	Transfer factor∗	Bioconcentration factor**	
		Fronds	Roots
Control	43.6 ± 7.9	51.5 ± 8.6	1.64 ± 0.53
Cd-50	34.1 ± 5.4	14.7 ± 0.99	0.52± 0.13
Cd-200	17.7 ± 1.25	14.2 ± 1.88	0.81 ±0.05
Ni-50	36.5 ± 7.7	31.2 ± 8.65	1.19± 0.42
Ni-200	15.8 ± 4.05	14.4 ± 4.61	1.27 ± 0.51
Zn-50	46.1 ± 5.15	35.8 ± 4.24	0.83± 0.12
Zn-200	26.6 ± 2.05	19.6 ± 1.21	0.79 ± 0.11
Pb-50	55.9 ± 3.85	27.5 ± 1.33	0.56 ± 0.06
Pb-200	45.2 ± 1.45	25.1 ± 1.48	0.56 ± 0.04

∗Ratio of arsenic concentration in frond to that in root.
**Ratio of arsenic concentration in plant tissue to that in the soil.
***Values presented as means ± standard error

3.3.5 Impact on Arsenic in Soils

It is widely recognized that the total metal concentration of soil is not a good predictor of metal bioavailability. Water-soluble metal concentrations, on the other hand, are a better indicator of soil metal availability for plant uptake (McBride, 1994). Water-soluble arsenic (0.2 mg kg^{-1}, Table 3-1) was much lower than the total As (131 mg kg^{-1}; Table 3-1).

Concentrations of water-soluble metals in the soil were determined at 5 and 8 weeks after plant transfer. Greater Cd, Ni, and Zn concentrations resulted in greater water-soluble Cd (i.e., 0.24 to 1.7 mg kg^{-1}after 5 weeks), Ni (i.e., 0.62 to 2.9 mg kg^{-1}after 8 weeks) and Zn (i.e., 0.10 to 0.14 mg kg^{-1} from 5 to 8 weeks) in the soil. Greater water-soluble Cd and Ni concentrations corresponded to lower plant biomass. Lead concentrations were generally below the detection limit. Zinc deficiency in this soil may be due to the low availability experienced in high pH soils (Welch et al. 1991).

Table 3-6. Impacts of plant growth on water-soluble and total soil arsenic concentrations at different sampling times after plant transfer

	Water-soluble arsenic ($mg\ kg^{-1}$)			Total soil arsenic ($mg\ kg^{-1}$)
Treatments	Zero day	5 weeks	8 weeks	8 weeks
Control	0.20a	2.12b	3.11a	86.9 a
Cd-50	0.18a	2.27b	3.53a	124 a
Cd-200	0.16a	2.22b	2.98a	114 a
Ni-50	0.11a	4.39a	3.55a	113 a
Ni-200	0.09b	4.41a	3.80a	107 a
Zn-50	0.13a	4.16a	3.57a	114 a
Zn-200	0.13a	3.54a	3.12a	97.7 a
Pb-50	0.29a	2.17b	2.61a	125 a
Pb-200	0.21a	1.90b	2.24a	123 a

Mean of four replicates and means with the same letter are not significantly different. **

It is expected that a plant will usually take up the most available metal fraction from the soil; that is, as more metal is being taken up by the plant, available metal concentrations tend to decrease (McGrath et al., 2000). However, this was not observed in our experiment. Water-soluble arsenic concentrations in all treatments were increased significantly ($P<0.05$) after 5 weeks of plant growth, more than 10-22 times greater than that of the concentration before planting (day zero) (Table 3-6). It seemed, though as plant arsenic uptake depleted water-soluble arsenic in the soil, the plant was capable of dissolving more arsenic into the soil solution, one of the characteristics of hyperaccumulators (McGrath et al., 2000). This might also lead to increased leaching of arsenic in extreme cases. Water-soluble arsenic concentrations in Ni- and Zn-amended soils at the end of the eight weeks decreased slightly than those at 5 weeks while others increased. Water-soluble metal concentrations except for that of nickel-amended soils increased with increasing metal concentration. Cd and Ni had significantly higher water-soluble concentrations than Zn and Pb at both 5 and 8 weeks after planting.

In addition to water-soluble arsenic, total arsenic concentrations in the soil were also determined (Table 3-6). It was reduced from the original 131 to 87 to 125 mg kg^{-1}, a 5 to 13% reduction in 8 weeks. Basically, *P. vittata* was effective in taking arsenic from the soil, although the presence of other metals reduced its accumulation capability.

3.3.6 Effects of Plant Arsenic Uptake and Presence of Metals on Arsenic Distribution in an Arsenic-Contaminated Soil

3.3.6.1 Plant arsenic removal

Plant biomass production coupled with plant elemental uptake is important for effective use of hyperaccumulators in phytoremediation (Reeves and Baker, 2000). The total amount of As a plant removed from soil (concentration x biomass) can be used to better determine the efficiency of a plant to concentrate As from the soil into its biomass.

Table 3-7 Arsenic removed by plant (mg/plant) after 8 weeks of growth

Treatment	Fronds	Roots	Total
Control	24.1 ± 1.8*	0.30 ± 0.65	24.4 ± 1.76
Cd-50	13.8 ± 1.1	0.77 ± 0.15	14.6 ± 1.04
Cd-200	8.68 ± 0.6	0.46 ± 0.07	9.13 ± 0.66
Ni-50	11.61 ± 0.8	0.39 ± 0.1	12.0 ± 0.73
Ni-200	6.55 ± 2.0	0.20 ±0.02	6.76 ± 2.02
Zn-50	18.8 ± 2.0	0.51 ± 0.06	19.3 ± 1.95
Zn-200	11.8 ± 0.6	0.59 ± 0.06	12.3 ± 0.62
Pb-50	18.6 ± 0.8	0.53 ± 0.07	19.2 ± 0.82
Pb-200	17.5 ± 0.4	0.80 ± 0.08	18.3 ± 0.44

*Mean ± Standard Error

As expected, *P.vittata* was efficient in taking up arsenic from the arsenic-contaminated soil and even in the presence of heavy metals. The plant took up 24.1 mg/plant arsenic in the

control (arsenic-contaminated soil) compared to 6.55 to 18.62 mg/plant in metal-spiked soils after 8-wks of growth (Table 3-7). This translated to arsenic concentration reduction of 8.9-28.4% compared to initial soil arsenic concentration of 131 mg kg-1. Tu and Ma (2002) in their study reported that *P.vittata* extracted up to 38 mg/plant after 20-weeks of plant growth. Among the four metals spiked, Ni resulted in the lowest plant arsenic removal, and Zn the highest, which contradicted with the nitrate effect (Table 3-7). This is because the amount of nitrate added with the metal decreased in the order of Ni (100%) > Zn (90.3%) > Cd (52.4%) > Pb (28.4%), which was calculated based on molecular weights. In other words, the highest nitrate addition resulted in the lowest plant arsenic removal.

Therefore, the type of metal spiked to the soil had more impact than the amount of nitrated added. This is supported by the fact that plant arsenic removal decreased with an increase in metal concentrations in metal-spiked soils despite the fact more nitrate was added at greater metal concentrations. Plants in Cd-200 treatment took up only 62% of that in Cd-50 treatment. Similarly, plants in Zn-200 treatment took up only 63% of that in Zn-50 even though Zn is an essential plant nutrient. Also, plants in Ni-200 treatment took up only 56% of that in Ni-50. While, plants in Pb-200 treatment took up 94% of that in Pb-50 suggesting that increase in lead concentration only slightly affected arsenic uptake. Due to the compounding effects of nitrate, it is difficult to assess how metal type affects plant arsenic removal. However, since the amount of nitrate added with Ni and Zn treatments were similar, it is possible to compare the effects of the two metals. Plant arsenic removal was greater under Zn treatment than Ni treatment, which is understandable since Zn is a plant nutrient. Even though more nitrate was added with Cd than Pb treatment, more arsenic was removed by the

plant with Pb treatment than Cd treatment. This suggests that Cd had more negative impact on plant arsenic removal than Pb.

3.3.6.1 Fractionation of arsenic in the soil

Environmental impacts of toxic metalloids like arsenic can be better understood by fractionating it into different fractions of availability (Balasoiu et al., 2001). As expected, stronger extractants, sulfuric acid (Ca-As; 45.4%), sodium hydroxide (Fe-As; 25.7%), and ammonium fluoride (Al-As; 24.0%) extracted much more arsenic (95%) than ammonium chloride (WE-As; 5.0%) in the arsenic-contaminated soil before plant uptake (Figure 3-5 and 3-6). This is consistent with previous reports that soil arsenic forms insoluble compounds with Al, Fe, and Ca and is largely unavailable to plant (Adriano, 2001). In a different study, approximately 29% of the total arsenic was extracted by ammonium chloride from soil previously equilibrated with arsenic while much smaller amounts were extracted in other soils (Jacobs et al. 1970).

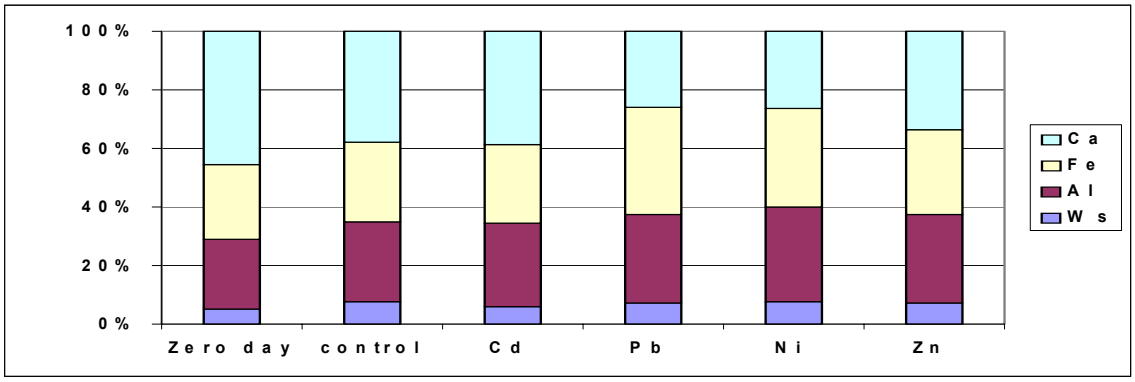


Figure 3-5. Fractionation of arsenic in a soil spiked with 50 mg/kg of various metals after 8weeks of plant growth

Fractionation before and after plant uptake in the control soil both showed that Ca-As was the predominant fraction, with Ca-As > Fe-As > Al-As > WE-As (Figure 4-1 and 4-2). This agrees with Adriano (1986) who reported that in calcareous soils, arsenic sorption is dominated by calcium minerals over iron and aluminum. The soil used in this experiment had

a soil pH of 7.6 and a total Ca content of 1.2%, thus calcium is expected to be dominant on the exchange sites in this soil. In an acidic sandy soil spiked with arsenic, the trend observed by Onken and Adriano (1997) was different, i.e. Fe-As > Al-As > Ca-As > WE-As. Using the same sequential extraction procedure, Johnston et al., (1979) also showed that arsenic is mainly associated with Fe (Fe-As >> Ca-As >Al-As >WE-As) in the soils they examined.

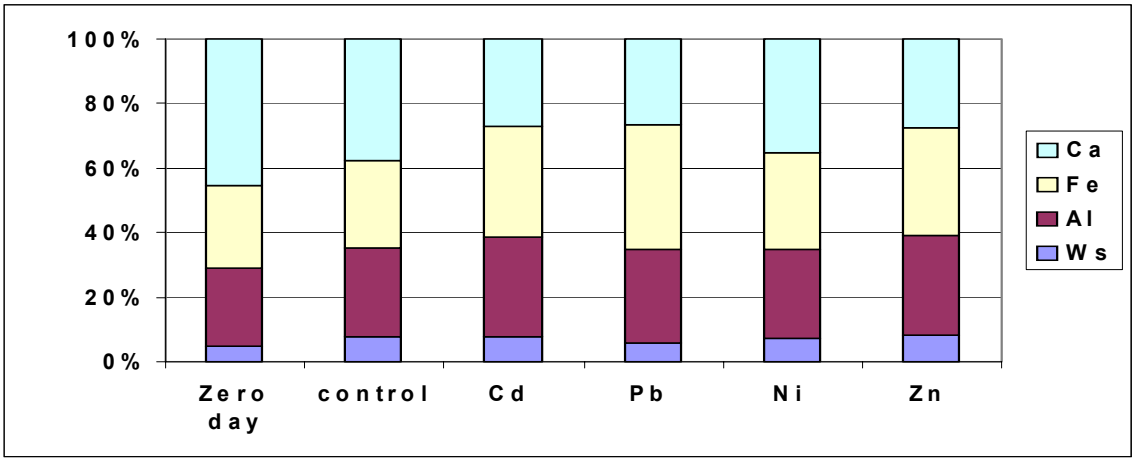


Figure 3-6. Fractionation of arsenic in a soil spiked with 200 mg/kg of various metals after 8 weeks of plant growth

Arsenic concentrations in all fractions decreased after 8 weeks of plant uptake in all the treatments (Table 3-8). The difference in each fraction was calculated between the arsenic extracted before and after 8 weeks of plant uptake. The amount of As reduced from each fraction through plant uptake followed the same trend as the amount of As present in each fraction, i.e. with greater reduction in the fraction having greater As concentration. The average amount of As reduction for Ca-, Fe-, Al-, and WE-As for all treatments was 61.8, 18.1, 17.3 and 2.66% (Table 3-8). Most of the arsenic taken up by the plant was from Ca fraction since it is the predominant fraction accounting for 45.4% of soil As before plant uptake. Statistical analysis showed that there were no significant differences in changes in WE-As fraction between treatments. This shows that the reduction in this fraction occurred similarly regardless of treatment. Changes in Al-As and Fe-As fractions were significantly

correlated (P=0.007). This might be because they are both insoluble and whatever solubilized one will also solubilize the other.

Table 3-8 Percent-soil arsenic reductions in each treatment after 8 weeks of plant growth.

Treatments	WE-As	Al-As	Fe-As	Ca-As
Control	2.9	21.0	24.0	51.0
Cd-50	3.1	16.2	23.7	57.0
Cd-200	2.0	16.6	16.7	64.7
Pb-50	2.0	14.7	16.6	66.7
Pb-200	2.4	19.7	20.0	57.9
Ni-50	2.6	17.1	22.0	58.3
Ni-200	1.8	16.1	16.9	65.2
Zn-50	2.9	17.5	14.7	64.9
Zn-200	4.2	16.9	8.10	70.8

3.3.6.2 Relationship between changes in soil arsenic fractions and plant arsenic removal

Among the four As fractions, Ca-As, Fe-As and Al-As can all be converted to WE-AS as the latter was taken by plants, i.e. WE-As changes continuously. Only changes in WE-As in all treatments significantly (P=0.007) correlated with arsenic removed by the plant after 8 weeks of growth (r=0.83). This fraction has also been demonstrated to be significantly correlated with plant growth (Woolson et al., 1971). Plant arsenic removal was also reported to be correlated with the WE-As in maize (Sadiq, 1986) in barley and ryegrass (Jiang and Singh, 1994). No significant correlation was obtained between the amount of plant As uptake and arsenic reduction in Al-, Fe-, or Ca-fraction (r=0.09 to –0.34). It was possible that arsenic taken up by the plant was from all four fractions and contribution from each fraction changed during the experiment.

3.3.6.3 Effect of plant arsenic removal on arsenic redistribution in the CCA-contaminated soil

It is important to understand how plant arsenic removal impacts arsenic distribution in the soil. Such information can be used to enhance plant arsenic uptake in the soil for the purpose of phytoremediation.

The distribution of As fractions in the CCA soil (control soil) changed slightly after 8-wks of plant arsenic uptake. WE-As increased from 5 to 8%, Al-As increased from 24 to 27%, Fe-As increased slightly from 26 to 27%, whereas Ca-As decreased from 45 to 38%. Similar trend was observed in the metal-spiked treatments. Onken and Adriano (1997) demonstrated that arsenic became more recalcitrant with time in soils without plants. An increase in WE-As fraction (Fig 3-5 & 3-6) and a decrease in Ca-As fraction (recalcitrant form) were observed in our experiment after 8-wk of plant arsenic uptake. The increase in WE-As fraction after plant arsenic uptake could be due to the fact that plant uptake depleted the water soluble arsenic and thus triggered arsenic release from the recalcitrant forms probably through root exudation of organic acids. Decrease in Ca-As fraction in all treatments suggests it was depleted by plant uptake (Table 3-8). The largest proportion (51 to 71%) of the changes in soil arsenic in all treatments was from Ca-As fraction, which is expected to be the most recalcitrant and unavailable for plant uptake. Calcium is probably of special importance to the fern because it prefers to grow in a lime environment (Jones, 1987). In an experiment using arsenate forms of comparable solubility (K, Na, and Ca) to grow the fern in an uncontaminated soil, calcium was more effective in increasing arsenic concentrations in the fronds (Tu and Ma, 2002). Calcium is an essential element that is known to promote root and leaf development (Follet et al., 1981), which is probably why it was more effective in increasing arsenic concentrations in the fronds. As a secondary

messenger in plant signaling, calcium also plays a vital role in plant growth and development, influencing plant response and adaptation to various environments (Yan et al., 2002).

3.3.6.4 Effect of metals on soil arsenic distribution

Most contaminated sites are heterogeneous and so it is important to know what effect these common co-contaminants in the soil will have on the efficiency of phytoremediation by examining soil arsenic distribution.

The presence of metals in the soil also changed the distribution of arsenic in the soil. After 8 weeks of plant growth, only Cd-50, Zn-50 and Ni-200 treatments had Ca-As as the dominant fraction. All other treatments had either Al-As or Fe-As as the dominant fraction. Concentrations of Al-As and Fe-As were significantly ($P<0.01$) greater in metal-spiked soils than the control after 8 weeks of plant uptake (data not shown). There was also a general increase in Fe-As fraction with an increase in metal concentration except in nickel-spiked soils after 8 weeks of plant growth. It is possible that the presence of these metals increased available Fe and Al in the soil by competing with them for exchange sites. It has been widely reported that arsenic has a high affinity for Fe and Al oxides (Woolson et al., 1973, Pierce and Moore, 1980, Takamatsu et al., 1982, Smith et al., 1998) and increasing exchangeable Fe and Al in soil solution would increase arsenic sorption and retention in these fractions in the soil. It is also possible that these metals form ternary complexes with the arsenate anion on Fe and Al oxidic surfaces (McBride, 1994) thereby reducing plant availability of the arsenate anion in the Fe-As and Al-As fractions. The formation of binuclear, inner-sphere complexes was postulated as a main mechanism for the sorption of arsenate onto ferrihydrite (Adriano, 2001).

3.3.6.5 Effect of plant arsenic uptake and metals on soil pH of the CCA soil

Soil pH is a master variable controlling soil chemical processes and reactions. (Mcbride, 1994). There was a general reduction in soil pH in all treatments after 8 weeks of plant growth probably due to the production of root exudates. Soil pH decreased from 7.6 before plant transfer to 6.78-7.19 after 8 weeks of plant growth. Changes in soil pH significantly and positively correlated with changes in the Ca-As fraction confirming that there was a depletion of calcium in the soils. Soil pH in Zn-50, Cd-200, and Zn-200 spiked soils were significantly lower than the control after 8 weeks of plant growth (Figure 4-3). Zn-200 treatment had the lowest soil pH after 8 weeks of plant growth. There might be more exudation of organic acids by the plants grown in this treatment. Exudation of phytic acid from plants has been implicated in plants exposed to high concentrations of zinc (Cakmak and Marschner, 1993). Except for nickel spiked soils, soil pH decreased with increasing metal concentration.

Soil pH also had an impact on the distribution of arsenic in the soil. There was a significant positive correlation ($P< 0.01$) between soil pH and Ca-As fraction in the soils after both 5 and 8 weeks of plant growth. This shows that Ca-As fraction increased with increasing soil pH. Soil pH was significantly negatively correlated ($P<0.05$) with Fe-As and Al-As, showing that Fe-As and Al-As increased with decreasing soil pH. Several scientists have also reported that arsenic distribution in soils is associated with pH (Akins and Lewis, 1976, Adriano, 1986, Bech et al., 1997). This is due to Fe and Al becoming more available to sorb arsenic in the soil as pH decreases whereas calcium sorption of arsenic increases as pH increases.

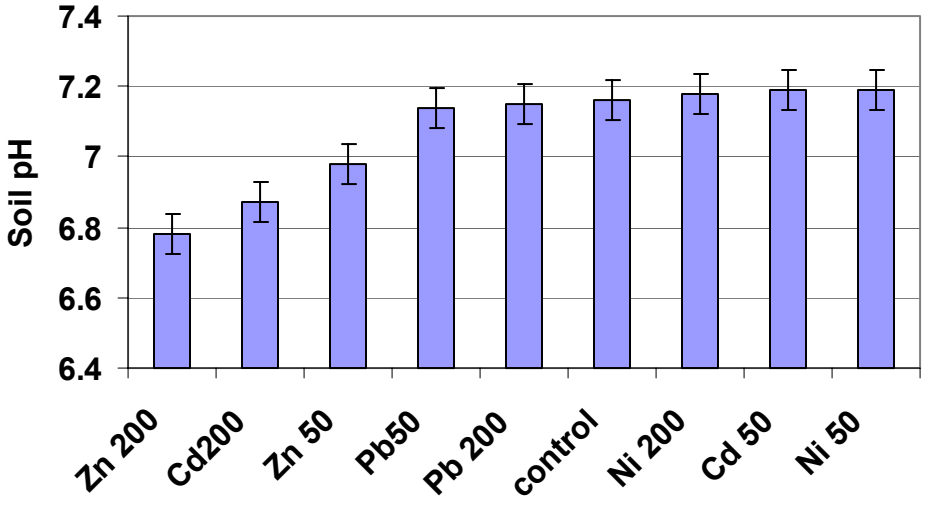


Figure 3-7. Soil pH after 8 weeks of plant growth in CCA soil spiked with various metals. Bars are SE of means.

3.4 Conclusion

Suitability of a plant for phytoremediation could be determined by its ability to produce a high aboveground biomass and high bioconcentration and transfer factors. *P. vittata* has fulfilled all of these characteristics in this experiment and also shown that it can hyperaccumulate arsenic and grow in the presence of other toxic metals though its hyperaccumulating efficiency was reduced. Further studies should be done to determine the phytoremediation potential of this fern in the field.

Fractionation of the CCA soil before and after plant growth in the control showed that Ca-As fraction was dominant with Ca-As > Fe-As > Al-As > WE-As. There was a change in the distribution of these fractions after plant arsenic uptake with WE-As, Al-As, and Fe-As increasing while Ca-As decreased. Increase in WE-As after plant arsenic-uptake after 8 weeks of growth could be due to the fact that plant uptake depleted the soluble arsenic, triggering the release of arsenic from the recalcitrant forms probably through root exudation of organic acids. The decrease in Ca-As in all treatments suggests it was depleted by plant

uptake and that calcium might play an important role in arsenic uptake by the fern. Only changes in WE-As in all treatments significantly correlated with arsenic removed by the plant after 8 weeks of growth. Soil pH also had an impact on the distribution of arsenic in the soil with Ca-As increasing with increasing soil pH and Fe-As and Al-As increasing with decreasing soil pH. There was a general reduction in soil pH from 7.6 to 6.78-7.19 after 8 weeks of plant growth in all treatments probably due to production of root exudates. The plant's capability in solubilizing soil arsenic from the recalcitrant fractions may have contributed to its arsenic hyperaccumulation characteristics.

CHAPTER 4
EFFECTS OF PHOSPHATE ROCK ON PLANT ARSENIC UPTAKE IN A MULTI-METAL SYSTEM.

4.1 Introduction

Our previous research has shown that the plant was able to accumulate arsenic in the presence of other metals albeit at reduced rate (Fayiga et al., 2004). Phosphate rock has been shown to immobilize metals in contaminated soils (Ma et al., 1995), and reduce metal uptake in sudax (*Sorghum bicolor L.*) (Laperche et al., 1997). On the other hand, phosphate has also been shown to increase arsenic availability in soil and plant uptake (Peryea, 1998). Therefore, P-induced metal stabilization and arsenic solubilization in soils should enhance arsenic uptake by *P.vittata* in the presence of other metals. Phosphate rock also supplies calcium and phosphate to the soil in addition to increasing soil pH thereby providing better growth condition for the fern. The main objective of this study was to investigate the effects of phosphate rock on plant uptake of arsenic, calcium, phosphorus in addition to Pb, Cd, and Zn.

4.2 Materials and Method

4.2.1 Soil and Phosphate Rock Characterization

A sandy soil collected from a garden was used in this experiment. The soil was air-dried, passed through 2mm sieve and analyzed for total concentrations of Pb, Cd, Zn and As. Soil pH was measured using a pH meter in a 1: 2 soil to solution ratio. Cation exchange capacity (CEC) was determined by the ammonium acetate method (Thomas, 1982). Organic matter was done by the Walkley Black method (Nelson and Sommers,

1982) and particle size by the pipette method (Day, 1965). The phosphate rock sample (PR, <60 μm), which is classified as ground concentrate, was obtained from PCS Phosphate, White Springs, FL. Phosphate rock and soil samples were digested using EPA Method 3050A with the Hot Block digestion System (Environmental Express, Mt. Pleasant, SC). Total Ca, Al, Mg, and Fe concentrations in the samples were analyzed using a flame atomic absorption spectrometer (Varian 220 FS with SIPS, Varian, Walnut Creek, CA). The selected physico-chemical properties of the soil and phosphate rock used in this experiment are listed in Table 4-1.

4.2.2 Greenhouse Experiment

The experiment consisted of five treatments; 1) control with no spiked arsenic (control), 2) spiked with 50 ppm As, 3) spiked with 50 ppm As and 50 ppm P (AsP) 4) spiked with 50 ppm As, 50 ppm Pb, Cd, and Zn (AsM), 5) spiked with 50 ppm As, 50 ppm Pb, Cd, and Zn, and 50 ppm P (PMAs). Arsenic was applied as sodium arsenate, the other metals as nitrates, and P as phosphate rock. The soil was fertilized with 2 g/kg Osmocote slow release fertilizer as a base fertilizer. After one-week equilibrium, one healthy fern with 5-6 fronds was planted in each pot (2.5 L, ϕ=15 cm) containing 1.5 kg of soil. These plants were propagated from spores in our laboratory using a method described by Jones (1987). Each treatment was replicated four times and arranged in a completely randomized design. The plants were grown for five weeks in a greenhouse where the average temperature varied from 14 (night) to 30 ^{o}C (day), with an average photosynthetic active radiation of 825μmol$m^{-2}s^{-1}$.

4.2.3 Plant and Soil Analysis

The harvested plants were separated into aboveground and belowground biomass, dried in the oven at 65^{o}C for 3 days and then ground into powder. Soil samples were air-dried and analyzed for soil pH, Mehlich-3 and total As, exchangeable Ca, and Mehlich-3 P. Exchangeable Ca in the soil was determined using a 1 N ammonium acetate extraction-centrifuge-decantation procedure (Thomas, 1982). Available P was extracted with the Mehlich-3 extractant, and determined using a modified molybdenum blue method to minimize arsenic interference (Carvalho et al., 1998). Soil and plant samples were digested with nitric acid using the Hot Block digestion System (Environmental Express, Mt. Pleasant, SC; EPA Method 3050A). Total As and Cd concentrations were determined with a graphite furnace atomic absorption spectrophotometer (Perkin Elmer SIMMA 6000, Perkin-Elmer Corp, Norwalk, CT) while Ca, Zn and Pb concentrations were analyzed on a flame atomic absorption spectrometer (Varian 220 FS with SIPS, Varian, Walnut Creek, CA).

4.3 Results and Discussion

4.3.1 Soil Characteristics

Selected chemical and physical characteristics of the soil used for the experiment are shown in Table 4-1. Arsenic, lead, cadmium and zinc are relatively low. The soil is a typical Florida sandy soil except for its neutral pH (Chen et al., 1999). The phosphate rock used contained 14.3% P, 34.3% Ca, and relatively low concentrations of Fe, Al, and magnesium.

Table 4-1. Selected properties of soil and phosphate rock used in this experiment

Soil	Concentration	Phosphate rock	Concentration (mg/kg)
% Sand	89.2	pH	7.1
% Silt	7.5	P (%)	14.3
% Clay	3.3	Fe (%)	0.63
CEC ($cmol_c$/kg)	17	Al (%)	0.66
Organic matter (g/kg)	31.5	Ca (%)	34.3
Soil pH	6.89	Mg (%)	0.22
Total As (mg/kg)	0.41		
Mehlich-3 Arsenic (mg/kg)	0.003		
Total P (mg/kg)	277		
Mehlich-3 P (mg/kg)	87.2		
Total Ca(mg/kg)	4769		
Total Cd (mg/kg)	0.13		
Total Pb (mg/kg)	9.52		
Total Zn (mg/kg)	105		

4.3.2 Effect of Phosphate Rock on Plant Arsenic Uptake

Addition of phosphate rock to contaminated soils in our experiment provides four potential benefits, i.e. reducing the availability of Cd, Pb and Zn, enhancing arsenic availability, providing plant nutrients Ca and P, and increasing soil pH. Therefore, use of phosphate rock, as a soil amendment should enhance the effectiveness of *P.vittata* in phytoremediation of arsenic–metal polluted soils.

As expected, in arsenic-treated soils, most of the arsenic taken up by *P.vittata* was concentrated in the fronds (89-93%) with arsenic concentrations in the roots ranging only from 78 to 126 mg kg^{-1} (Figure 4-1). This is consistent with previous reports (Tu and Ma, 2002, Zhang et al., 2002, Tu et al., 2002) and is typical of hyperaccumulators. The addition of metals reduced plant arsenic uptake by 32-63% though the plants were still able to accumulate 608-1,046 mg kg^{-1} arsenic in the fronds. In the absence of metals, phosphate rock slightly reduced plant arsenic uptake from 1631 to 1,530 mg kg^{-1} in the fronds. Phosphate added to a soil plays two roles due to the chemical similarity between phosphate and arsenic. On the one hand, it releases arsenic from the soil, and on the other hand, it also competes with arsenic for plant uptake (Tu and Ma, 2003a). The fact

that less P was taken up by *P.vittata* in the AsP treatment (50 mg kg^{-1} As + P) than in the As treatment (50 mg kg^{-1} As) suggests that P competed with arsenic for plant uptake (Figure 4-2). This is expected since both were added into the soil system and were both available for plant uptake. We would expect a release of arsenic in the soil if the soil were naturally contaminated with most of the arsenic strongly sorbed in the soil.

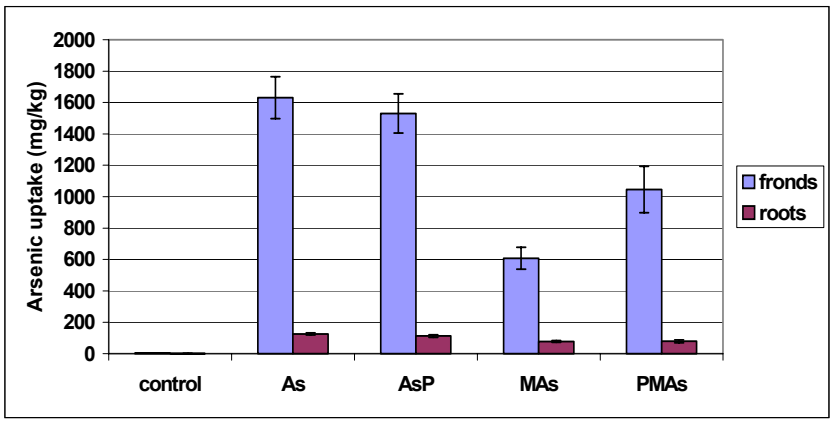


Figure 4-1. Arsenic uptake in *P.vittata* grown in soil spiked with arsenic, phosphorus and metals. As-arsenic, P-phosphorus, M-metals. Phosphate rock increased arsenic uptake in P.vittata in the presence of other metals. Bars are SE of means.

In the presence of other metals, however, phosphate rock significantly increased plant arsenic uptake, increasing from 608 to 1,046 mg kg^{-1} in the fronds (Fig. 4-1). This shows the ability of phosphate rock to aid in arsenic accumulation by *P.vittata* in a multi-metal system. Boisson et al. (1999) also reported an increase in arsenic uptake by plants (*Zea mays* cv. Volga and *Phaseolus vulgaris* cv. Limburgse vroege) after applying hydroxyapatite to a soil contaminated with Zn, Pb, Cu, Cd and As. They suggested that the increased phosphate concentration in the soil solution might be responsible. In this experiment, phosphate rock probably reduced the toxic effects of the metals on the fern, thereby enhancing the arsenic uptake by the fern.

4.3.3 Calcium and Phosphorus Uptake by *P. vittata*

Calcium and P are essential nutrients for plant growth and they both are major constituents of phosphate rock. Thus addition of phosphate rock should increase plant uptake of P and Ca, which were observed only in some treatments (Fig. 4-2 & 4-3).

Unlike arsenic distribution in *P.vittata*, more P was concentrated in the roots than in the fronds except in the control (Fig. 4-2) where more P was present in the fronds (65%). Compared to the control, the presence of arsenic increased P accumulation in the roots (Fig. 4-2). This result is consistent with those of Tu and Ma (2003b) who reported that arsenic uptake by *P.vittata* significantly increased plant P concentrations in the roots. They also suggest that the ability of *P.vittata* to retain more P in the roots in the presence of arsenic stress may constitute one of its arsenic detoxification mechanisms. Due to the chemical similarity between phosphate and arsenate, arsenic is toxic to plants because of its capability to interfere with plant P metabolism (Tu and Ma, 2002). With more P concentrated in the roots, it may lessen the arsenic-induced toxicity to *P.vittata*, making it more tolerant to arsenic.

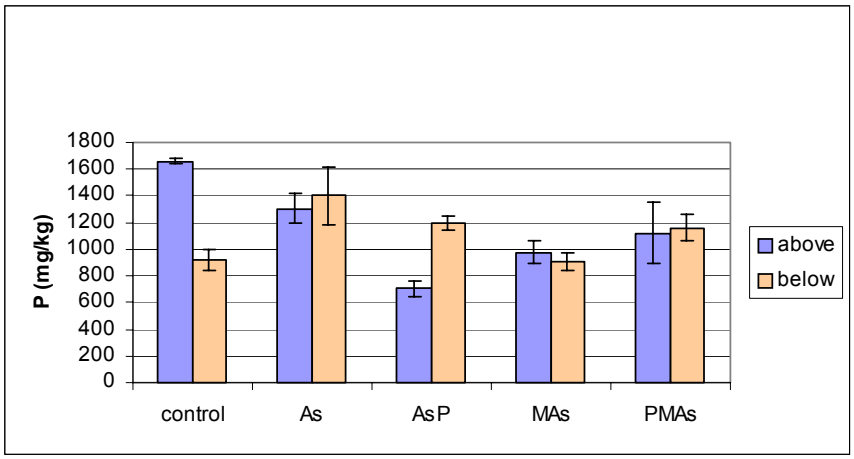


Figure 4-2. Phosphorus uptake in *P. vittata* grown in soil spiked with different combinations of arsenic, phosphorus and metals. As=arsenic. P=phosphorus. M=metals. Bars are SE of means.

Contrary to P accumulation in the roots, P accumulation in the fronds was lower in the presence of arsenic than in the control (Fig. 4-2), i.e. arsenic has reduced P translocation in *P. vittata*. This is partly because more arsenic was translocated by the plant in the presence of arsenic (Fig. 4-1). Arsenic concentrations in the fronds were linearly, but negatively correlated (r=-0.43, P=0.0565) to phosphorus concentrations in the fronds, suggesting more arsenic translocation reduced P translocation in the plant. Plant arsenic uptake has also been shown to reduce root and shoot P concentrations in tomato plants (Carbonell-Barrachina et al., 1998) and in the shoots of red clover (Marscher et al., 2002).

Follet et al. (1981) reported that plant parts vary greatly in their Ca content depending on both plant species and their growth conditions. A greater percentage (57-60%) of Ca taken up by *P. vittata* remained in the roots than in the fronds (Fig. 4-3), which is typical of most plants since Ca is immobile. The presence of arsenic enhanced plant Ca uptake in all treatments, especially in the roots. For example, calcium concentrations in the roots increased from 1.4% in the control to 2.3% in the As treatment. Tu and Ma (2002) reported that among different arsenate forms with comparable solubility (K, Na, and Ca), Ca was more effective in increasing arsenic concentrations in the fronds of *P. vittata*, which is consistent with our data. While P didn't impact plant Ca accumulation in the absence of metals (treatments As vs. AsP), it significantly increased Ca accumulation in the roots in the presence of metals (treatments AsMP vs. AsM). The highest Ca accumulation was found in the fronds in AsMP treatment (50 mg kg^{-1} As, metals and P), which was approximately 2.9%. Up to 4%

calcium was reported in tobacco leaves while 2% in soybean and alfalfa leaves (Follet et al. 1981).

Similar to the relation between As and P, Ca concentrations in the fronds were linearly, but negatively correlated with P concentrations in the fronds. This shows an antagonistic relationship between calcium and phosphorus in *P. vittata*.

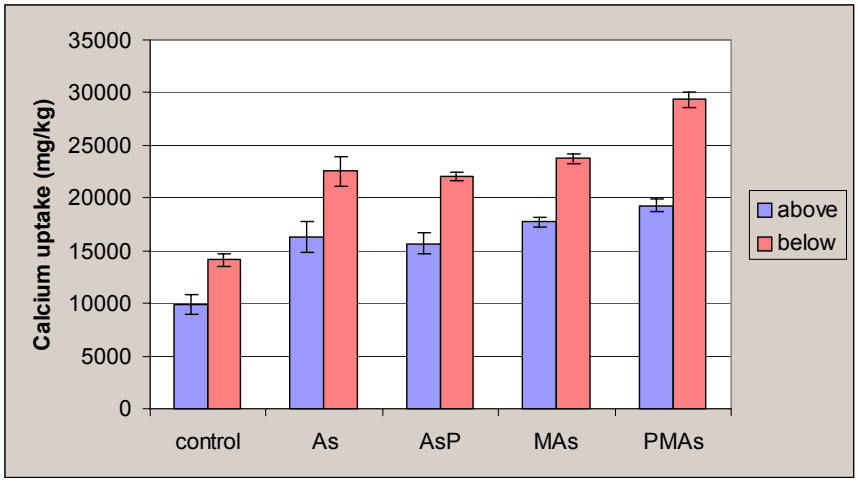


Figure 4-3. Calcium uptake in *P. vittata* grown in CCA soil spiked with various metals. As=arsenic. P=phosphorus. M=metals. Bars are SE of means.

4.3.4 Metal Uptake by *P. vittata*

Reduced metal uptake has been reported with the use of phosphate in metal impacted soils. Laperche et al. (1997) reported that lead content in shoot tissue of sudax (*Sorghum bicolor* L. Moench) decreased as the quantity of added apatite increased. Cai et al. (2002) demonstrated lead immobilization in a site contaminated primarily due to past lead battery recycling activities using a mixture of H_3PO_4 and phosphate rock. Phosphate treatment significantly reduced lead contents in the shoots of St. Augustine grass (*Stenotaphrum secundatum*), which grows on the site. In this study, phosphate rock significantly reduced the lead and cadmium concentrations in the fronds of *P.vittata* (Table 4-2). As expected, more Pb was concentrated in the roots than in the fronds. This

is because little Pb was translocated to the fronds. However, in a study conducted by Basta et al. (2001), phosphate rock did not reduce the concentrations of cadmium and zinc in lettuce grown in a smelter contaminated soil.

Table 4-2. Effects of phosphate rock and arsenic on metal uptake by *P. vittata*

Treatment	Zn (mg/kg)		Cd (mg/kg)		Pb (mg/kg)	
	above	below	above	below	above	below
Control	69.2a*	48.4a	< 0.1b	0.13b	5.9b	10.1b
As	43.0a	41.5a	<0.1b	0.15b	4.67b	9.01b
AsP	49.1a	41.9a	<0.1b	0.13b	5.45b	9.84b
AsM	56.4a	65.6a	13.0a	27.4a	13.5a	25.9a
AsMP	55.1a	65.9a	3.45b	28.8a	4.1b	30.6a

*Means with the same letters are not significantly different at P<0.05

4.3.5 Effect of Phosphate Rock and Metals on Soil Arsenic, Ca, and P

The use of phosphate rock in different soil types as a fertilizer has been widely studied. Available arsenic in the control soil, extracted using Mehlich-3 extractant, increased from 0.003 to 0.04 mg kg^{-1} after 5 weeks of plant growth (data not shown). This shows that *P.vittata* was able to solubilize arsenic from the soil. Among the four treatments, arsenic in the metal-spiked soil (AsM) was least available (Fig. 4-4), which might explain the lowest plant uptake of arsenic in treatment AsM (Fig. 4-1). As expected, slightly more available arsenic was found with phosphate rock addition (i.e., As vs. AsP, and AsM vs. AsMP). Phosphorus was most available in the metal-spiked soils probably because the metals were applied as nitrates (Fig. 4-5). High nitrate concentration in solution could displace phosphate, making it more available in the soil. Though more available P was observed in the metal-treated soils (Fig. 4-5), plants failed to take up more P in these treatments (Fig. 4-2), possibly due to metal toxicity to the plant.

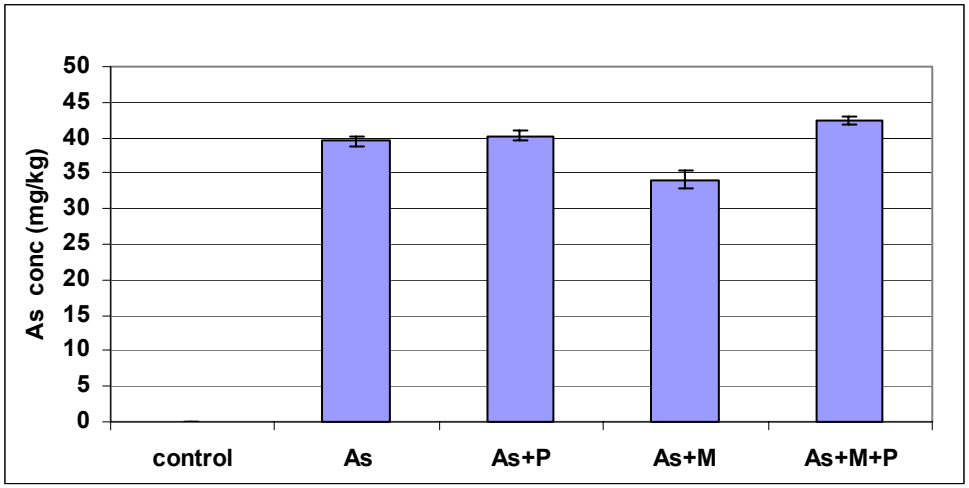


Figure 4-4. Mehlich-3 extractable arsenic in soil after plant harvest. As-arsenic, Phosphorus, M-metals Bars are SE of means.

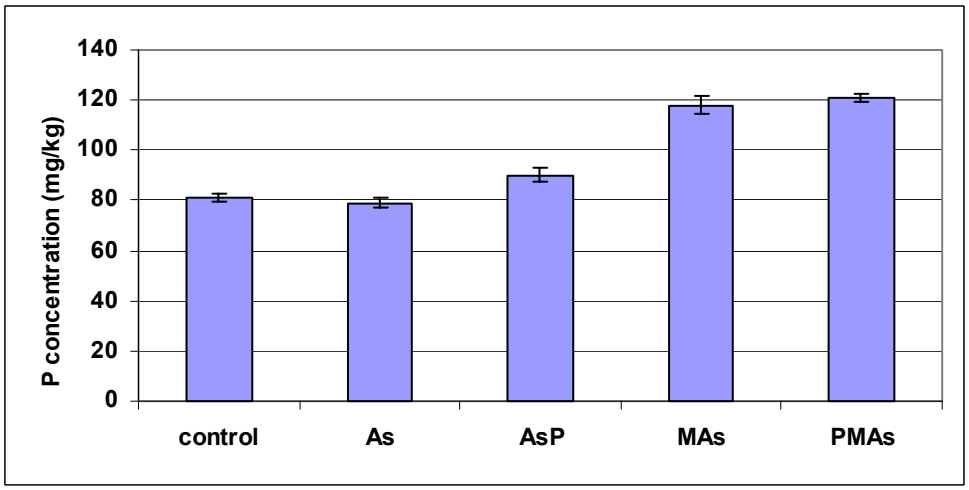


Figure 4-5. Mehlich-3 extractable P in the soil after plant harvest. As-arsenic, P-phosphorus, M-metals. Bars are SE of means.

Arsenic uptake was significantly, but positively correlated with both total soil arsenic (r=0.75) and Mehlich-3 arsenic (r=0.69) in the soil providing a link between availability and uptake. Several extractants have been used to estimate the bioavailability of arsenic. Due to the similarities between phosphorus and arsenic, the Mehlich-3 extractant normally used to extract phosphorus was used in this study. This reagent in this study extracted up to 85% of the total arsenic in the soil. It was also significantly

positively correlated (R^2=0.95) with total soil arsenic, which is probably due to the fact that arsenic was added to the soil as soluble salt and it was therefore more available.

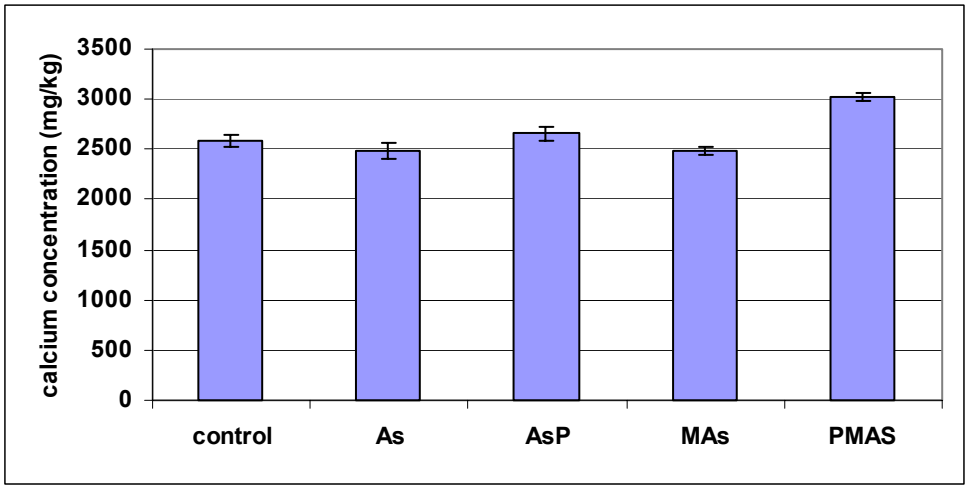


Figure 4-6. Available calcium in the soil after plant harvest. As=arsenic. P=phosphorus. M=metals. Bars are SE of means.

Phosphate rock had little effect on soil pH (data not shown) probably because of the small quantity of P (50 mg kg^{-1}) added and because the soil used had a neutral pH. Basta et al. (2001) reported that rock phosphate added at 100g kg^{-1} had little effect on soil pH in a soil with near neutral pH. Phosphate rock is generally more available in a soil with low pH (Yost et al., 1982). Similar to P, phosphate rock treated soils had higher available calcium in the soil than in other treatments (Fig. 4-6). The available calcium content (Fig. 4-6) in the AsMP treatment was the highest probably due to the acidifying effect of the metals increasing dissolution of phosphate rock.

4.4 Conclusions

This study has shown that phosphate rock significantly increased arsenic uptake and decreased lead uptake by *P.vittata* in a multi-metal system. *P.vittata* was also able to grow in a soil spiked with cadmium, zinc and lead at 50 mg kg^{-1}. Phosphate rock increased both arsenic and calcium availability in the soil. Arsenic uptake by the fern was

positively correlated with both total and available arsenic in the soil. Phosphorus uptake was significantly lower in the arsenic treated soils whereas Ca uptake was significantly higher in arsenic treated soils. Phosphate rock is recommended as an effective soil amendment for phytoremediation of arsenic contaminated soils especially in arsenic–metal polluted soils

CHAPTER 5
ARSENIC UPTAKE BY TWO FERN TYPES IN DIFFERENT ARSENIC-CONTAMINATED SOILS

5.1 Introduction

Glutathione (GSH) is very important in the adaptive mechanism of plants exposed to stressful environmental conditions having been implicated as the substrate, for phytochelatin biosynthesis under metal (Steffens 1990) and arsenic (Hartley-Whitaker et al. 2001) stress. Thus, plant exposure to toxic metals may deplete levels of glutathione due to the synthesis of these metal-binding peptides (phytochelatins) for which glutathione serves as a precursor (Rauser 1987). Zinc and nickel treatments were shown to lead to a decrease in the total glutathione contents of two pigeonpea cultivars (MadhavaRao and Sresty 2000).

This study attempted to evaluate and compare arsenic accumulation of these two ferns in different As contaminated soils.

5.2. Materials and Method

5.2.1 Soil Characterization

The soils used in this experiment were collected from various sites in the United States. A sandy soil collected from a garden in Gainesville, Florida was used as the control in this experiment. Soil referred to as golf course soil was collected from a golf course in Miami, Florida that had hot spots contaminated with arsenic from the use of arsenical herbicides. Arsenic contaminated soil from cattle dip vat site in Gainesville, Florida was collected and referred to as cattle dip vat soil. The mining soil was collected

from South Carolina while CCA contaminated soil was collected from an abandoned CCA wood preservation site in Archer, Florida.

The soils collected were air-dried, and analyzed for total Ca, K, P, Pb, Cd, As, and Zn concentrations. Soil pH was measured using a 1:2 soil to water ratio. Cation exchange capacity (CEC) was determined by a displacement sodium acetate/ammonium acetate method using sodium as the index cation (Thomas, 1982). Exchangeable Ca and K in the soil before and after plant growth were determined using 1 N ammonium acetate extraction-centrifuge-decantation procedure (Thomas, 1982). Available P before and after plant growth was extracted with Mehlich-3 extractant, and determined using a modified molybdenum blue method to minimize arsenic interference (Carvalho et al., 1998). Organic matter content was measured by the Walkley Black method (Nelson and Sommers, 1982) and particle size by the pipette method (Day, 1965). Aluminium and Fe in the different soil types were extracted by acid ammonium oxalate in the dark (McKeague and Day, 1966).

Mineralogy of the clay fraction was done for different soil types. The different fractions were separated by centrifugation following sodium saturation to promote dispersion. The clay fraction was prepared for x-ray diffraction by depositing 250 mg as a suspension on ceramic tiles under suction. Magnesium and glycerol saturation was done on the tiles and samples scanned at 2^{o} 2θ min-1 with CuKα radiation. Selected physical-chemical properties of the soil are listed in Table 5-1.

5.2.2 Greenhouse Experiment

The five different arsenic contaminated soils were each fertilized with 3.3 g/kg Dynamite (18-6-8-1.2-0.02-0.05-0.20-0.06-0.02: %N-P-K-Mg-B-Cu-Fe-Mn-Mo) slow

release fertilizer containing micronutrients as a base fertilizer. After one-week equilibrium, one healthy fern with 5-6 fronds was planted in each pot (2.5 L, ϕ=15 cm) containing 1.5 kg of soil. Each treatment was replicated four times and arranged in a completely randomized design. Three-month-old *Pteris vittata* and *Pteris cretica* ferns were procured from a nursery (Milestone Agriculture, Inc., FL, USA). The plants were grown for six weeks in a greenhouse where the average temperature varied from 14 (night) to 30 ^{o}C (day), with an average photosynthetic active radiation of 825μmol$m^{-2}s^{-1.}$

5.2.3 Plant and Soil Analysis

The harvested plants were separated into aboveground and belowground biomass, dried in the oven at 65^{o}C for 3 days, weighed and then ground into powder. Soil samples were air-dried and analyzed for soil pH, Mehlich-3 and total As, exchangeable Ca, K, and Mehlich-3 P. Soil and plant samples were digested with nitric acid using the Hot Block digestion System (Environmental Express, Mt. Pleasant, SC; EPA Method 3050A). Total and Mehlich-3 extractable As concentrations were determined with a graphite furnace atomic absorption spectrophotometer (Perkin Elmer SIMMA 6000, Perkin-Elmer Corp, Norwalk, CT) while Ca, Fe, Al, and K concentrations were analyzed on a flame atomic absorption spectrophotometer (Varian 220 FS with SIPS, Varian, Walnut Creek, CA).

5.2.4 GSH Analysis

The method of Hausladen et al. (1990) was used in GSH analysis. Freeze-dried roots and shoots of *P.vittata* and *P.cretica* were homogenised in 3 ml of 2% metaphosphoric acid containing 2 mM EDTA and polyvinylpolypyrrolidone by using a pre-cooled mortar and pestle and then centrifuged at 10,000 x g for 10 min. The pH of the extract was brought to about 5.5 with 10% sodium citrate. Three working solutions were

prepared; 1) 0.3 mM NADPH, 2) 6 mM DTNB and 3) approximately 50 units of glutathione reductase per ml prepared by using 100mM sodium phosphate buffer (pH 7.5) containing 6.3 mM Na-EDTA. Total gluthathione content was estimated by mixing 700µl of solution 1 and 100 µl of solution 2 and the sample extract of 200 µl to give a final volume of 1.0 ml, directly in a cuvette with 1cm light path and equilibrated to 30oC. To this solution 10 µl of solution 3 was added and then the absorbance was monitored at 412 nm. Concentration of GSH was estimated from standard curves.

5.2.5 Fractionation of Arsenic

Arsenic fractionation procedure used by Onken and Adriano (1997) was modified and used in this experiment. The procedure is briefly summarized below using 1g of soil and 25 ml extraction solution. Fractions of WE-As, Al and Fe-As, Ca-As and residual arsenic were obtained by determining supernatants extracted using 1M NH_4Cl (shaken for 30 min), 0.2M acid ammonium oxalate buffer pH 3.1 (4 h) in the dark, 0.5 M H_2SO_4 (17 h), and HNO_3/H_2O_2 respectively. For each step, the suspensions, after shaken for the specific times, were centrifuged at 3500 rpm for 10 minutes. A standard reference material (SRM 2710) Montana soil was also fractionated along with the soil samples.

5.2.6 Statistical Methods

The experiment is a single-factored experiment with four replications arranged in a completely randomized design. Treatment effects were determined by analysis of variance. Mean separation (Duncan) was done using the SAS software (2003). Linear correlation coefficients were also determined using the SAS software.

5.3. Results

5.3.1 Background Properties of Soils Used

Selected properties of the different soil types used in the study are shown in Table 5-1. The control soil with its high CEC and organic matter content seems to be the best for plant growth. Arsenic was most available in the CCA soil probably because it had the highest soil pH. High total potassium in mining soil is due to the presence of mica in the soil. The cattle dip vat soil had the highest oxalate extractable iron (4555 $mgkg^{-1}$) and aluminum (545 $mgkg^{-1}$) concentration that might be toxic to growing plants. Though the mining soil had the highest exchangeable calcium, it also had a high concentration of oxalate extractable iron (1626 $mgkg^{-1}$) and about 1000 $mgkg^{-1}$ lead in the soil that might limit plant growth.

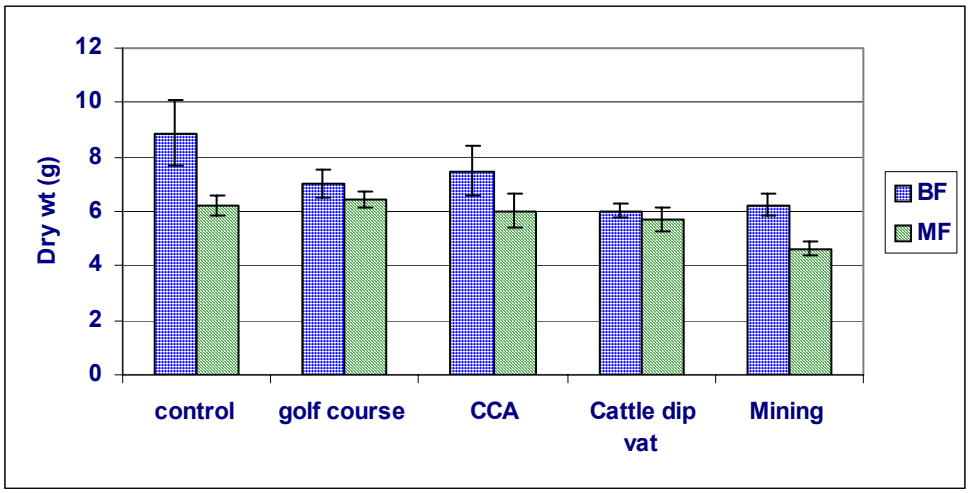
Table 5-1. Selected properties of soils used in this experiment

Property	Control	Golf course	CCA	Cattle dip vat	Mining
Sand (%)	89.2	94	88.2	84	84.7
Silt (%)	7.5	4	9.1	14	15.2
Clay (%)	3.3	2	2.7	2	0.1
Soil pH	6.66	6.80	6.94	6.76	6.75
CEC (Cmolc/kg)	20.4	10.7	13.4	16.8	12.0
OM (gkg-1)	31.5	7	11	26.5	4.2
Total As(mgkg-1)	0.41	19	131	291	294
Av As (mgkg-1)	0.003	4.16	33.7	31.5	9.89
Total Ca (mgkg-1)	4769	38134	12157	15999	9327
Ex Ca (Cmolc/kg)	11	11	19	9	25.9
Total K (mgkg-1)	82.5	35.3	39.6	96.3	504
Ex K (Cmolc/kg)	0.11	0.02	0.10	0.03	0.04
Total P (mgkg-1)	478	529	342	429	452
Av P (mgkg-1)	87.2	0.85	21.8	14	3.9
Total Pb (mgkg-1)	9.52	4.87	8.10	8.15	1382
Total Cd (mgkg-1)	0.13	0.12	0.08	0.49	0.12
Total Zn (mgkg-1)	105	15	0.81	259	37.6
Fe(Ox) (mgkg-1)	207	392	267	4555	1626
Al(Ox) (mgkg-1)	345	86	260	545	104
Clay minerals	Q, K, V	Q, K	Q, K, V	Q, K, S	Q,K,M,E

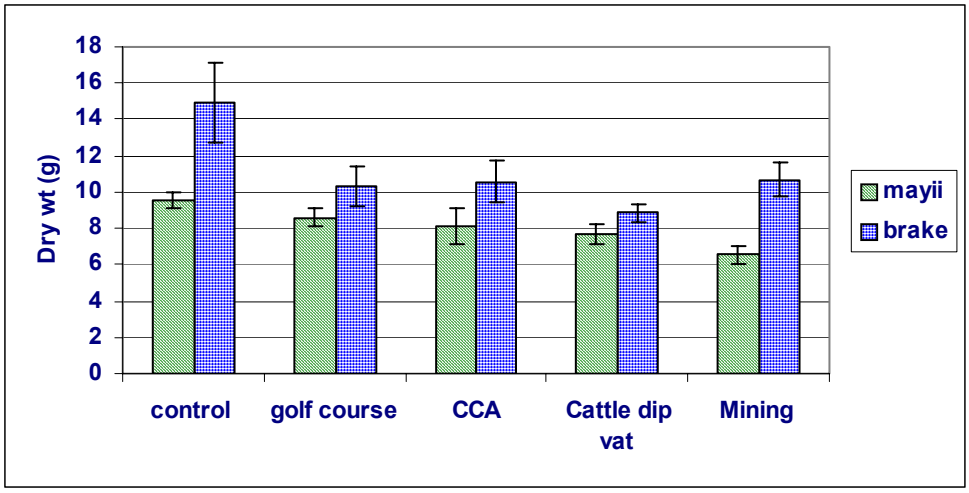
CEC=cation exchange capacity, OM=organic matter, EX=exchangeable, Ox=oxalate, Q=quartz, K=kaolinite, V=vermiculite, S=smectite, M-mica, E=expansible phyllosilicates

5.3.2 Plant Biomass

The aboveground biomass is very important in phytoremediation because it is harvested periodically and the plants are then allowed to grow back. *P. vittata* had significantly higher aboveground and total biomass than *P. cretica* at $P < 0.01$.



A



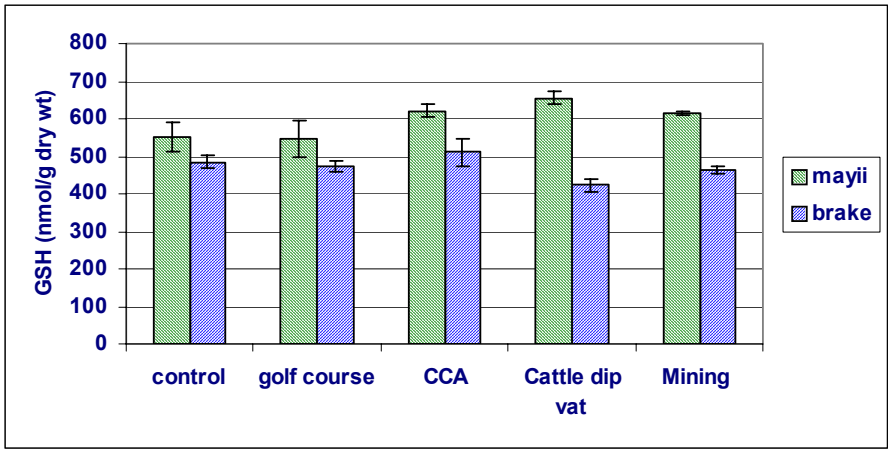
B

Figure 5-1. Plant biomass charts of *P. vittata* and *P.cretica* after 6 weeks of plant growth in different arsenic contaminated soils. A) Aboveground plant biomass of *P. vittata* (BF) and *P. cretica* (MF) after 6 weeks of plant growth in different arsenic-contaminated soils. Higher biomass in control is due to higher organic matter and cation exchange capacity of the soil. *P.vittata* has more biomass than *P. cretica* in control and mining soil. B) Total plant biomass of *P. vittata* (Brake) and *P. cretica* (Mayii) after 6 weeks of plant growth in different arsenic-contaminated soils. Bars are SE of means.

There were significant (P=0.05) differences between the aboveground biomass in the different arsenic contaminated soils. The trend in the above ground biomass (Fig. 5-1A) in the different arsenic contaminated soils for *P. vittata* was CCA >golf course>mining>cattle dip vat. There was a different trend for *P. cretica* with golf course>CCA>cattle dip vat>mining soil. The aboveground and total biomass (Fig. 6-1A & B) of *P. vittata* in the uncontaminated soil was the highest.

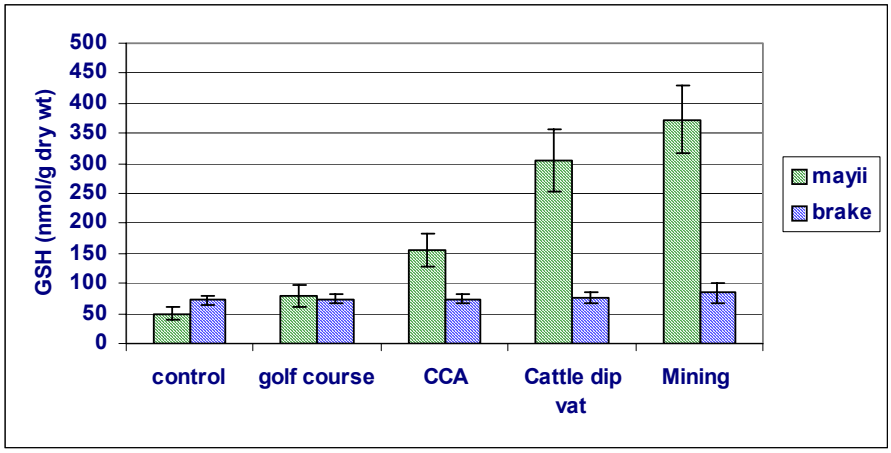
5.3.3 Glutathione Content

GSH concentrations in the fronds were significantly higher than in roots of both fern types at P<0.01. *P. cretica* had a significantly (P<0.001) higher GSH content in the fronds and roots (Fig. 6-2A) than *P. vittata*. There was no significant difference between the fronds GSH content in the different soil types. There were however significant differences between the root GSH concentrations in different soil types at P<0.01.



A

Figure 5-2 . The GSH concentrations in A (fronds) and B (roots) of *P.vittata* (Brake) and *P. cretica* (mayii) after 6 weeks of plant growth in different arsenic-contaminated soils. Bars are SE of means.



B

Figure 5-2. Continued

GSH concentrations in the roots (Fig. 5-2B) of *P. cretica* increased as the arsenic concentration and stress factors in the soils increased while *P.vittata* had about the same GSH concentration in all soil types. There was no correlation between plant arsenic concentrations and GSH concentrations in both fern types.

Table 5-2. Arsenic uptake by two fern types in different arsenic-contaminated soils

Soil type	µg/plant		
	Frond	Root	Total
	Pteris vittata		
Control	16.4d	5.9d	22.3d
Golf course	89.5c	43.5c	133c
CCA	1155a	702a	1857a
Cattle dip vat	500bc	278bc	778bc
Mining	780ab	458ab	1238ab
	Pteris cretica		
Control	12.5c	8.2c	20.7d
Golf course	196b	58.9b	254c
CCA	769a	224a	993a
Cattle dip vat	297b	158a	455bc
Mining	400b	205a	605b

Means with the same letter are not significantly different for each fern specie

5.3.4 Arsenic and Nutrient Uptake in *Pteris vittata* And *Pteris cretica*

Pteris vittata had significantly higher As uptake in the fronds and roots than *P. cretica* (Table 5-2) in all soil types except in the golf course soil. Ferns growing in the

CCA soil had the highest arsenic concentration while the golf course soil had the lowest arsenic concentration.

Arsenic uptake in both fern types followed the trend CCA>Mining> Cattle dip vat>Golf course>control. *P. cretica* significantly ($P<0.01$) took up more calcium in the fronds (Table 5-3) than *P. vittata*. In soils planted with *P. cretica*, there were no significant differences between the frond and root calcium concentrations in different soil types at $P<0.05$. In soils planted with *P. vittata*, there were also no significant differences between the frond calcium concentrations in different soil types at $P<0.05$. But in the roots, *P. vittata* growing in the mining soil had significantly the highest Ca concentration.

Pteris cretica had significantly ($P<0.01$) (Table 5-3) more potassium than *P. vittata* in the fronds while *P. vittata* had significantly more than *P. cretica* in the roots. In soils planted with *P. cretica*, there were no significant differences between the frond and root potassium concentrations in different soil types at $P<0.05$. While in soils planted with *P.vittata*, there were significant ($P<0.05$) differences between the frond potassium concentrations in different soil types. But in the roots, there were no significant differences in the potassium concentrations in different soil types.

Phosphorus accumulated (Table 5-4) in the roots of ferns grown in all soil types except the mining soil. *Pteris cretica* took up more phosphorus in their fronds and roots than *P. vittata* except in the mining soil.

There were also significant differences between the phosphorus concentrations in the fronds ($P<0.001$) and roots ($P<0.05$) of ferns growing in different soil types. A significant interaction between fern type and soil type was observed in phosphorus concentrations in the fronds.

Table 5-3. Calcium and potassium accumulation by two fern types in different arsenic-contaminated soils

Soil type/fern type	Frond Ca (mg/kg)	Root Ca (mg/kg)	Frond K (%)	Root K (%)
P. vittata	ns			ns
Control	6341	5967b	1.7c	1.69
Golf course	5305	9375b	1.75bc	2.4
CCA	5394	6299b	1.91ab	2
Cattle dip vat	6624	6275b	1.72c	2.31
Mining	6632	30733a	1.9a	1.89
P. cretica	ns	ns	ns	ns
Control	6310	6528	2.27	0.9
Golf course	8378	7205	2.32	1
CCA	7656	6883	2.7	1.18
Cattle dip vat	9464	7195	2.34	1.39
Mining	7447	15310	2.49	1.36

Means with the same letter are not significantly different for each fern specie.
ns= no significant difference between soil types

There were significant ($P<0.05$) differences between the frond and root P/As ratio of both ferns growing in different soil types. CCA soil had the lowest P/As ratio in the fronds. The roots P/As molar ratios were much higher than in the fronds. P/As ratio ranged from 80-939 in the fronds and from 163-2483 in the roots of *P. vittata* growing in the arsenic contaminated soils. P/As ratio ranged from 130-421 in the fronds and from 197-1158 in the roots of *P. cretica* also growing in the arsenic contaminated soils.

Phosphorus concentrations in the roots were significantly ($P<0.05$) positively correlated with root arsenic concentrations and with root potassium concentrations. Calcium and potassium concentrations in the fronds were also significantly ($P<0.05$) positively correlated.

Table 5-4. P/As ratio of two fern types in different arsenic contaminated soils

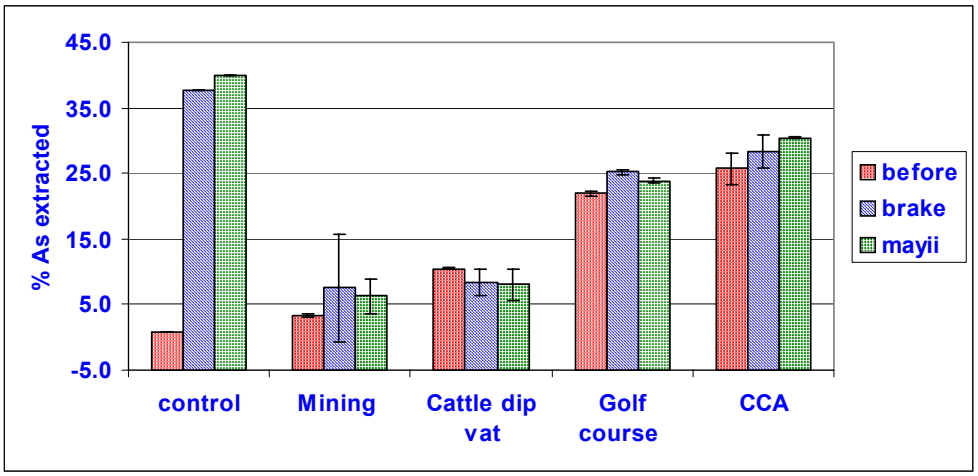
	Frond As	Root As	Frond P	Root P	Frond	Root
	mmolAs		mmolP		P/As molar ratio	
P. vittata				ns		
Control	0.02c	0.01d	77.1b	129	3127a	9978a
Golf course	0.17b	0.17c	150b	314	939b	2483b
CCA	2.05a	3.07a	135b	435	80c	163c
Cattle dip vat	1.11ab	1.31b	129b	402	136c	309c
Mining	1.67a	1.37b	1070a	443	717b	349c
P. cretica						
Control	0.03d	0.03c	74.5c	251	2780a	7625a
Golf course	0.41c	0.37b	154c	389	421b	1158b
CCA	1.71a	1.39a	215ab	378	130c	304c
Cattle dip vat	0.70c	1.04a	209b	420	324b	430c
Mining	1.15b	1.43a	269a	274	239bc	197c

Means with the same letter are not significantly different (comparing soil types).
ns= no significant difference between soil types

5.3.5 Effect of Different Fern Types on Selected Soil Properties

Mehlich –3 extractable As increased (Fig. 5-3a) with time in all soils except the cattle dip soils under both plants. This effect was most prominent in the control soil where a 30% increase was observed after 6 weeks of plant growth. Arsenic extracted was more in the CCA soil than in the other arsenic contaminated soils. Plant arsenic concentrations were significantly ($P<0.001$) positively correlated (Table 5-5) with Mehlich-3 arsenic and phosphorus ($P<0.01$) in the soil.

Available phosphorus (Table 5-6) in all soil types had drastically increased after six weeks of plant growth with CCA soil having significantly the highest P available. Figure 5-3b shows the relationship between soil pH and percentage of total soil arsenic extracted by Mehlich-3 in the soils before plant transfer. This line graph shows that available As is dependent ($R^2=0.96$) on soil pH. Soil pH was significantly correlated with available arsenic even after plant transfer.



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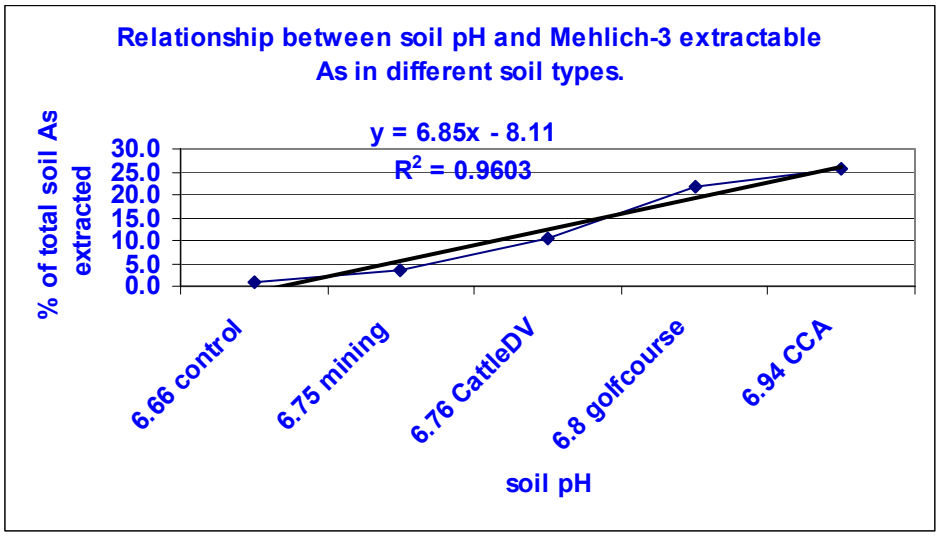


B

Figure 5-3 A) Mehlich-3 extractable As in different arsenic contaminated soils before and after plant growth. CCA soil has the highest percentage of arsenic extracted. B) Relationship between soil pH and Mehlich-3 arsenic in different arsenic contaminated soils. Cattle DV=cattle dip vat. Bars are SE of means.

Table 5-5. Pearson Correlation Coefficients

	Ex. K	Soil pH	Total soil As	Plant As
Av. As	-0.19	0.46	0.30	0.72
(probability)	0.30	0.008*	0.09	<0.0001*
Ex. Ca	0.48	-0.81	0.70	0.17
(probability)	0.005*	<0.0001*	<0.0001*	0.34
Av. P	-0.26	0.37	-0.18	0.59
(probability)	0.16	0.04*	0.34	0.0004*

*Significant at probabilities shown

Table 5-6. Selected soil properties after 6 weeks of plant growth

	Soil pH		Available P	mgkg-1	Ex. K	mgkg-1
	Brake	Mayii	Brake	Mayii	Brake	Mayii
Control	6.15d	5.82d	63.5b	44.7b	74.2a	67.7a
Golf course	6.94b	6.91b	57.8b	51.4a	44.2b	43.8ab
CCA	7.13a	7.14a	89.6a	54.5a	39.1b	32.6b
Cattle dip vat	6.90b	6.99b	49.6c	59.5a	65.4a	46.6ab
Mining soil	6.58c	6.62c	54.1b	54.7a	58.2a	56.0a

Means with the same letter not significantly different

Soil pH was also significantly (Table 5-5) correlated with exchangeable calcium ($P<0.001$), and available phosphorus ($P<0.05$) in the soil after plant harvest. For both fern types, CCA still had significantly the highest soil pH after plant harvest.

After six weeks of plant growth, total soil arsenic concentrations were slightly reduced in the different soils except the mining soil. There was greater than 80% mass balance (Table 5-7) for most of the pots used in the experiment.

Calcium was the only plant nutrient not added through the dynamite timed release fertilizer and so the effects we observed were solely from the different soil types. A significant correlation existed only between the calcium uptake in the plant and exchangeable calcium in the soil before plant transfer for both fern types. There was however no significant relationship between exchangeable calcium in the soil and plant arsenic uptake.

The exchangeable K remaining (Table 5-6) in the soils with *P. vittata* was higher than in soils with *P. cretica*. This is not surprising since *P. cretica* took up more potassium than *P. vittata*. Exchangeable potassium in arsenic contaminated soils had lower values than the control for both fern types after plant harvest. Exchangeable potassium in the soils before plant transfer was significantly correlated with plant arsenic uptake (Fig. 5-4) in both fern types.

Table 5-7. Total soil arsenic concentrations and mass balance

Soil type	Total soil As (mg/kg)	mg/pot	mg/plant	sum	Background (mg/pot)	Recovery (%)
	Pteris vittata					
Control	0.37	0.56	0.02	0.58	0.77	75.5
Golf course	15.3	22.9	0.13	23.1	28.5	81.0
CCA	112	168	1.86	170	196	86.4
Cattle dip vat	221	331	0.78	332	436	76.1
Mining	307	461	1.24	462	441	105
	Pteris cretica					
Control	0.49	0.74	0.02	0.76	0.77	98.8
Golf course	14.9	22.3	0.25	22.6	28.5	79.3
CCA	123	184	0.99	186	196	94.4
Cattle dip vat	281	421	0.46	422	436	96.7
Mining	308	462	0.61	463	441	105

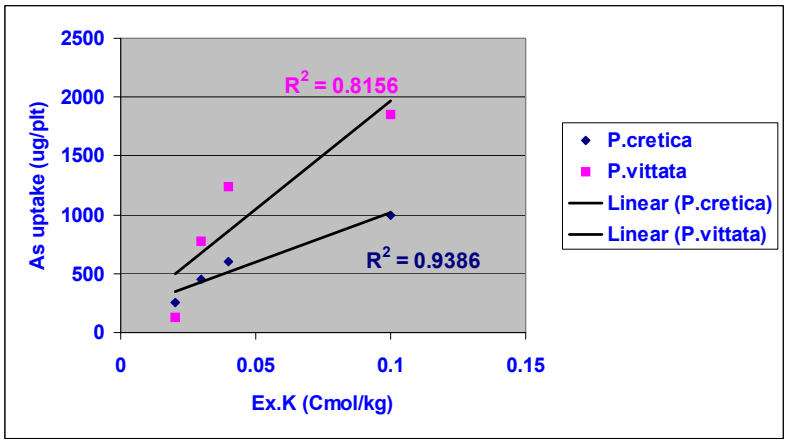


Figure 5-4. Effects of exchangeable potassium before plant transfer on plant arsenic uptake

5.3.6 Fractionation of Arsenic in Different Arsenic-Contaminated Soils

Despite various sources of arsenic contamination, the amorphous iron and aluminum arsenic bound fraction dominated (Fig. 5-5) in the different arsenic contaminated soils used for this experiment before plant transfer. The percentage of arsenic (73-75%) in this fraction was about the same in the golf course, CCA, and cattle

dip vat soils despite the fact that they had different total arsenic concentrations. The uncontaminated soil however had most of its arsenic in the residual fraction. The highest water-soluble and exchangeable arsenic concentration was in the CCA soil. The percentage of water-soluble arsenic in the different soil types before plant transfer was positively correlated (r=0.89) to the Mehlich-3 arsenic concentrations.

After plant transfer, the amorphous iron and aluminum arsenic fraction still remained the dominant fraction in arsenic contaminated soils except the golf course soil which now had the calcium fraction as the dominant fraction (Fig. 5-6a & 5-6b). This trend was observed in soils with both fern types. After plant transfer, the arsenic

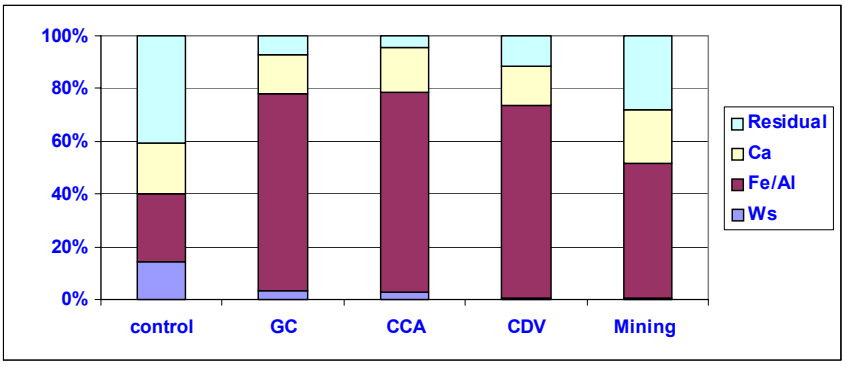
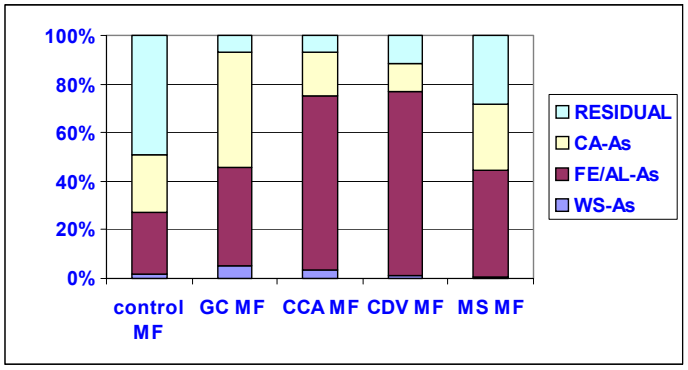
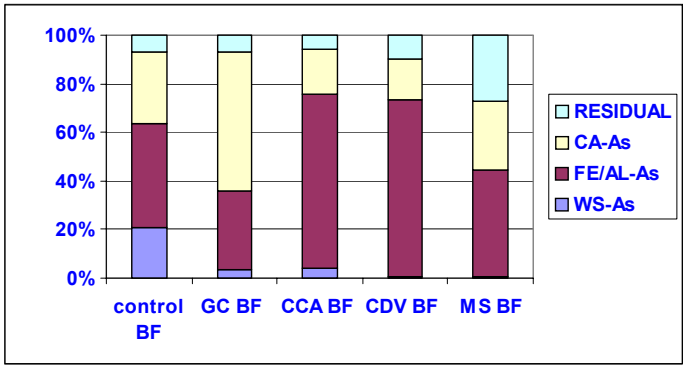


Figure 5-5. Fractionation of different arsenic contaminated soils before plant transfer. GC- golf course soil, CCA-chromated copper arsenate, CDV- cattle dip vat soil.

concentrations in the amorphous iron and aluminum fraction significantly decreased in soils with both fern types. There was significant interaction ($P<0.01$) between time and soil type on this fraction showing that the effect of time depended on the soil type. The concentration of arsenic in the residual fraction decreased with time in the cattle dip vat soil with both fern types. The concentrations of arsenic in the water-soluble fraction before planting were significantly correlated ($P<0.01$, $r=0.66$) to plant arsenic concentrations after plant harvest.



A



B

Figure 5-6. Fractionation of different arsenic contaminated soils after plant transfer. GC=golf course soil. CCA=chromated copper arsenate, CDV= cattle dip vat soil. MF=mayii fern. A) *P. cretica.* B) *P. vittata.*

5.4. Discussion

Plant biomass is a very important factor in the phytoremediation technology since efficiency of phytoremediation is determined by the ability to concentrate the metal in the aboveground biomass that is then harvested repeatedly to remove the contaminant. *P.vittata* had higher aboveground and total biomass than *P. cretica*, which makes it a better candidate for phytoremediation than *P. cretica*. About 10-14g/plant (dry wt.) was recorded in this experiment for four month-old *P.vittata*. Tu and Ma (2002) reported 2.9g/plant in soil spiked with 50mgkg^{-1}As after 12 weeks of growth. The high biomass recorded here might be because the soils are naturally contaminated with less available arsenic in the soil. In this study, the total biomass of ferns in the uncontaminated soil was

the highest probably because the soil had the highest cation exchange capacity and organic matter, which facilitated plant growth. This is further confirmed by the data that shows that biomass of *P.vittata* was not affected by the arsenic concentrations since it had the same biomass in the mining soil and the golf course soil.

The low biomass of *P. cretica* recorded in the cattle dip vat soil is probably due to iron toxicity because of its high oxalate extractable Fe and Al concentrations that could potentially affect plant growth. Some *P. cretica* ferns growing in this soil were actually dying when harvested while *P. vittata* ferns were still alive and well. The biomass of *P. cretica* was significantly lowest in the mining soil probably due to a combined toxicity of Fe and Pb in the soil. *P. vittata* had the same total biomass in both the CCA and mining soil, giving further credibility to the adaptability of this fern to metal stress.

In this study, both ferns had constitutively some glutathione since the ferns growing in the uncontaminated soil had a reasonable amount present. Meharg (1994) explained that plants need constitutive mechanisms to enable them to colonize highly metalliferous soils. Though *P. cretica* had a higher constituent GSH level than *P.vittata*, it does not imply more resistance to oxidative stress as the biomass data suggests. In some cases, the controlled response to an environmental stress might require direct responses of the glutathione systems, whereas in other cases it may not (Tausz, 2001). Since *P. vittata* took up more arsenic than *P. cretica* and GSH concentrations were not correlated with arsenic in plant tissues, GSH availability may not be a major determinant in the detoxification of arsenic in these two ferns. Hatton et al. (1996) discovered that giant foxtail (*Setaria faberia* Herrm) contained significantly higher concentrations of GSH than did corn (*Zea mays* L. var. Artus) and suggested that GSH availability is not a major

determinant in the detoxification and selectivity of either the chloroacetanilides or atrazine in these two species.

Though glutathione is a constituent of all plant organs, its concentration differs between organs and under different environmental conditions (Rennenberg, 2001). Glutathione concentrations in both fern types used in this experiment were higher in the fronds than in the roots. Several other scientists have reported that glutathione concentrations are lower in the roots compared to the leaves (Klapheck, 1988; Arisi et al. 1997; Koricheva et al. 1997).

Total glutathione concentrations in both ferns growing in arsenic contaminated soils ranged from 400 to 650 nmol/g dry wt. Total glutathione concentrations in birch seedlings subjected to simulated heavy metal and acid rain deposition ranged from 0.8 – 1.4μmol/g dry wt (Koricheva et al. 1997). GSH concentrations in four *Holcus lanatus* clones exposed to a range of arsenate concentrations ranged from 0.2 to 1.2μmol/g dry wt (Hartley-Whitaker et al., 2001).

Also in the roots of *P. cretica*, GSH concentrations increased with increase in total soil arsenic concentrations and other stress factors. This might be because plant roots are the first point of contact for toxic metals in the soil (Hartley-Whitaker, 2001). The roots of *P. vittata* however had the same GSH concentrations in both the uncontaminated and contaminated soils. This suggests that *P. vittata* is more efficient in maintaining its constitutive functions and quenching reactive oxygen species in the roots.

This is most probably the reason *P. vittata* took up more arsenic than *P. cretica* in all soil types except the golf course soil. The higher biomass of *P. vittata* might also be responsible for the higher arsenic uptake. *P. vittata* took up to 1857μgAs/plant while *P.*

cretica took up to 993μgAs/plant after six weeks of plant growth in CCA soil. A similar result was reported by Tu and Ma (2002) with 1280μgAs/plant accumulated by *P. vittata* after 8 weeks of plant growth in the same CCA soil. Plant arsenic uptake was significantly positively correlated with available (Mehlich-3) arsenic after harvest with plants in CCA soil taking up the most arsenic. Woolson et al. (1971) also reported that correlation was better between available arsenic and plant growth than with total arsenic and plant growth. Available arsenic in the soil increased with an increase in soil pH probably due to an increase in arsenic desorption in the soil.

Bioavailability of arsenic in these soil types was further examined by sequential extractions to separate arsenic into operationally defined chemical associations. The method of Onken and Adriano (1997) was modified in this study to include a step that is specifically associated with iron and arsenic. Ammonium oxalate buffer extraction of arsenic contaminated soils in a previous experiment showed that arsenic extraction and Fe dissolution were simultaneous (Gleyzes et al., 2002). In this study we observed that the oxalate fraction dominated in the different arsenic contaminated soils used for this experiment before and after plant transfer. This result is consistent with the report of Wenzel et al (2001) who also showed that arsenic was most prevalent in the oxalate fraction in twenty different arsenic contaminated soils in Austria. This is due to the high affinity of arsenic for amorphous Fe and Al oxides (Pierce and Moore, 1980, Takamatsu et al., 1982, Smith et al., 1998).

A significant correlation was observed between the concentrations of arsenic in the water-soluble and exchangeable (WE-As) fraction before planting and plants arsenic concentrations after plant harvest. Plant arsenic concentrations were also reported to be

correlated with the WE-As fraction in maize (Sadiq, 1986) in barley and ryegrass (Jiang and Singh, 1994). This is expected since this is the pool readily available to the plant.

Data showing that exchangeable potassium in the soils before plant transfer was significantly correlated with plant arsenic uptake in both ferns suggests that maybe potassium nutrition of these fern types may have a role to play in facilitating arsenic uptake. This is further confirmed by the data after harvest that shows that exchangeable potassium in arsenic contaminated soils planted with both fern types had lower values than the uncontaminated soil. The plants growing in the arsenic contaminated soils must have taken up more potassium in the soil resulting in lower values at harvest.

The reduction in total soil arsenic in soils planted with *P. vittata* cannot be explained solely by plant arsenic uptake due to the incomplete mass balance recorded in these soils. There could have been volatilization of arsenic because the experiment was done in the summer and it was so hot. However, we recorded better mass balance in soils planted with *P. cretica*. In CCA soil planted with *P. cretica*, at the end of 6 weeks of plant growth, only 0.8% of arsenic in the soil was taken up by plant. At this rate, it will take about 13 years to clean up this soil. Though the golf course soil might not take that long, the arsenic uptake by these two ferns will need to be improved upon to speed up the rate of cleanup for them to be used in phytoremediation.

It has been shown that arsenate is taken up by the phosphate transport systems in *Holcus lanatus*, *Deschampsia cespitosa* and *Agrostis capillaris* (Meharg and Macnair, 1991, 1992). Recently, this has also been confirmed to be the uptake system in the arsenic hyperaccumulator *P. vittata* (Wang et al., 2002). P uptake in these ferns was examined to further understand effect of arsenate on phosphate accumulation in the plant tissues of

these ferns. Phosphorus concentrations in the root were significantly correlated with arsenic uptake in the roots further confirming the fact that these two anions are taken up by the same uptake system. Though as Wang et al. (2002) explained, phosphate seems to have a higher affinity to the uptake system in the roots than arsenate. This might explain why, in this study, both fern types had higher phosphorus concentrations in the roots than in the shoots except in the mining soil.

In a separate study, Tu and Ma (2003b) also reported greater concentrations of phosphorus in the roots than in the fronds with phosphorus concentrations in the fronds ranging from 2201 to 5425 mgP/kg. The higher phosphorus concentration observed in the fronds of *P. vittata* in the mining soil might be due to the low available phosphorus in the soil. P deficiency increases the capacity of plant roots to take up phosphate in plants that have the capacity to synthesize additional transporter molecules (Drew et al., 1984). Wang et al (2002) in their own study reported phosphorus concentrations in the fronds that ranged from 4,000 to 8,000 mgP/kg dry wt while root P concentrations ranged from 4000 to 12000mgP/kg. We obtained comparable results from this study with frond phosphorus concentrations ranging from 4000 – 6000 mgP/kg dry wt except for an high P concentration (3.3%) in fronds of *P. vittata* grown in the mining soil and root P concentrations in both ferns ranged from 8000 to 18000mgP/kg. P/As molar ratios in both ferns were higher in the roots than in the fronds of both fern types. This observation was also made by Tu and Ma (2003b) in their experiment with different combinations of arsenate and phosphate. They concluded that P/As molar ratio of greater than 1.0 in the fronds seemed to be necessary for normal growth of the fern probably because phosphorus also helps to reduce arsenic toxicity to the plant.

5.5 Conclusions

Pteris vittata survived in these different soil types polluted with different sources of arsenic while *P. cretica* did not grow well in cattle dip vat and mining soil probably due to heavy metal stress. *P. vittata* took up more arsenic than *P. cretica* in all soil types except the golf course soil. *P.vittata* also had higher biomass than *P. cretica*, which makes it a better candidate for phytoremediation than *P. cretica*. GSH availability in these two fern types may not be a major determinant in the detoxification of arsenic in their plant tissues.

CHAPTER 6
EFFECT OF DIFFERENT ARSENIC SPECIES ON ARSENIC UPTAKE BY DIFFERENT FERN TYPES IN WATER

6.1 Introduction

Specifically, this study attempted to investigate: 1) the effects of arsenic species and concentrations on arsenic uptake by two arsenic hyperaccumulators *P. vittata* and *P. cretica*; 2) arsenic uptake, distribution and reduction in *P. vittata* and *Nephrolepis exaltata (Boston fern)*, a non-hyperaccumulator.

6.2 Materials and Method

6.2.1 Experiment One: Effectiveness of Arsenic Uptake from Water by Two Hyperaccumulator Fern Species under Different Arsenic Sources and Concentrations.

The effectiveness of two *Pteris* species to take up arsenic from different sources was evaluated in a randomized complete block design, with a 2x2x2 factorial scheme with two plant species, exposed to two sources of arsenic, at two concentrations. Three-month-old *P. vittata* and *P. cretica* were transferred into a hydroponics system and grown in a 20% Hoagland-Arnon solution (Hoagland and Arnon, 1938) for 4 weeks. The arsenic sources were 1 and 10 mg L^{-1} of monomethylarsonic acid (MMA) and sodium arsenite (As (III)). Each treatment was replicated three times. Samples of solution in which the ferns were grown were taken twice a week and analyzed for arsenic. The 4-month-old plants were then harvested, dried, ground, digested, and analyzed for arsenic.

6.2.2 Experiment Two: Arsenic Uptake and Speciation in a Hyperaccumulator and in a Non-Hyperaccumulator

The change in arsenic uptake and speciation with time in two fern species was evaluated in a randomized complete block design with a 2x2 factorial scheme. Two plant species, two month-old *Pteris vittata* and *Nephrolepis exaltata* S. hyper accumulator and non-hyperaccumulator species, respectively, were grown in 2 different time lengths (1 and 15 days). Each treatment was replicated four times. The plants were transferred into a hydroponics system and grown in Hoagland-Arnon solution for 1 and 15 days in a 20% Hoagland–Arnon solution and then treated with 5 mg L^{-1} arsenic as As (V) and 20 mg L^{-1} arsenic as As (III). Half of the ferns were harvested after 1 day and the other half after 15 days. Speciation of As in the fern fronds and roots was determined immediately after harvesting by extracting plant samples ultrasonically in 1:1 methanol/ water twice, using the method of Zhang et al. (2002). Arsenate and arsenite were separated using an As speciation cartridge (Metal Soft Center, Highland Park, NJ), which retains arsenate (Meng et al., 2001). The samples were then analyzed for total As and arsenite. The plants were also dried, ground, digested and analyzed for arsenic.

6.3 Results

6.3.1 Effect of Different Plant Species, Arsenic Sources, and Concentrations on Arsenic Uptake

The main effects of the three factors were significant. *P. vittata* took up significantly higher arsenic in the fronds than *P. cretica* for both arsenic species and concentrations (Table 6-1). The ferns took up more arsenic from the MMA treatment than the arsenite treatment. The arsenic uptake in both fronds and roots of both fern species increased with the dose (Fig. 6-1) of arsenic supplied.

Table 6-1. Plant arsenic uptake in *P. vittata* and *P. cretica* ferns grown in arsenic contaminated nutrient medium for 4 weeks

Fern specie	MMA *(mg/L)			As III** (mg/L)		
	0	1	10	0	1	10
			Fronds (mg/kg)			
P. vittata	10.4aA	558bA	1666cA	10.4aA	457bA	1075cA
P. cretica	4.0aB	79.6aB	627bB	4.0aB	201bB	249bB
			Roots (mg/kg)			
P. vittata	3.1aB	131bA	357cB	3.1aB	82.2bA	362cB
P. cretica	6.3aB	107bB	347cB	6.3aB	41.9bB	331cB

Mean of three replicates. Means within the horizontal rows followed by the same lower-case letter are not significantly different at P<0.05. Means on the vertical followed by a capital letter compare fern type; and similar letters are not significantly different at P<0.05. MMA*=Monomethyl arsenic acid. As III **= Arsenite.

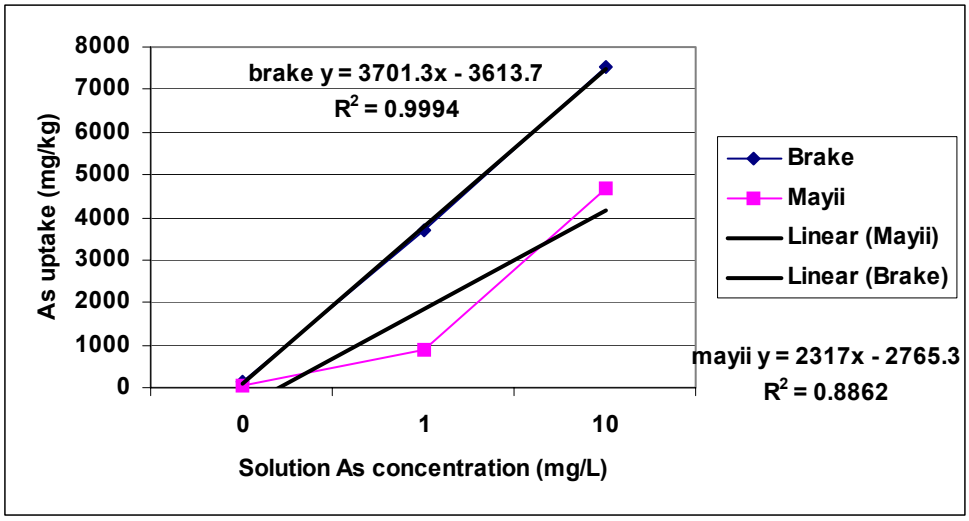


Figure 6-1. Total arsenic uptake in fronds and roots of both *P. vittata* and *P. cretica* exposed to different levels of arsenic in hydroponics after 4 weeks

There was a significant (P<0.05) interaction between plant specie and source of arsenic for arsenic uptake in the fronds, which implies that arsenic uptake from different sources of arsenic varied with plant specie. *P. vittata* took up more MMA than arsenite in

its fronds (Table 6-1) at both concentrations. *P. cretica* took up more arsenite than MMA at arsenic concentration of 1mg L^{-1} while it took up more MMA than arsenite at 10 mgL^{-1}. The interaction between plant specie and dose interaction is significant at $P<0.05$ showing that arsenic uptake at different solution arsenic concentrations varied with plant specie. There was also a significant interaction between source and dose, which shows that arsenic uptake in different sources of arsenic varied with dose applied. Because there was a high variability observed in the data set, the data was lognormalised. Statistical analysis of these lognormalized values revealed a three-way interaction ($P<0.01$) between plant specie, source and dose of arsenic applied.

Among all treatments, *P. vittata* had the highest transfer factor when exposed to arsenite at 1mg L^{-1}. At both concentrations, *P. vittata* also had higher transfer factors and bioconcentration factors (Table 6-2) than *P. cretica*.

The solution concentration of arsenic was reduced by about 60% by *P. cretica* and 70% by *P. vittata* in low arsenite concentration while it was reduced by about 50% by *P. cretica* and 60% by *P. vittata* in the low MMA concentration after four weeks. In the high concentration (10 mg L^{-1}) solution arsenic was reduced by 40% by both ferns in the arsenite treatment and by about 45% in the MMA treatment. Even though the percentage of the reduction in solution arsenic appears to be much smaller when the ferns were exposed to a high concentration (10 mg L^{-1}) of arsenic, the ferns took up a lot more arsenic in these treatments. In the low arsenic (1mg L^{-1}) treatment, the concentration of arsenic remaining (Fig. 6-2) in solutions where *P. vittata* was grown was lower than that for *P. cretica*. This is confirmed by the arsenic uptake that was shown in Fig. 6-1 with

P. vittata taking up more arsenic than *P. cretica*. In the high (Fig. 6-3) arsenic treatment (10 mg L-1), about 60% of arsenic was remaining in all treatments.

Table 6-2. Transfer and bioconcentration factors of *P. vittata* and *P. cretica* in arsenic-contaminated water after 4 weeks

Fern type	MMA* (mg/L)			As III* (mg/L)	
	0	1	10	1	10
		Transfer factors*			
P.vittata	3.35b	4.42ab	4.82ab	5.56a	2.97b
P.cretica	0.63cd	0.06d	1.81c	4.80ab	0.75cd
		Bioconcentration factors** (frond)			
P.vittata	62b	1115a	309b	1143a	211b
P.cretica	4b	155b	98b	402b	44b
		Bioconcentration factors** (root)			
P.vittata	3.05c	263a	66ab	206ab	71ab
P.cretica	6.35c	208ab	54.3b	107ab	58.1b

Mean of three replicates. Means with the same letter are not significantly different at P<0.05
* Ratio of arsenic concentration in frond to that in root.
** Ratio of As concentration in plant tissue to that in the solution.
MMA=monomethyl arsenic acid. As III=arsenite.

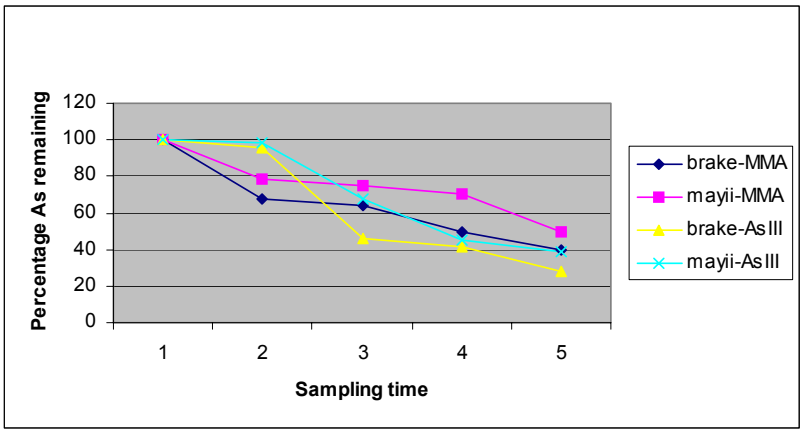


Figure 6-2. Percentage arsenic remaining in solution of 1 mgAs^{-1} after 4 weeks in hydroponics

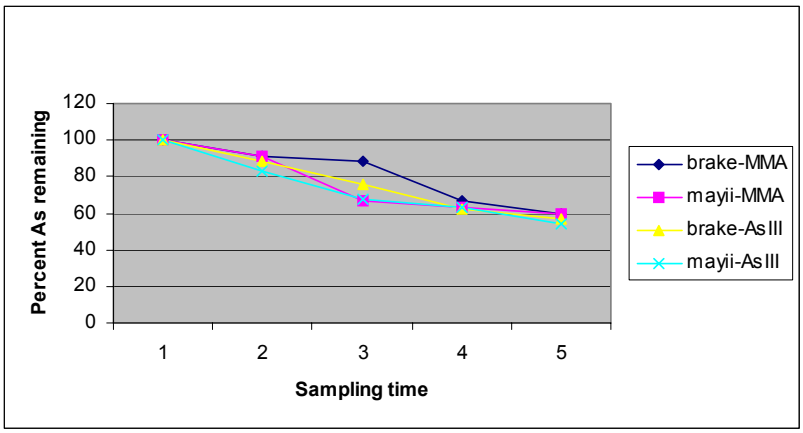


Figure 6-3. Percentage of arsenic remaining in solution of 10 mgAs/L after 4 weeks in hydroponics

6.3.2 Arsenic Uptake and Speciation in *P. vittata* and *N. exaltata*

Arsenic uptake and transfer factors of. *P. vittata* under different arsenic sources were greater than that of *N. exaltata* at different days. *P. vittata* took up in its fronds four times more arsenic in the arsenate solution and eight times more arsenic in the arsenite treatment than *N. exaltata*.

Time was a highly significant ($P<0.01$) factor in the arsenic uptake and transfer factors of the ferns. Both ferns took up more arsenic after fifteen days than at one day. *N. exaltata* took up more arsenate than arsenite in its fronds and roots after fifteen days even though the concentration of arsenite was more. There was a significant interaction between fern type and time for arsenic uptake and transfer factors.

Table 6-3. Plant arsenic concentrations in *P.vittata* and *N. exaltata* at different time periods

	1 day			15 days		
	frond	root	Transfer factor*	frond	root	Transfer factor
			5 mg/L As V			
P. vittata	38.2 b	26.5 b	1.44 b	282 a	50.6 b	5.57 a
N. exaltata	4.81 b	25.7 b	0.19 b	70.6 b	156 a	0.45 b
			20 mg/L AsIII			
P. vittata	267 b	205 a	1.30 b	542 a	254 a	2.13 a
N. exaltata	38.7 c	128 b	0.30 b	66.3 c	144 b	0.46 b

Mean of four replicates. Means with the same letter are not significantly different at $P<0.05$ *Ratio of arsenic concentration in frond to that in root. As III=Arsenite. As (V)=Arsenate.

This shows that the effect of time on arsenic uptake and transfer factor varied with fern type. The transfer factor (Table 6-3) of *P. vittata* was higher after 15 days for both arsenic species than at 1 day.

The one-day arsenic uptake in the fronds of *P. vittata* was about 13% of the arsenic (5 mgL^{-1} As (V)) taken up in 15 days, while *N. exaltata* took up about 6.81% of the arsenic (70.6 mg kg^{-1}) taken up in 15 days in 5 mgL^{-1} As (V). The 1-day arsenic uptake in the fronds of *P. vittata* was about 49% of the arsenic (20 mgL^{-1} As (III)) taken up in 15 days, while *N. exaltata* took up about 58.4% of the arsenic (66.3 mg kg^{-1}) taken up in 15 days in 20 mgL^{-1} As (III). The *P. vittata* accumulated arsenic in its fronds, while *N. exaltata*

retained most of the arsenic taken up in the roots when exposed to both arsenic species and concentrations.

Table 6-4. Percentage of As III in the fronds and roots of *P.v ittata and N. exaltata* after exposure to 5 mg L^{-1} AsV or 20 mg L^{-1} AsIII for 1 or 15 d

Treatment	One day		15 days	
	Fronds	Roots	Fronds	Roots
		5 mg/L AsV		
P.vittata	BDL	BDL	83.7	26.9
N. exaltata	BDL	BDL	12.5	BDL
		20 mg/L AsIII		
P.vittata	BDL	40.8	83	26.8
N. exaltata	BDL	31.7	24.4	10.3

Mean of four replicates. BDL=below detectable limit.

When exposed to As (V), there was no detectable arsenite present (Table 6-4) in the fronds and roots of both *P. vittata* and *N. exaltata* after one day. There was however, 40.8% As III in the roots of *P. vittata* while *N. exaltata* had 31.7% As III in its roots after exposure to As (III) for 1 day. After 15 days, *P. vittata* had the same percentage (Table 6-4) of As III when exposed to both As (V) and As III in its fronds (83%) and roots (27%). The *N. exaltata* had 12.5% As III in its fronds and no detectable arsenite in its roots after 15 days when exposed to As (V). In arsenite treatment, however, it had 10.3% As III in the roots and 24.4% As III in the fronds after 15 days.

6.4 Discussion

In this study, arsenic transfer factor (TF; the ratio of arsenic concentrations in fronds to roots) was used to measure the effectiveness of plant arsenic translocation,

while arsenic bioconcentration factor (BCF; the ratio of arsenic concentrations in plant to solution) was used to measure the effectiveness of plant arsenic accumulation in the biomass.

Though *P. cretica* has been identified and reported as an arsenic hyperaccumulator (Ma et al., 2001; Zhao et al., 2002), the effect of varying arsenic concentrations and species on arsenic accumulation in comparison with *P. vittata* have not been studied. In this study, *P. vittata* accumulated more arsenic than *P. cretica* in the fronds and the roots (Table 7-1). *P. vittata* also had greater arsenic TF and BCF than *P. cretica*. This implies that *P. vittata* was more effective than *P. cretica* in translocating As from the roots to the fronds in addition to taking up more arsenic from solution. The highest TF and BCF of 5.6 and 1143 were observed for *P. vittata* at 1 mg L^{-1} AsIII.

A greenhouse experiment by Zhao et al. (2002) comparing arsenic accumulation of *P. vittata* and *P. cretica* showed similar capacity in their arsenic accumulation probably because different cultivars of *P. cretica* (albo-lineata vs. wimsetti vs. Mayii) and different systems (soil vs. hydroponic) were used in these studies. Huang et al., (2004) reported that at similar plant ages, both *P.vittata* and *P. cretica* had similar arsenic phytofiltration efficiency and were able to rapidly remove arsenic from water to achieve arsenic levels below the drinking water limit of 10 μgL^{-1}. However, the use of the term phytofiltration efficiency is not very clearly stated in this study, and bioconcentration and transfer factors that are normally used to measure efficiency of phytoremediation were not reported in this study.

Arsenic accumulation by both plants increased as arsenic concentration in solution increased except in the fronds of *P. cretica* where they remained unchanged with

increasing AsIII concentration. This may indicate limited translocation capacity of *P. cretica* for AsIII compared to *P. vittata*. Carbonell-Barrachina et al. (1998) reported that arsenic concentrations in two wetland plants significantly increased with increasing As application rates, regardless of the arsenic chemical form.

Statistical analysis of log-normalized values of plant arsenic concentration revealed a three-way interaction ($P<0.01$) between plant species, and arsenic concentrations and species. This shows that the effect of arsenic species and concentrations on plant arsenic uptake depended on plant species. Plant arsenic uptake is directly related to arsenic species in the environment. In a hydroponic experiment, O'Neil (1990) showed arsenic accumulation in the roots of bean plant (*Phaseolus vulgaris*) is in the order of AsIII>MMA. Similar preference in plant arsenic uptake has been observed in two rice cultivars (Marin et al., 1992) and two wetland plant species (Carbonell et al., 1998) in hydroponic experiments. However, in our experiment with As hyperaccumulators, the ferns accumulated and translocated more MMA than As III. This may be because AsIII is more toxic to plant than MMA (Sachs and Micheals, 1971) as AsIII reacts with sulfhydryl groups of enzymes and tissue proteins, leading to inhibition of cellular function and death (Meharg & Hartley-Whitaker, 2002).

Arsenic concentrations and transfer factors in the fronds of *P. vittata* were greater than those of *N. exaltata*. Higher transfer factors in *P. vittata* show it was able to translocate more arsenic in its fronds while *N. exaltata* retained most of the arsenic in its roots. Efficient As translocation has been reported as one of the hyperaccumulation mechanisms of *P. vittata* (Ma et al., 2001), which is confirmed in this experiment. In this study, we also observed that *Nephrolepis exaltata* had arsenic TF of <1, which is typical

of non-hyperaccumulators as discussed earlier. Similar results were reported by Tu and Ma (2003) after exposing both ferns to As for two days, *P. vittata* accumulated more arsenic in its fronds than *N. exaltata* and transfer factor of *N. exaltata* was lower than one and less than those of *P.vittata*. The reduced arsenic translocation and accumulation in non As-hyperaccumulating plants may be attributed to suppression of the high-affinity phosphate uptake system (Meharg and Macnair, 1991).

Another mechanism of arsenic hyperaccumulation by *P. vittata* is its ability to reduce arsenate to arsenite, which is probably transported as a chelate to the fronds (Ma et al., 2001). It has been proposed that under the reducing conditions of plant cells, As (V) is readily reduced to As III in hyperaccumulators and complexed by organic ligands such as thiols to avoid damage to plant cells when the plant is exposed to arsenic (Zhang et al., 2002). Arsenic concentrations and speciation of two plants were compared after exposing them to AsV or AsIII for 1 or 15 days.

However, no AsIII was detected in the fronds regardless of arsenic species applied after one day implying that an appreciable amount of arsenic is not reduced in one day. This might explain why the ability of *P. vittata* to translocate arsenic increased with time as shown by the transfer factors. When exposed to arsenite, in one day, arsenite was present in the roots of both ferns probably because it had not yet been converted to arsenate which is most probably the form arsenic is transported in the ferns since it was not present in the fronds. By 15 days, the percentage of arsenite present in roots of both ferns had decreased suggesting that it had been converted to arsenate.

This is consistent with previous reports that arsenate is the dominant form in the roots of *P. vittata* when exposed to arsenic while arsenite is the predominant form in the

fronds. Zhang et al., (2002) reported that 60 to 74% of the arsenic in the fronds was present as arsenite while only 8.3% was present as arsenate in the roots of *P. vittata*. Lombi et al. (2002) also reported that 75% of the As in the fronds was present as arsenite. Tu et al (2003) reported that 94% arsenic in the fronds was present as arsenite.

In this study, after 15 days, 83% of the arsenic was present as arsenite in the fronds regardless of the arsenic specie and concentration that *P. vittata* was exposed to. The speciation of arsenic in the fronds and roots of the hyperaccumulator (*P. vittata*) was not affected by arsenic solution concentration after 15 days. Percentage of arsenite in both fronds and roots of the non-hyperaccumulator (*N. exaltata*) however increased with arsenic solution concentration. In the control, like in the previous experiment there was a redistribution of arsenic from the fronds to the roots after 15 days in *P. vittata*.

6.5 Conclusion

Arsenic hyperaccumulators *P. vittata* and *P. cretica* were both efficient in accumulating MMA and AsIII from solutions and translocating arsenic from roots to fronds with *P. vittata* being much more efficient than *P. cretica*. Compared to non arsenic-hyperaccumulator *N. exaltata*, *P. vittata* had significantly greater arsenic translocation and reduction capacity, suggesting the role of arsenic translocation and reduction in arsenic tolerance by *P. vittata*.

CHAPTER 7
EFFECT OF DIFFERENT NUTRIENT ELEMENTS ON ARSENIC UPTAKE BY *P. vittata* IN WATER

7.1 Introduction

The role of plant nutrition in arsenic uptake is not very clear. Calcium (Ca) is probably of special importance to *P. vittata* because it prefers to grow in an environment dominated by limestone (Jones, 1987). Tu and Ma (2002) in a soil experiment reported that Ca is effective in increasing arsenic concentrations in the fronds of *P. vittata* and enhancing arsenic translocation from the roots to the fronds. It is, however, unclear if it is the soil alkalinity or Ca that actually facilitates plant arsenic uptake. Komar (1999) reported a positive correlation between accumulation of potassium (K) and arsenic. He attributed increased plant biomass production to increased plant K levels. Komar (1999) reported a positive correlation between arsenic and potassium accumulation (r^2=0.58) and related this to increased plant biomass production typically associated with increases in plant potassium levels. A recent study investigated arsenic distribution in the fern and reported that arsenic and potassium in the upper epidermis were found to correlate positively (Lombi et al, 2002). The effects of Ca and K on arsenic uptake of *P. vittata* in hydroponics are unknown.

It was also interesting to see if pre-exposing the fern to arsenic will either facilitate or hinder arsenic uptake in different solution arsenic concentrations, since there has been no previous study on this.

Hence, this paper discusses the effects of nutrition and exposure to arsenic on arsenic accumulation by *P. vittata* in hydroponics.

7.2 Materials and Method

7.2.1 Effect of Arsenic Pre-Exposure on Arsenic Accumulation in *P. vittata*

Arsenic uptake in *P. vittata* that was already exposed to arsenic and had already accumulated arsenic was evaluated. The experimental design was a randomized complete block in a 2x2 factorial scheme. Two-plant accumulation, preloaded and unloaded plants, exposed to two arsenic concentrations, low arsenic concentration (300 μg L^{-1}) and high arsenic concentration (2mg L^{-1}). The control treatment was also evaluated. Each treatment was replicated four times.

Three-month-old *P. vittata* plants were grown in chromated copper arsenate contaminated soil and allowed to take up arsenic for about three months hereafter referred to as the preloaded ferns. The unloaded ferns and the control were also grown for the same length of time in potting soil. This 6-month-old ferns were grown for 2 weeks in 50% Hoagland–Arnon solution with vigorous aeration and replenishment twice a week. Samples of solution in which the ferns were grown were taken at different times (every 3 days). The plants were then harvested, dried, ground, digested and analyzed for arsenic.

7.2.2 Effect of Calcium and Potassium on Arsenic Uptake in Water

This study evaluated the effect of different calcium and potassium doses on arsenic uptake. The treatments were arranged in a randomized complete block design with a 2x2x2 factorial scheme. Three-month-old *P. vittata* procured from a nursery (Milestone Agriculture, Inc., FL, USA) was treated with 2 levels of calcium (2 and 4 mM), 2 levels of potassium (3 and 6 mM) and 2 levels of arsenic (10 mgL^{-1} (130 μM), 50 mg L^{-1}

(650μM)). Each treatment including a control was replicated four times. The ferns were grown for one week with vigorous aeration. The plants were then harvested, dried, ground, digested and analyzed for calcium, potassium and arsenic.

7.2.3 Effect of Nutrition on Arsenic Uptake in Water

This experiment was designed to 1) compare the effect of 3 levels of Hoagland solution 10%, 20%, 30% on arsenic uptake 2) evaluate the effect of $CaCO_3$ on As uptake and 3) determine the effect of different nutrient elements Ca, K, NO_3, NH_4, and P on As uptake in 20% Hoagland solution containing 1 mgL^{-1} As. $CaCO_3$ (32 mgL^{-1}) was tested at two Hoagland solution strength 10 and 20%. Each of the nutrient elements were added at levels representing an additional 20% Hoagland solution strength, Ca (32mgL^{-1}), K (46.8 mgL^{-1}), NO_3 (174 mgL^{-1}), NH_4 (50 mgL^{-1}), and P (3.1 mgL^{-1}). The treatments were arranged in a randomized complete block design with each treatment replicated four times. *P. vittata* plants were allowed to grow in these treatments for 4 weeks and then harvested. Wet plant biomass of ferns used for the experiment was taken before and after the experiment to monitor increase in plant weight with these treatments. Solution samples of treatments were taken before and after the experiment and analyzed for pH, dissolved organic carbon, (DOC) and As concentrations. DOC was analyzed using total organic carbon analyzer (Shimadzu, TOC- 5050 A).

7.2.4 Experimental Procedure

In all the above experiments, the plants were first transferred into a hydroponics system and grown in Hoagland - Arnon solution with temperature ranging from 23 to 28 ^{o}C and 70% relative humidity for about a week to acclimatize to an aquatic system before being exposed to arsenic. An assembly of cool-white fluorescent lamps supplied a 14-hour photoperiod with an average photon flux of 825 μmol m-2 s -1.

7.2.5 Plant Analysis

The harvested plants were separated into aboveground (fronds) and belowground biomass and dried in the oven at 65oC for 3 days and then ground into powder. Plant samples were then digested with nitric acid using the Hot Block Digestion System (Environmental Express, Mt. Pleasant, SC; EPA Method 3050A). Total As concentrations were determined with a graphite furnace atomic absorption spectrophotometer (Perkin Elmer SIMMA 6000, Perkin-Elmer Corp, Norwalk, CT) while calcium and potassium contents were analyzed on a flame atomic absorption spectrophotometer (Varian 220 FS with SIPS, Varian, Walnut Creek, CA).

7.2.6 Statistical Methods

Treatment effects were determined by analysis of variance according to the general linear model procedure of the Statistical Analysis System. Duncan mean separation test were used to separate treatment effects using SAS software.

7.3 Results

7.3.1 Effect of Preloading on Arsenic Remediation in Water

In 2 weeks, preloaded ferns exposed to high arsenic concentration (2 mg L^{-1}) reduced solution arsenic concentrations by about 70%, while the unloaded ferns reduced it by about 60%. Preloaded ferns exposed to low arsenic concentration (0.3 mg L^{-1}) reduced solution arsenic concentrations by about 40%, while the unloaded ferns reduced it by about 60%. There was a significant ($P<0.05$) interaction between the effect of plant type and dose applied, showing that the effectiveness of the plant species to take up arsenic from the solution varied with the dose of arsenic applied. The unloaded ferns were more effective at low concentration, while the preloaded ferns were most effective at high

concentration. The preloaded in high As concentration had only about 20 % arsenic remaining in solution after the experiment.

There was a significant interaction between plant type and dose of arsenic on arsenic uptake in the roots. The arsenic uptake in the roots of the preloaded ferns at high As concentration was higher than at low As concentration. Preloaded ferns had significantly more arsenic in the fronds and roots than the unloaded ferns. There was no significant difference in arsenic uptake (Fig. 7-1) in the fronds of the preloaded ferns for both concentrations. Arsenic uptake in the fronds and roots of the unloaded ferns significantly increased with increase in solution arsenic concentration.

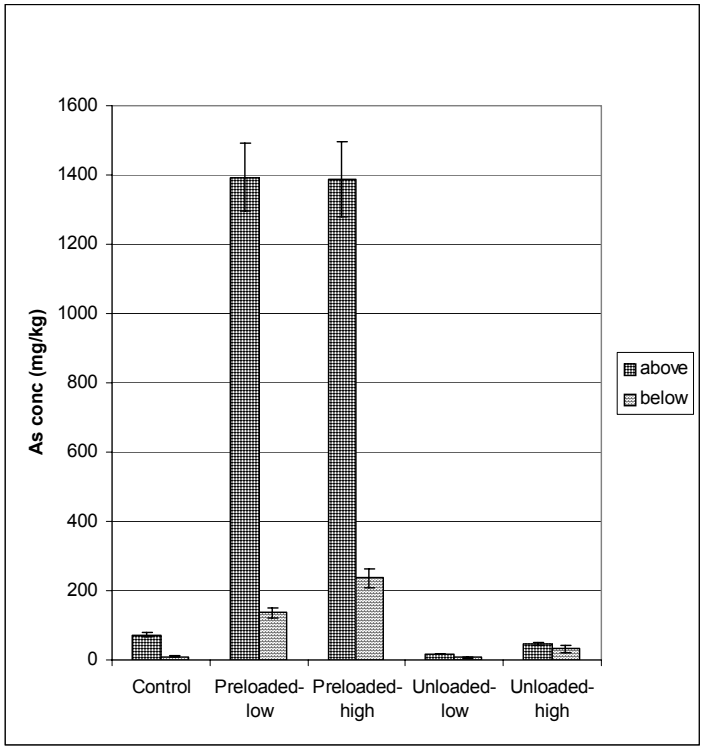


Figure 7-1. Effect of preloading on As uptake in *P. vittata* exposed to different As solution in water. Bars are SE of means.

Table 7-1. Effect of preloading on transfer and bioconcentration factors of *P. vittata*

Treatment	Transfer factor**	Bioconcentration factor***	
		Fronds	Roots
Control	7.09 ± 0.83*	7254 ± 1061	86.3 ± 10.6
PF-Low As	11.64 ± 2.9	4804 ± 684	468 ± 100
PF-High As	6.19 ± 0.57	2392 ± 371	407 ± 90.5
UF-Low As	2.88 ± 0.42	134 ± 8.85	50.8 ± 11
UF-High As	3.02 ± 1.1	83.0 ± 4.99	25.1 ± 6.0

PF=preloaded fern. UF=unloaded fern. *Mean ± Standard Error (n=4). **Ratio of arsenic concentration in frond to that in root. ***Ratio of As concentration in plant tissue to that in the solution.

Bioconcentration factor (Table 7-1) of the fronds and the roots decreased with increase in solution arsenic concentration for both preloaded and unloaded ferns.

7.3.2 Effect of Calcium and Potassium on Arsenic Uptake in Water

The effect of calcium on the arsenic uptake of brake fern depended on solution arsenic concentration. At 10 mg L^{-1} As, arsenic accumulation in the fronds of *P. vittata* increased with increasing calcium concentration while at the arsenic concentration of 50 mg L^{-1} the accumulation decreased with increasing calcium concentration. Similar trend was observed in the roots of *P. vittata*.

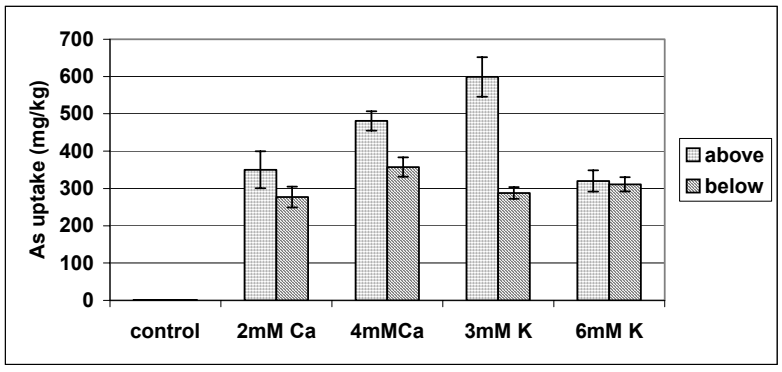


Figure 7-2. Effect of calcium and potassium on arsenic uptake in *P. vittata* exposed to 10 mgAs/L. Bars are SE of means.

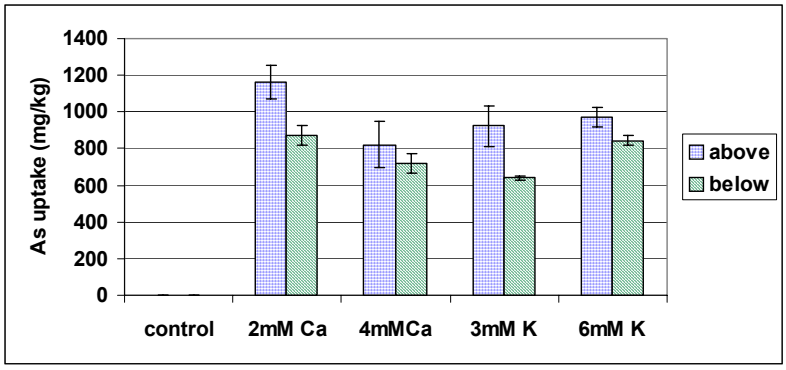


Figure 7-3. Effect of calcium and potassium on arsenic uptake in *P. vittata* exposed to 50 mgAs/L. Bars are SE of means.

P. vittata had the highest arsenic uptake at 3mM K in 10 mg AsL^{-1} while in 50 mg AsL^{-1} As accumulation was highest in 2mM Ca solution (Fig. 7-2 and 7-3). The arsenic uptake by *P. vittata* increased with solution arsenic concentration. The effect of potassium on arsenic uptake also depended on solution arsenic concentration. At 10 mgL^{-1}As, the arsenic in the P. vittata fronds decreased with increase in potassium concentration while there was no difference in As uptake at 50mgAsL^{-1}.

Calcium uptake in the fronds increased with calcium concentration while potassium uptake decreased with increasing potassium solution concentration. A significant positive correlation (r= 0.61, P=0.0002**) was observed between the different levels of potassium applied and potassium uptake in the roots. This means that increasing potassium solution concentration increased root uptake of potassium in *P. vittata.* The highest calcium uptake in the fronds of *P. vittata* was in the 4mM treatment in 10mgL^{-1} arsenic. The highest potassium uptake in the fronds of *P. vittata* occurred in the 3mM treatment in 10mgL^{-1} arsenic. Calcium accumulated in the roots while potassium accumulated in the fronds of *P. vittata*. There was a significant negative correlation (r= - 0.51, P=0.003**)

between potassium uptake and calcium uptake in the roots of *P. vittata*. There was no significant difference in the effect of calcium and potassium on arsenic uptake.

7.3.3 Effect of Different Hoagland Solution Strength and Different Nutrients

7.3.3.1 Arsenic accumulation and transfer factor

There were no significant differences between the arsenic accumulation in the fronds and roots of *P. vittata* at the different Hoagland solution strengths compared, mostly due to the high variability in data collected. The calcium carbonate treatment at 20% Hoagland solution strength had significantly higher arsenic concentrations in the fern fronds than the 10% treatment. Only the addition of calcium carbonate to the 20% Hoagland solution significantly increased arsenic concentration in the fern fronds over the control. Addition of KCl, $CaCl_2$, NH_4Cl, and PO_4^{2-} to the 20% Hoagland solution strength decreased the amount of arsenic (total As) accumulated in the fern. Transfer factors (Table 8-2) of calcium carbonate and sodium nitrate treated ferns were significantly higher than the control.

Table 7-2. Effect of different nutrients on arsenic uptake (mg/kg) and transfer in *P. vittata*

	Frond As	Root As	Transfer factor
CaCl2	49.2d	28.2b	1.74c
KCL	80.6bcd	27.8b	2.90bc
NaNO3	129.9b	35.8b	3.63ab
NH4CL	47.3d	17.3b	0.37bc
NaH2PO4	65.4cd	24.7b	2.64bc
CaCO3	212.7a	42.5b	5.01a
Control	124.2 bc	70.5a	1.76c

Means with the same letters are not significantly different

7.3.3.2 Plant biomass

There were significant differences between the plant biomass taken before and after the experiment for the various Hoagland solution strength tested. The plant biomass after 4 weeks was significantly higher than when the experiment started. There were also significant differences between the biomass before and after the experiment for the various nutrients tested. Potassium and calcium carbonate treated ferns had higher increase in plant biomass than the control (Fig. 7-4).

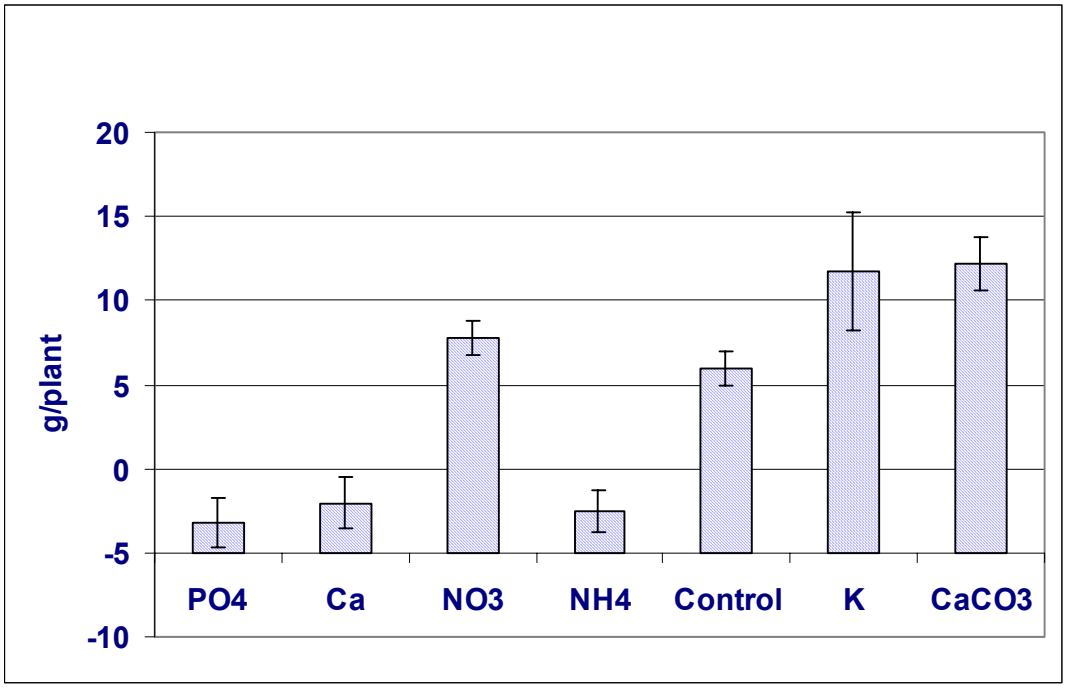


Figure 7-4. Increase in plant biomass grown in Hoagland strength with different plant nutrients. Bars are SE of means.

7.3.3.3 Solution arsenic concentrations

After 28 days, the 10% Hoagland solution had the most reduction in solution arsenic concentrations. The ferns in this treatment had reduced the solution arsenic to about 200 ppb, a 75% reduction. The more the Hoagland strength, the less the reduction in solution arsenic. When calcium carbonate was added however, there was more arsenic

reduction with the 20% Hoagland solution strength. In the case of the different nutrients, phosphate treatment (Fig. 7-5) had the highest arsenic remaining in solution. The calcium carbonate treatment greatly reduced the arsenic in solution by about 75% while the nitrate treatment reduced by about 65% in 4 weeks.

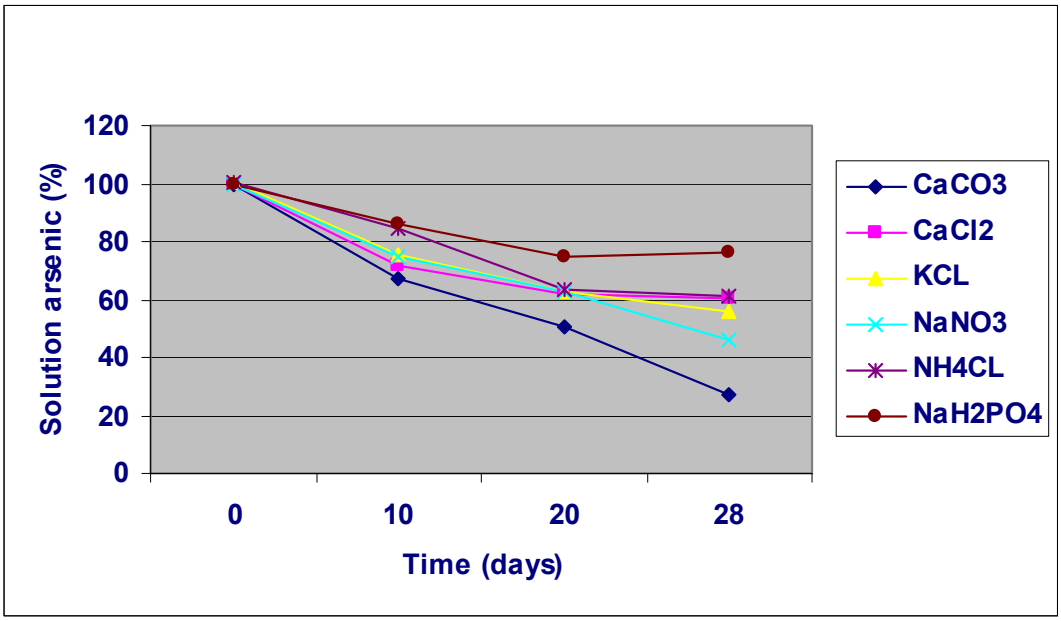


Figure 7-5. Percent arsenic remaining in solution after 4 weeks

7.3.3.4 Change in solution pH and dissolved organic carbon (DOC)

Solution pH was measured before and after the experiment. Solution pH in 10 and 20% Hoagland strength increased (Fig. 7-6) while it decreased in 30% Hoagland solution strength. There were also changes in solution pH with the various nutrients (Fig. 7-7). There was a 0.8 decrease in pH with the ammonium treatment while there were increases in pH with the other nutrients except calcium.

Dissolved organic carbon (DOC) decreased (Fig. 7.8) with increase in Hoagland solution strength after 4 weeks though there were no significant differences between

the different Hoagland strength tested. The nitrogen treated ferns had the lowest DOC solution concentration while calcium and potassium had the same.

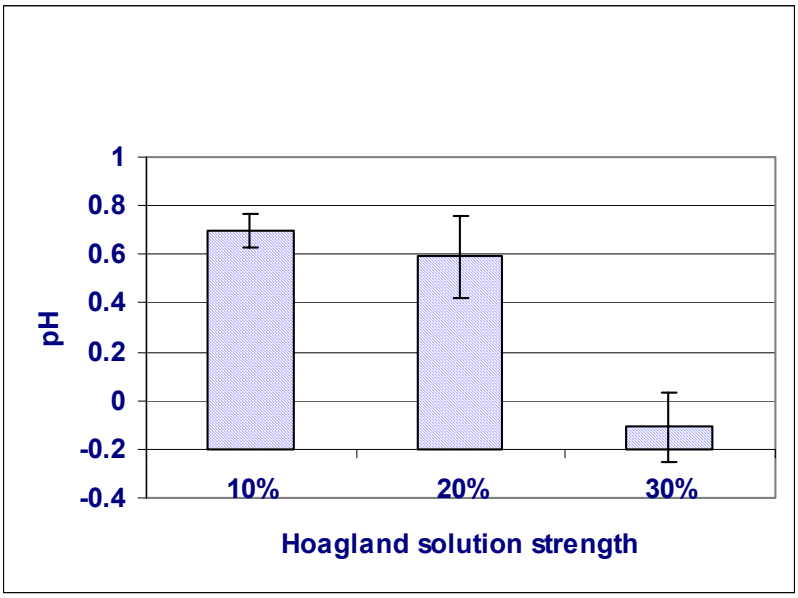


Figure 7-6. Change in solution pH after four weeks of plant growth in 1mgAs/L. Bars are SE of means.

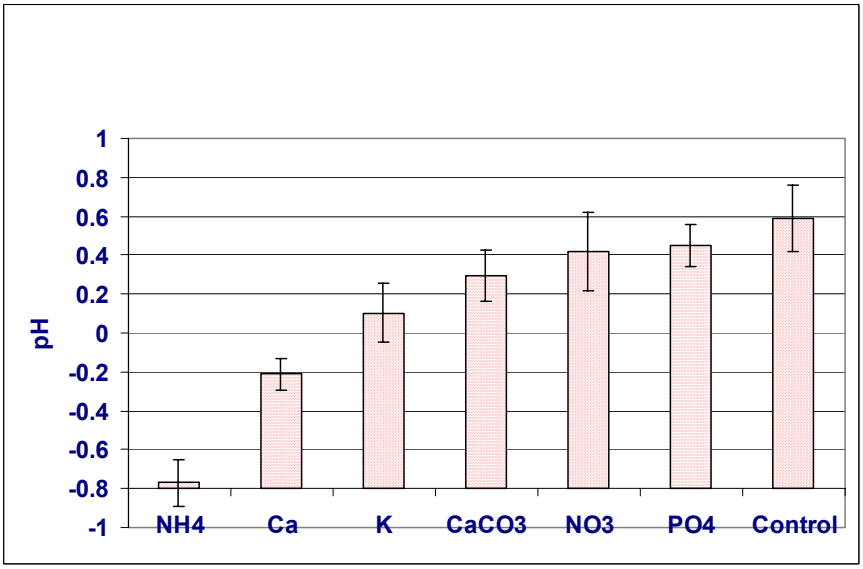


Figure 7-7. Change in solution pH of plants grown in Hoagland solution with added nutrients in 1 mgAs/L. Bars are SE of means.

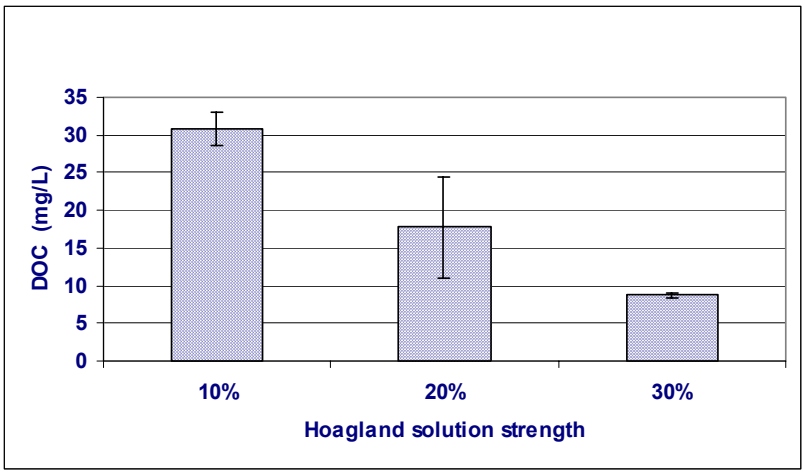


Figure 7-8. Dissolved organic carbon in different Hoagland solution strength. Bars are SE of means.

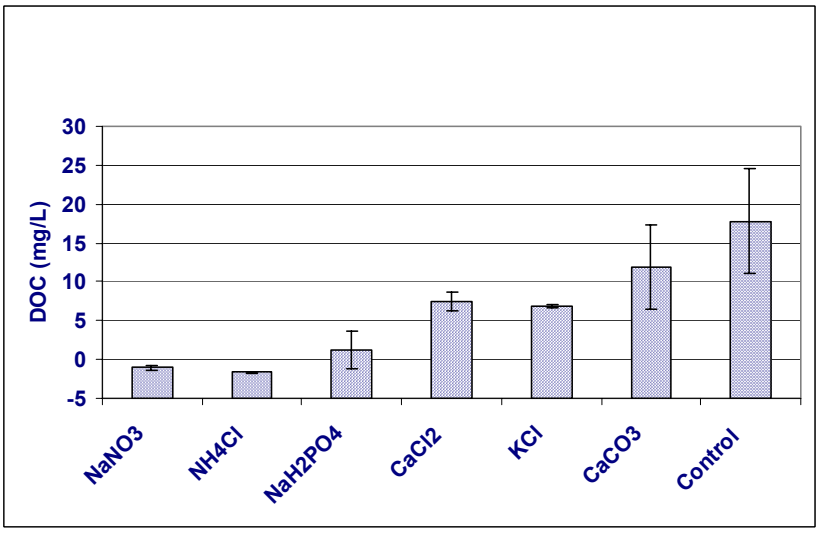


Figure 7-9. Dissolved organic carbon in Hoagland solution with added nutrients. Bars are SE of means.

7.4. Discussion

Arsenic uptake was very little (17 and 46 $mgkg^{-1}$ As) for the unloaded ferns because of the relatively low concentration of arsenic in the water when compared to soil concentrations. Bioconcentration factors of 2392 and 4804 were recorded in this study with preloaded ferns. Bioconcentration factors up to 30 000 was reported in the roots of sunflower plants (Helianthus annus L.) used in the rhizofiltration of uranium from water

contaminated with 21 – 874 μgL^{-1}uranium (Dushenkov et al., 1997). Plutonium bioconcentration factors ranged from 2.07 to 74.74 for shoots and from 16.4 to 698 for roots in Indian mustard and sunflower in nutrient solutions (Lee et al., 2002).

There was a significant increase in arsenic uptake with increasing solution arsenic concentration. This shows that the more arsenic is present in solution the more it was taken up by the fern and confirms that the tolerance mechanism of this fern is not that of avoidance or exclusion but an inner mechanism to detoxify arsenic most probably by chelation by ligands and compartmentalization or sequestration of arsenic away from sites of metabolism in the cytoplasm (Cai and Ma, 2003).

The increase in arsenic uptake observed with increase in calcium concentration at low arsenic concentration may be due to the effect of calcium on root growth that could have helped arsenic uptake. Calcium is known to promote root and leaf development (Follet et al., 1981). At $50mgL^{-1}$ arsenic, there was a decrease in arsenic uptake with increase in calcium concentration because of oversaturation, confirmed by V-MINTEQ that probably led to precipitation on the root. This is possible since calcium; an immobile element was accumulated in the roots of the fern. Accumulation of calcium was also observed in the root zone of rice grown in rhizoboxes and this was attributed to excess supply by mass flow (Hylander et al., 1999).

Potassium accumulated in the fronds of *P. vittata* suggesting that it is more translocated in the plant than calcium and could be mostly associated with arsenic uptake as a counter cation. A recent study investigated arsenic distribution in the fern and reported that arsenic and potassium in the upper epidermis were found to correlate positively (Lombi et al, 2002). No significant correlation was observed between arsenic

and potassium uptake or with calcium uptake in this study. In this study, calcium uptake in the fronds increased with increasing calcium concentration while potassium uptake decreased with increasing potassium in solution. However potassium content of the leaf increased with increasing potassium in solution. Nowak et al. (2002) reported that leaf potassium content of *Nephrolepis exaltata* S. increased with increasing concentration of nutrient solution while leaf calcium concentrations decreased with increasing content of nutrient solution. A negative correlation between calcium and potassium uptake in the roots of *P. vittata* suggests an antagonistic relationship between these two nutrient elements in the *P. vittata*.

Comparing the effect of various nutrients, addition of calcium carbonate to the nutrient solution significantly increased plant biomass and arsenic uptake above the other nutrients. Though the transfer factor of the calcium carbonate treatment and the nitrate treatment were not significantly different implying that both could be used to facilitate arsenic uptake of *P. vittata* in water. Though the greatest reduction in solution arsenic occurred with the calcium carbonate treatment. This is very good for the phytofiltration of arsenic contaminated water in areas where limestone is present in the rock strata.

Though the less the Hoagland solution strength, the more the reduction in solution arsenic, 20% Hoagland solution appears to be the best for our laboratory experiments though the high variability in data did not make this very clear.

Nitrogen nutrition has been said to be responsible for cation/anion uptake ratio that greatly affects rhizosphere pH (Marschner and Romheld, 1983). The result of our experiment with the two different nitrogen sources also confirms this report. *Pteris vittata* fed with the nitrate source increased solution pH while with ammonium there was a decrease

in solution pH. In the soil, fertilization of plants grown on arsenic contaminated soil with nitrate as the nitrogen source potentially increases rhizosphere pH and probably enhances arsenic accumulation in plant tissues (Fitz and Wenzel, 2002).

The presence of dissolved organic carbon in Hoagland solution that has only inorganic carbon present is an indication of root exudation of carbon containing low and high-molecular-weight solutes. Root exudation could be caused or increased by various forms of stress such as mechanical impedance, anaerobiosis, drought, and mineral nutrient deficiency (Marschner, 1995). Release of root exudates in this experiment appears to be due to stress from deficiency of the various nutrients since the control without any added nutrient had the highest concentration of dissolved organic carbon. The nitrogen fed ferns with the least dissolved organic carbon was the least stressed suggesting that nitrogen is very critical to proper nutrition of the fern. Phosphorus is the second important nutrient for the fern, if applied at rates that will not inhibit arsenic uptake. The data also confirms an earlier experiment that showed that calcium and potassium had about the same effect on the fern nutrition. It seems that calcium carbonate did very little to relieve the fern from stress compared with the other nutrients. I think it was able to facilitate arsenic uptake by increasing CO_2 availability to the roots thereby increasing plant biomass that also led to more arsenic taken up by the plant.

7.5. Conclusion

Pre-exposure to arsenic increased the ability of *P. vittata* to accumulate arsenic from the solution containing the higher arsenic concentration (e.g., 2 mg L^{-1}). However, its ability was reduced when growing in a solution containing lower arsenic concentration (e.g., 0.3 mg L^{-1}). Regardless of arsenic concentrations, more K was concentrated in the fronds while more Ca in the roots. Greater Ca hindered arsenic translocation in *P. vittata*

when arsenic concentrations were increased from 10 to 50 mg L^{-1} while K increased arsenic translocation. Calcium carbonate facilitated plant growth and arsenic uptake in *P. vittata.* Low DOC in solutions with nitrogen treated ferns suggests that this nutrient is very critical to the growth of the fern, which would also facilitate arsenic uptake.

CHAPTER 8
SUMMARY AND CONCLUSIONS

8.1 Summary

The effects of Cd, Ni, Pb, and Zn on arsenic accumulation by *Pteris vittata*, an arsenic hyperaccumulator, were investigated in a greenhouse study. *P. vittata* was grown in an arsenic-contaminated soil (131 mg kg^{-1} arsenic), which was spiked with 50 or 200 mg kg^{-1} Cd, Ni, Pb, or Zn (as nitrates) for eight weeks. *P. vittata* was effective in taking up arsenic (up to 4,100 mg kg^{-1}) and transporting it to the fronds, but little of the metals. Arsenic bioconcentration factors ranged from 14 to 36 and transfer factors ranged from 16 to 56 in the presence of the metals. Both were reduced with increasing metal concentration. Fern biomass increased as much as 12 times compared to the original dry weight after 8 wks of growth (up to 19 g per plant). Greater concentrations of Cd, Ni, and Pb resulted in greater catalase activity in the plant. Most of arsenic in the plant was present as arsenite, the reduced form, indicating little impact of the metals on plant arsenic reduction. This research demonstrates the capability of *P. vittata* in hyperaccumulating arsenic from soils in the presence of heavy metals.

Understanding how plant arsenic removal impacts arsenic distribution in the soil can be used to enhance the efficiency of phytoremediation. Chemical fractionation of the arsenic-contaminated soil spiked with various metals (used in the former experiment) was performed before and after 8 weeks of plant growth based on sequential extractions using NH_4Cl (water-soluble plus exchangeable, WE-As), NH_4F (Al-As), NaOH (Fe-As), and H_2SO_4 (Ca-As). Arsenic in the soil was present primarily as the recalcitrant forms with

WE-As being less than 10% in the soil. Fractionation analysis before and after plant arsenic uptake in the control soil with no metals spiked showed that Ca-As was the dominant fraction, with Ca-As > Fe-As > Al-As > WE-As. Arsenic was redistributed into more available forms after plant arsenic uptake with percentages of WE-As, Al-As, and Fe-As increasing while Ca-As decreased in the soil after 8 weeks of plant growth. The actual concentrations of Al-As and Fe-As were significantly ($P<0.01$) greater in all metal-spiked soils than the control at 8 weeks. Only changes in WE-As in all treatments significantly ($P=0.007$) correlated with plant arsenic removal by the plant after 8 weeks. There was a general reduction in soil pH from 7.6 before plant transfer to 6.78-7.19 after 8 weeks of plant growth in all treatments probably due to production of root exudates. The plant's capability in solubilizing soil arsenic from the recalcitrant fractions may have contributed to its arsenic hyperaccumulation.

The effects of phosphate rock on arsenic and metal uptake by the arsenic hyperaccumulating Chinese Brake fern (*P. vittata*), in the presence of Cd, Pb and Zn, were examined in a greenhouse experiment. Five treatments were used, including 1) control with no arsenic, 2) spiked with 50 ppm As, 3) spiked with 50 ppm As and 50 ppm P as rock phosphate (AsP), 4) spiked with 50 ppm As, 50ppm Pb, Cd, and Zn (AsM), 5) spiked with 50 ppm As, 50 ppm Pb, Cd, and Zn, 50 ppm P as rock phosphate (AsMP). Chinese brake fern was able to grow in a soil spiked with cadmium, zinc and lead at 50 mg kg^{-1}. Phosphate rock increased both arsenic and calcium availability in the soil. Phosphate rock significantly increased arsenic uptake and reduced lead uptake by *P. vittata* in the multi-metal system. Arsenic uptake by the fern was positively correlated with both total and available arsenic in the soil. Phosphorus uptake was significantly

lower in the arsenic treated soils whereas Ca uptake was significantly higher in arsenic treated soils. Phosphate rock facilitated arsenic uptake in the multi-metal system showing that it can be used in the phytoremediation of arsenic-metal polluted soils as a cost-effective soil amendment

A greenhouse study was again conducted to evaluate and compare arsenic uptake from different arsenic contaminated soils by two arsenic hyperaccumulators, *P.vittata* and *P.cretica*. These plants were grown for six weeks in golf course, chromated copper arsenate (CCA), cattle dip vat, mining and an uncontaminated soil. *P. vittata* had significantly higher aboveground and total biomass than *P. cretica*. *P. vittata* also had higher As uptake than *P. cretica* except in the golf course soil. Arsenic uptake in both fern types followed the trend CCA>Mining> Cattle dip vat>Golf course>control. In arsenic contaminated soils used in this study, P/As molar ratio in the fronds ranged from 80 to 939 in *P. vittata* and from 130 to 421 in *P. cretica*. Plant arsenic concentrations were significantly positively correlated with Mehlich-3 arsenic in the soil. Soil pH was also significantly correlated with Mehlich-3 (available) arsenic before and after plant transfer. Plant As uptake was significantly correlated with exchangeable potassium in the soil before plant transfer. GSH availability is not implicated as a detoxification mechanism in these ferns. The ability of *P. vittata* to accumulate As and tolerate metal toxicity better than *P. cretica* makes it a better candidate for phytoremediation of arsenic contaminated soils.

The effectiveness of two *Pteris* species (As hyperaccumulators) to take up arsenic from different sources was evaluated in a hydroponic set-up, with two plant species, two arsenic species and concentrations. *P. vittata* was more efficient than *P. cretica* with

significantly greater arsenic accumulation and translocation. The percentage of As III increased from below detectable limits to 83% in the fronds of *P. vittata* as arsenic exposure increased from 1-d to 15-d, indicating arsenic reduction from AsV to AsIII in the fronds of *P. vittata* did not occur appreciably after 1-day exposure. Changes in arsenic accumulation and speciation with time between two fern species, hyperaccumulator *P. vittata* and non-hyperaccumulator *N. exaltata,* were evaluated in another hydroponics experiment. *P. vittata* took up more arsenic than the non-hyperaccumulator, *N. exaltata*. *P. vittata* accumulated arsenic in the fronds while *N. exaltata* accumulated it in the roots.

Pre-exposure to arsenic increased the ability of *P. vittata* to accumulate arsenic from solution containing higher arsenic concentration (e.g. 2 mg L^{-1}). However, its ability was reduced when growing in a solution containing lower arsenic concentration (e.g. 0.3 mg L^{-1}). Greater Ca hindered arsenic translocation in *P. vittata* when arsenic concentrations were increased from 10 to 50 mg L^{-1} while K increased arsenic translocation. The greatest reduction in solution arsenic occurred when calcium carbonate was added to the solution. This is very good for the phytofiltration of arsenic contaminated water in areas where limestone is present in the rock strata. *P.vittata* fed with the nitrate source increased solution pH while with ammonium there was a decrease in solution pH. Low DOC in solutions with nitrogen treated ferns suggests that this nutrient is very critical to the growth of the fern, which could also facilitate arsenic uptake. The nitrate fed ferns significantly took up more arsenic than the ferns fed with the other nutrients. Results from this study should help to optimize arsenic removal by *P. vittata* in a hydroponic system.

8.2 Conclusion

Pteris vittata hyperaccumulated arsenic and grew in the presence of other toxic metals though its hyperaccumulating efficiency was reduced. Addition of phosphate rock to a mixed-metal-arsenic treated soil enhanced arsenic uptake by the fern. Phosphate rock is recommended as an effective soil amendment for phytoremediation of arsenic contaminated soils especially in arsenic–metal polluted soils. *P. vittata* took up more arsenic than *P. cretica* in the different arsenic contaminated soil types except the golf course soil. Arsenic uptake of both fern types in water was dependent on source and dose of arsenic applied. Pre-exposure to arsenic facilitated arsenic uptake in high arsenic solution concentration (2mg/L). There were no statistical differences in the effect of calcium and potassium on arsenic uptake in *P. vittata*. There was a significantly higher reduction in solution arsenic when calcium carbonate was added to the solution. This is very good for the phytofiltration of arsenic contaminated water in areas where limestone is present in the rock strata.

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BIOGRAPHICAL SKETCH

I am the last to be born into a family of six kids (not by my parents plan but by God's divine purpose). I started my education at a very early age and had my primary, secondary and higher education in Nigeria. I graduated from the University of Ibadan, Nigeria in 1987 with a bachelor's degree in Education (chemistry, biology) and completed a Master's degree in Science Education in 1989. I taught Integrated Science for two years at the International School, University of Ibadan, Ibadan, Oyo state, Nigeria. I got another job at the Federal College of Forestry under the Forestry Research Institute of Nigeria in 1991 as a Chemistry lecturer. I went back for another master's degree in Soil Science at the University of Ibadan in other to be more relevant and productive at my job and finished in 1998. I moved to the United States in December 2000 as a permanent resident and was admitted to the University of Florida (Gainesville) to do a PhD in soil and water science in fall 2001.