THE EFFECT OF ARSENIC SPECIATION ON ARSENIC UPTAKE AND FATE IN THE PRESENCE OF THE HYPER-ACCUMULATING SPECIES *PTERIS CRETICA*

by

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THE EFFECT OF ARSENIC SPECIATION ON ARSENIC UPTAKE AND FATE IN THE PRESENCE OF THE ARSENIC HYPER-ACCUMULATING SPECIES *PTERIS CRETICA*

Ana Maria Benavides, M.S.

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Understanding arsenic uptake mechanisms employed by the arsenic hyper-accumulator *Pteris cretica* is important to optimize its arsenic accumulation capability to further improve the treatment process for small-scale or point of use applications in the developing world.

Since most of the approaches for arsenic control using Phytoremediation as a treatment technology that have been conducted using arsenate as arsenic source[26], the present study was carried using arsenite to determine the role of speciation in arsenic uptake by the hyper-accumulator *Pteris cretica*.

It was determined that trivalent arsenic oxidation plays an important role on the overall arsenic uptake. When trivalent arsenic was added to the aerated solution, only about 50% of the initial arsenite concentration was oxidized to arsenate regardless the exposure time. On the other hand, in the presence of *Pteris* ferns, arsenite is completely oxidized as it is being removed from the solution. In order to further elucidate the fate of arsenite in the presence of plant roots, experiments were conducted with *Boston* fern (*Nephorlepis exaltata)* a non hyper-accumulating fern that is sensitive to arsenic [35]. It was observed that the total concentration of arsenic in solution remains constant but arsenite is converted to arsenate.

Given that these plant species do not have the ability to oxidize arsenite [24], our hypothesis is that the microbial community of the rhizosphere is responsible for arsenite oxidation as a symbiotic relationship with the plant, which has never been evaluated by other groups working with this technology.

Studies in the presence of non-accumulating Boston fern were conducted to evaluate this hypothesis by adding an antibiotic cocktail to the arsenic solution together in an effort to suppress microbiological activity in the rhizosphere and a reduction in arsenite oxidation rates was observed.

These results provide evidence of a symbiotic relationship between arsenic oxidizing microorganisms and arsenic hyper-accumulating plants, and suggest that the oxidation step is crucial on the arsenic uptake process when arsenic is present as arsenite.

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1.0 INTRODUCTION

Water supplies in many regions of the world are contaminated by the carcinogenic metalloid arsenic (As). Though the U.S. Environmental Protection Agency (EPA) recently reduced the Maximum Contaminant Level (MCL) from 50 μg/L to 10 μg/L, water in some developing countries exceeds this standard. Therefore, highly efficient, sustainable technologies that require low maintenance and have low capital costs must be developed.

Phytoremediation is an emerging treatment technology that relies on plants to remove unwanted substances from water or soil. In some cases, a plant plays host to symbiotic bacteria that facilitate the conversion of a contaminant to its less toxic form. In other cases, a contaminant is sequestered by adsorption to its root surface, translocated and volatized for release into the air or stored in its upper biomass.

Several *Pteris* fern species are effective at removing arsenic from soil and water and the mechanism of arsenic hyper-accumulation is of great interest because to most plants, inorganic arsenic species are very toxic. Despite the fact that trivalent arsenic species can be about ten times more toxic than pentavalent species[42], most of the approaches for arsenic control have been conducted using arsenate as arsenic source [26].

2.0 LITERATURE REVIEW

2.1 ARSENIC IN NATURAL WATERS

2.1.1 Arsenic in the environment

Arsenic is present in different concentrations in the environment, with natural levels of arsenic in sediments usually ranging between 3 and 10 mg/kg [29] depending on the mineral. Soil concentrations tend to be higher than those in rocks and sediments because of soil heterogeneity and, it has been estimated that global arsenic concentration in soils is about 5 mg/kg [29]. These, concentrations may be affected by several factors, including composition and electrochemical properties of the soil.

Water is the most important means of arsenic transport in the environment. Arsenic predominates in aquatic environments in places with high geothermal activities. Soil erosion and leaching contribute between 612 and 2380 $*10^8$ g/yr to the oceans[42].

Several anthropogenic activities, including coal combustion, irresponsible disposal of mine tailings, glassware and ceramic industries, petroleum refining, dyes and pesticides contribute to elevated arsenic levels in the environment. One of the most important sources of arsenic contamination is leaching of naturally occurring arsenic into water aquifers resulting in runoff of arsenic into surface waters.

The interactions between water and atmospheric precipitation, soil erosion and leaching, volcanic emissions, agricultural runoff, and industrial effluents are key processes affecting arsenic distribution into the aquatic system. Figure 2.1 is a schematic of arsenic cycle, where different transformations among air, water and land can be observed.

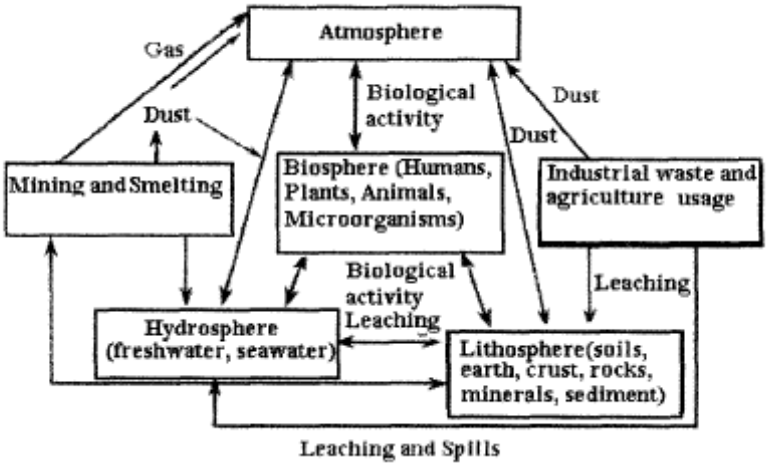


Figure 2.1 Arsenic Cycle [29]

Organic arsenic forms are usually produced through biological activity in surface waters and water impacted by industrial pollution [31].In natural aquatic environments, arsenic species can either bound to sediments or be absorbed by microorganisms. Once absorbed, this species can be metabolically converted to organorasenicals by reduction to arsenite and addition of methyl groups [42].

2.1.2 Arsenic contamination

Arsenic is a metalloid of well known toxicity and is widely distributed in the environment as it is the twentieth most abundant element in the earth's crust [7], fourteenth in seawater and the twelfth most abundant in the human body [29]. It can be found in the atmosphere, soils and rocks, water, and organisms in different chemical compounds, both inorganic and organic. It is

mobilized by a series of natural processes such as weathering reactions, biological activity, volcanic emissions and anthropogenic activities [31].

Arsenic contamination of fresh waters has been reported in different places around the world with typical concentrations below 10 μg/l [31].Although water supplies in the U.S are not characterized by high arsenic concentrations, a survey conducted in 1985 by the American Water Works Association (AWWA) for inorganic contaminants unveiled 43 violations for arsenic with concentrations ranging from 0.52 to 0.190 mg/l [42] especially in northern and southern areas of the western U.S. [11]. As can be observed in Figure 2.2, a number of areas with high arsenic contamination of groundwater have been located around the world, especially in Argentina, Chile, Mexico, China, Hungary, Romania, Taiwan, and recently in Bangladesh, West Bengal, and Vietnam. Arsenic associated with geothermal activities has also been identified in several areas, including Japan, New Zealand, Kamchatka, Iceland, France, and El Salvador. Mining related arsenic contamination has also been identified in different parts of the world, including Ghana, Greece, Southwest England, Thailand, and the U.S.

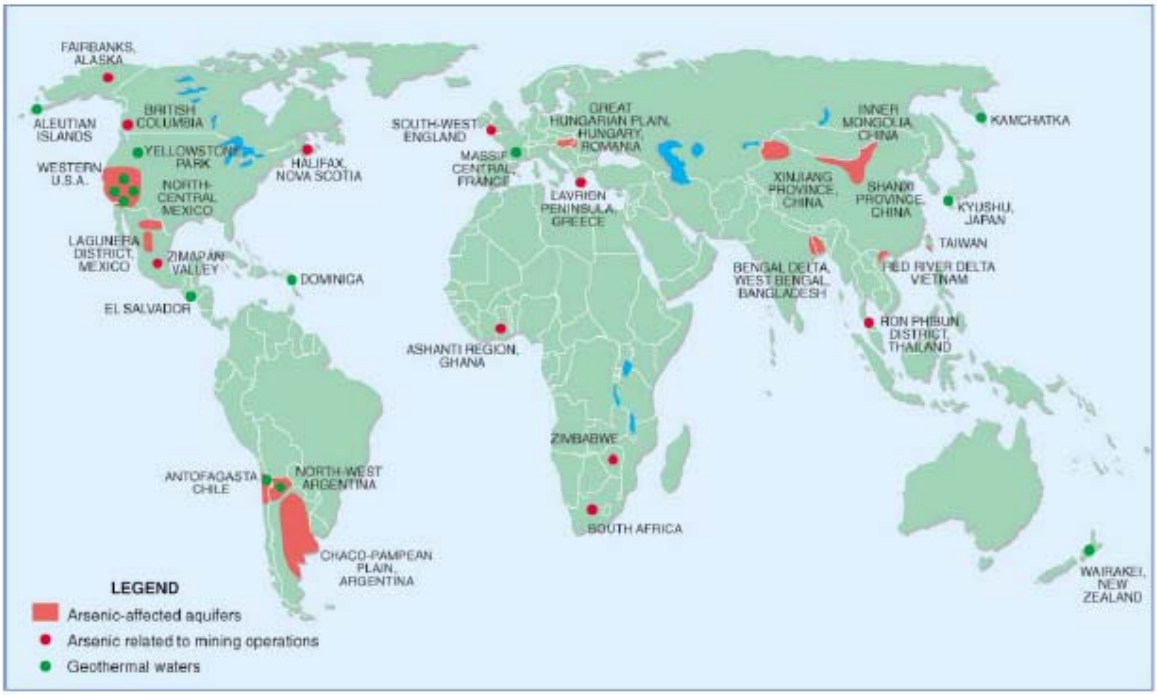


Figure 2.2 Arsenic contamination around the world [31]

2.1.3 Arsenic Chemistry

Arsenic (atomic number 33) is frequently associated with metal ores of copper, lead and gold and it occurs in several oxidation states (-3, 0, +3, +5) under different redox conditions. Arsenate (As V) is the pentavalent form of arsenic, and it exists as AsO_4^{-3}, $HAsO_4^{-2}$, and $H_2AsO_4^{-}$. Arsenite (As III), the trivalent form of arsenic commonly occurs in the following species $As(OH)_3$, $As(OH)_4^{-}$, $AsO_2(OH)^{-2}$, and AsO_3^{-3}.[42] In general, pentavalent species predominate in oxygen rich environments while trivalent species dominate under reducing conditions, such as groundwater [42].

Methylated or "organic arsenic" species are typically detected in natural waters at concentrations below 1μg/l. Consequently, organic arsenic forms are not considered to be of significance for drinking water treatment[42].

Redox potential and pH are two essential factors that determine arsenic speciation, influencing the occurrence, distribution and mobility of arsenic compounds. Figure 2.3 illustrates the distribution of aqueous arsenic species and its relationship with pH and Eh. Under oxidizing conditions, $H_2AsO_4^-$ dominates at low pH, while at high pH, $HAsO_4^{-2}$ is the dominant form. Under reducing conditions and at pH below 9.2, uncharged arsenite species H_3AsO_3 dominate [31].

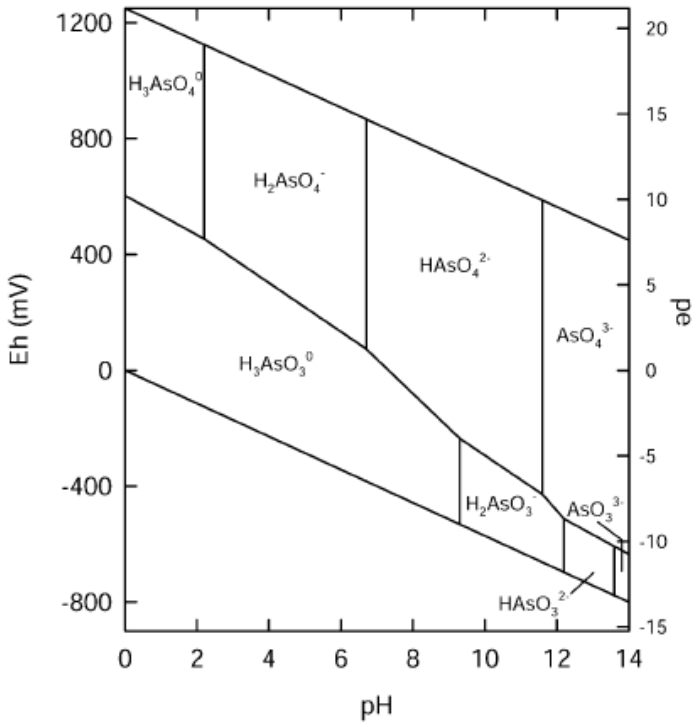


Figure 2.3 Eh-pH diagram for aqueous As species at 25˚C and 1 bar total pressure.[31]

2.1.4 Health impacts of Arsenic

Both trivalent and pentavalent forms of arsenic are toxic, but the mechanisms of action can differ. Arsenite interrupts protein functions by binding the sulfhydryl groups but it does not act as mutagen, while arsenate interferes with the oxidative phosphorilation and ATP synthesis

[28].The toxicity of arsenic depends on its oxidation state, chemical form and solubility. Trivalent species can be about ten times more toxic than pentavalent species; the toxicity scale decreases as follow: arsine > inorganic As(III) > organic As(III) > inorganic As(V) > organic As(V) > arsonium compounds and elemental arsenic [42].

Arsenic can cause acute and subacute poisoning that can be recognized by nausea, vomiting, diarrhea, erythema, tingling of extremities, and nervous weakness. Long term arsenic exposure can result in chronic arsenic intoxication known as arsenicosis, which may harm respiratory, digestive, renal, and circulatory systems [29]. Other clinical effects of arsenic poisoning include damage of the peripheral nerve bodies, melanosis, hyperkeratoisis and circulatory disorders.

When ingested, arsenic distributes throughout the internal organs of the body. The lethal range of inorganic arsenic in the human adult has been estimated to be 1- 3 mg arsenic/kg [29]. The most documented effect of chronic arsenic intoxication is its carcinogenicity. Some studies have demonstrated that arsenic is a carcinogen usually associated with skin, lung and bladder cancers [28]. As a result, arsenic has been recognized as a Class I human carcinogen, and it is a major concern due to its extensive use.

2.1.5 Regulation History

Due to the carcinogenicity and other adverse health effects associated with arsenic and its compounds, regulatory agencies have set a Maximum Contaminant Level (MCL) for drinking water with the purpose of protecting consumers from effects of long term and chronic exposure to arsenic.

Health and regulatory agencies have had a position regarding arsenic in drinking water since 1958. The World Health Organization (WHO) included arsenic in the category of toxic substances and the allowable concentration was established as 200 μg/L at that time. In 1963, arsenic was included under the same category, but had a more severe limit of 50 μg/L established. [45] In 1974, the Safe Drinking Water Act (SDWA) introduced maximum contaminant levels (MCL) as national drinking water standards, where Environmental Protection Agency (EPA) was designated as the agency responsible for periodically reviewing these standards. In 1975 EPA regulated the maximum arsenic level of 50 μg/L in drinking water.

Studies conducted on the carcinogenicity of arsenic and its long term health effects and the fact that arsenic compounds were classified by the International Agency for Research on Cancer (IARC) as recognized human carcinogens in Group I [45] have driven a reassessment of the arsenic MCL of 50 μg/L. As a result, WHO established 10 μg/L as a provisional guideline for arsenic in drinking water in 1993. EPA promulgated a revised standard to set the new MCL for arsenic of 10 μg/L in 2002. Water distribution systems were required to comply with this standard by January 23, 2006 [11].

Therefore, new drinking water treatment alternatives must be considered to include new treatment systems or retrofit existing facilities to comply with the latest regulations for drinking water.

2.2 DISTRIBUTION OF ARSENIC SPECIES IN WATER BODIES

Concentration of arsenic in water bodies varies depending on arsenic source and availability, the geochemical environment, the past and present hydrogeology. Under natural conditions, the

highest concentrations of arsenic are commonly found in the groundwater environment resulting from water-rock interactions. High arsenic concentrations have been found under both oxidizing and reducing conditions; therefore these conditions are not necessarily related to areas of high arsenic concentration in the rocks [31].

The overall redox potential of natural waters is often controlled by major elements with arsenic redox equilibrium responding to the changes in oxygen, carbon, nitrogen, sulfur and iron. Although thermodynamic equilibrium calculations predict the predominance of arsenate (AsV), in all but strongly reducing conditions, such theoretical behavior is not often followed. This is because different redox couples in natural waters can affect the redox potentials and lead to thermodynamic disequilibrium and ultimately modify the time periods required for changes between oxidation states [31].

Different studies have documented stability of Arsenate (AsV)/ Arsenite (AsIII) ratios over long periods of time, ranging form several days to a year, suggesting relatively slow oxidation rates of arsenite (AsIII) [31]. The rates of arsenite oxidation and arsenate reduction in natural environments can be affected and controlled by microorganisms. One example of this was documented in southwestern U.S.A., where arsenite (AsIII) in geothermal waters input to streams was rapidly oxidized with a pseudo first order half-life as short as 0.3 hours, which was attributed to bacterial intervention [31].

2.2.1 Water in Bangladesh

The contamination of groundwater by arsenic in Bangladesh represents one of the largest poisonings in history in terms of the population exposed. Surface water sources in Bangladesh have been historically contaminated with microorganisms, producing considerable rates of

disease and mortality. During the 1970's the United Nations Children's Fund (UNICEF) in a combined effort with the Department of Public Health Engineering to reduce the incidence of water-borne diseases, installed tube-wells that were drilled into the ground at a depth less than 200m, covered and outfitted with a cast iron or steel hand pump to encourage the use of groundwater as drinking water supply. At the time those tube-wells were installed arsenic was not recognized as a drinking water problem; thus standard testing procedures did not include tests for arsenic[33].

During the 1990's, a number of studies revealed that 30-35 million people in Bangladesh and about 6 million people in West Bengal were exposed to arsenic concentration above 50μg/L[31]. The contaminated aquifers are generally 100-150 meters deep and contain sand, silts and clay sediments deposited by the Ganges, Brahmaputra and Meghna rivers. These aquifer sediments are covered by a layer of clay and silt that restricts the oxygen transfer to the aquifers. The combination of the restricted oxygen and the presence of organic matter results in an environment with highly reducing conditions that facilitates arsenic mobilization[31]. As indicated in Figure 2.4, the geographic distribution of arsenic in groundwater is very heterogeneous as a consequence of the diverse sedimentary characteristics in the area as well as the depth of the wells.

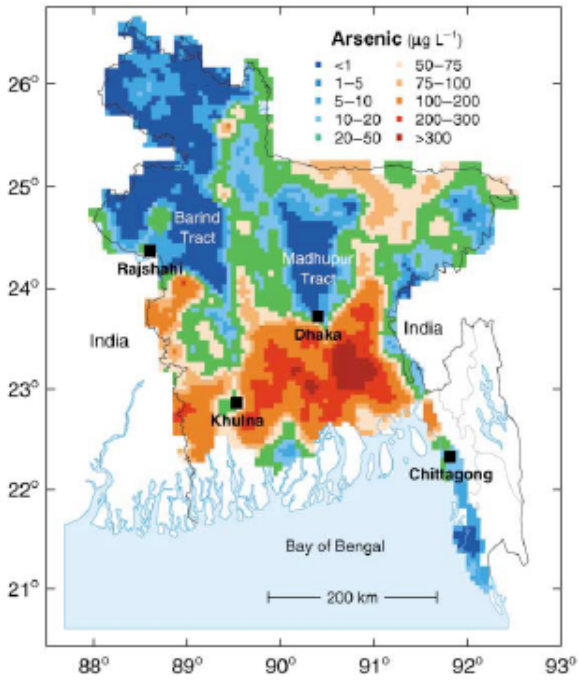


Figure 2.4 Arsenic distribution in groundwater in Bangladesh [31]

A survey conducted of Bangladesh tube-wells estimated that the levels of total arsenic concentration vary from 2.5-846 µg/L in the groundwater samples. Although arsenate was the predominant form of arsenic in some wells, arsenite was the predominant form of arsenic in most wells, representing 67-99% of total arsenic in these waters. Moreover, it was reported that maximum arsenic concentrations is found at depths between 20 and 50 meters while samples shallower than 10 meters and deeper than 150 meters were essentially free of arsenic[1].

2.3 ARSENIC REMOVAL METHODS

Several treatment processes for arsenic removal have demonstrated their effectiveness. These methods can be divided into different categories depending on the mechanisms used: coagulation/filtration, lime softening, adsorption, ion exchange, activated alumina and membrane

technologies [14]. However, these alternatives can generate large volumes of hazardous waste and are generally characterized by high capital costs as well as operation and maintenance costs, and involve chemical handling during the process. The main concern regarding these technologies is the economic viability, especially for the developing world [41].

Table 2.1 presents a list of arsenic removal technologies and their maximum removal rates as determined by the U.S. EPA.

Table 2.1 Treatment Technology Maximum Achievable Removal Percentages

Adapted from [10]

Treatment Technology	Maximum percent removal
Coagulation / filtration	95
Enhanced Coagulation/ Filtration	95
Coagulation Assisted Microfiltration	90
Lime Softening (pH> 10.5)	90
Enhanced Lime Softening (pH> 10.5)	90
Ion exchange (sulfate , 50 mg/L)	95
Activated Alumina	95
Reverse Osmosis	>95
Greensand Filtration (20:1 iron:arsenic)	80
POU Activated Alumina	90
POU Ion Exchange	90

An emerging technique known as phytoremediation, which consists of using plants to remove undesired substances from contaminated sites, appears to be promising for arsenic removal, especially due to its low cost.[4, 41]

Initial research on phytofiltration of contaminants from water was initiated with the studies of wetlands for water purification; however, in this case precipitation of contaminants into sediments made the recovery of contaminants problematic. Later investigations focused on the ability of aquatic plants to remove metals from water and the results were promising, especially in the treatment of uranium, lead and cesium from contaminated waters [14]. More recently, an arsenic hyper-accumulating fern (*Pteris Vittata L.*) has been identified by scientists

at the University of Florida and has been used by different research teams in an effort to comprehend the factors affecting arsenic phytofiltration [20].

2.3.1 An Arsenic Hyperaccumulating Fern

An abandoned wood preservation site in Central Florida was investigated whereby, soil and plant samples from this chromated copper arsenate contaminated site were analyzed. During the course of the study, 14 plant species were analyzed and it was discovered that the species *Pteris vittata L.* (Chinese Brake fern) contained extremely high arsenic concentrations when compared to other species [20]. The analyzed Brake fern originating from the contaminated site contained about 1,440 - 7,520 mg/kg of arsenic, while Brake ferns from uncontaminated site contain 12 - 64 mg/kg of arsenic. Both arsenic concentrations were elevated in comparison with other plants growing at uncontaminated sites that usually contain less than 3.6 mg/kg of arsenic [20].

Ma et al. 2001 determined that *Pteris vittata* had the ability to tolerate soil conditions containing arsenic levels of up to 1500 mg/kg through a mechanism that successfully translocates arsenic from the roots to the fronds. Laboratory experiments confirmed that Brake ferns planted in soil containing 1500 mg/kg of arsenic experienced from 29 to 15,860 mg/kg of arsenic concentration in the fronds within a two week period, while the concentration in the roots was reported to be considerably lower and never surpassed 303 mg/kg [20].

Different physiological studies have suggested that metal hyper-accumulation is an adaptive response to the environment and it is believed that the ecological significance of hyper accumulation is for plant defense [28].It has also been suggested that early terrestrial plants might have evolved in the presence of hot springs, which contained elevated arsenic levels. As a result, ferns may have had to survive with high levels of arsenic and thereby developed a hyper-

accumulation mechanism with the additional benefit of obtaining protection from insect pests, disease, and herbivores[21].

Hyper-accumulating plants usually have several advantages, including their ability to support rapid growth, efficient root uptake system, and root to shoot translocation [43]. In particular, *Pteris vittata* is a mesophyte with worldwide distribution, especially in warm and tropical areas. In the US, it natively grows in southeast and southern California [28]. *Pteris vittata* is not the only recognized arsenic hyper-accumulator. Different studies have demonstrated that other *Pteris* plant species hold the ability of hyper-accumulate arsenic, including *Pteris cretica, Pteris longifolia, Pteris umbrosa, Pteris multifida,* and *Pteris oshimensis* [43, 46].

2.3.2 Arsenic uptake and transport

Arsenic is a nonessential element for plants. At high concentrations it can interfere with metabolic pathways, inhibit growth and even lead to death. Inorganic arsenic is extremely toxic to most plant species. Therefore, the mechanism of arsenic hyper-accumulation has been widely studied since its understanding could improve its features for phytoremediation [36, 37].

Since arsenic is electronically similar to phosphorus, it has been hypothesized that arsenate is taken up by the phosphate transport system in the plant because it might be difficult for the plant to differentiate between arsenate and phosphate [28, 44]. In addition, after entering the plant, arsenate may substitute phosphate in ATP synthesis, which will interfere with the metabolic pathways causing toxicity for the plant [37]. Competition between arsenate and phosphate has also been observed in various species including plants, fungi and bacteria [28].

Arsenate uptake and plant toxicity depend on phosphorus/arsenic ratio (P/As) and phosphate nutrition. Different studies have reported that arsenate reduces phosphate uptake in the

plants, even if the uptake system has higher affinity for phosphate[37]. A molar P/As ratio of 12 is recommended to protect plants against toxicity. However, it has been demonstrated that at the same P/As ratio arsenate is less toxic at high phosphate concentrations because at low phosphate concentration more arsenate is taken up by the plants[37].

Confirmation of the hypothesis on arsenic uptake mechanism was provided by Poynton et al.[26] The use of radio-labeled arsenic allowed them to prove that phosphate inhibited arsenic uptake in a competitive way. Poynton also showed that arsenate influx in *Pteris vittata* and *Pteris cretica* was considerably larger than in *Nephrolepis exaltata* (Boston fern), a non hyper-accumulating species that is sensitive to arsenic. It was also concluded that arsenic is taken up by means of the phosphate transport system.

Laboratory studies revealed that growth enhancement of *Pteris vittata* can be achieved at moderate doses of arsenate (< 200 mg/Kg) since these arsenic amounts caused an increase on phosphorus concentration in the fronds[38]. It was also observed that at low arsenic levels (<0.5 mg/Kg), arsenic was concentrated in the younger fronds, while at moderate to high arsenic levels (>0.5 mg/ Kg) arsenic concentrates in the older fronds. These experiments demonstrated that arsenic acts as a nutrient in *P. vittata* and its transport is favored in young plant tissues[36, 38]. When exposed to high arsenic concentrations, *Pteris vittata* did not show signs of phosphate deficiency, which suggested that this plant is able to take up and use phosphate even in the presence of arsenic[37].

It has been reported that phosphorus deficiency increases the ability of phosphate uptake in the roots. In a similar experiment, it was demonstrated that when *Pteris vittata* is nutritionally deprived of phosphorus for eight days, arsenate influx increases 2.5 fold [44]. Thus, the plant

responds to phosphorus deficiency by synthesizing additional transporter molecules in the plasma membranes of root cells which ultimately enhance phosphate or arsenate uptake[44].

Other arsenic species including arsenite are also known to be accumulated by the *P. vittata* fern. [20, 22] However it has been shown that arsenite influx is considerably low, occurring at a rate about one tenth of arsenate influx [44]. It has been hypothesized that arsenite is oxidized to arsenate before being taken up by the fern [20].However, the exact arsenite uptake mechanisms remain unclear to date.

2.3.3 Factors influencing arsenic uptake

Since the discovery of arsenic hyper accumulating ferns, the concentration of arsenite (AsIII) has been reported to be greater in the fronds than in the roots.[20] Confirmation of the findings of Ma et al., was performed by using X-ray absorption near edge spectroscopy analyses (XANES) to determine the physiological speciation of arsenic and its location within the plant. Lombi et al. (2002) reported that about 75% of the arsenic present in the fronds was in the trivalent oxidation state[19].

Additional studies confirm that arsenite is the predominant form of arsenic in the fronds of *P. vittata* [12, 44] while arsenate predominates in the roots. From these observations, it is deduced that arsenate reduction may occur in the fronds [40].Corroboration of the previous hypothesis was provided by a study conducted by Pickering et al. (2006), with the use of X-ray absorption spectrometry (XAS). Through XAS imaging, it was possible to verify that the roots predominantly contain arsenate (90 ± 4%) while the leaves predominantly contain arsenite (95 ± 1%). Additionally, the direct visualization of arsenic forms in intact fern tissues allows further

understanding of how arsenic is transported through the plant. It revealed that *P. vittata* transports untransformed arsenate to the fronds where is primarily stored as arsenite[25].

Generally, plants use different intracellular and extracellular mechanisms to enhance nutrient uptake. The external mechanisms include the exudation of substances that can bind the metal ions and change the rizosphere pH. Consequently, this action affects metal speciation or the production of ion exchange sites on the cell walls, both acting to facilitate the uptake [28].

Root exudates are plant metabolites released to the root surface or into the rizosphere, they are classified into two categories, high molecular weight (HMW) and low molecular weight (LMW); the first includes mucilage and ectoenzymes and the second consists of organic acids, sugars, phenols and amino acids [39].

Although root exudates depend on the inherent plant biology and the external environment, the quantity and chemical make up can be different form plant to plant. In order to understand the role of these metabolites Tu S. et al.(2004a) compared the nature of root exudates in *Pteris vittata* fern and the non hyper accumulating specie *Nephrolepis exaltata* L. (Boston fern) on the mobilization of aluminum arsenate, iron arsenate, and chromated copper arsenate. It was found that the predominant components of the root exudates were two LMW organic acids, phytic acid and oxalic acid for both fern species. Citric acid, ascorbic acid, and fumaric acid were also found in smaller proportions[39].Both ferns had comparable phytic acid exudation in the absence of arsenic. On the other hand, the *Pteris fern* exuded 46-106% more phytic acid when exposed to arsenic. Moreover, the *Pteris* fern exuded about 3 to 5 times more oxalic acid than the Boston fern under the experimental conditions used in this study [39].

These root exudates have been observed to dissolve significant amounts of arsenic from minerals. Root exudates from *Pteris* fern mobilized 3-4 times more arsenic from aluminum

arsenate, 4-6 times more arsenic from iron arsenate, and 6-18 times more arsenic from chromated copper arsenate than Boston fern exudates. [39]

On the other hand, microorganisms have been documented to impact mobility and speciation of metals through catalytic reactions that ultimately impact metal bioavailability. Under natural conditions, the majority of plants have symbiotic mycorrhizal fungus that can stimulate nutrient uptake in their host plants. The effect of these associations appears to be specific to each metal and plant[17].

A number of prokaryotes are known for their ability to generate energy from metabolic reactions involving arsenic, some are dissimilatory arsenate respiring organisms and others are heterotrophic arsenite oxidizers that can exist in a wide range of habitats[16, 23]. The microbial processes that directly impact arsenic speciation and mobility include methylation, demethylation, oxidation, and reduction, which are used for detoxification and energy generation. In methylation, arsenate or arsenite is converted into a number of different intermediate methylated compounds, including monomethyl arsenate (MMA(V)), [6] methylarsonite (MMA(III), dimethylarsinate (DMA(V)), dimethylarsinite (DMA(III)), and trimethylarsine oxide; the end products of methylation include volatile arsines, monomethylarsine, dimethylarsine, and trimethylarsine[2, 6, 13, 27].

Due to the elevated arsenic resistance and efficient arsenic accumulation characteristics of the hyper accumulating ferns, it is possible that its rizosphere encloses a specific microbial community that can be connected to arsenic phytoremediation, however the presence of arsenic metabolizing organisms and its association with hyper accumulating plants has not been studied to the date.

3.0 MATERIALS AND METHODS

3.1 REAGENTS AND STOCK SOLUTIONS

Experimental stock solutions and standards were prepared using deionized (DI) water from the environmental lab using a Barnstead™ Nanopure system. All glass and plastic equipment including bottles, beakers, flasks, pipettes, and test tubes were soaked overnight in a polyethylene basin containing 10% v/v Fisher Scientific™ Nitric Acid diluted in DI water, then triple rinsed with tap water and triple rinsed with DI water to prevent interference during chemical analysis.

Arsenate stock solution was prepared by dissolving 0.625 g of sodium hydrogen arsenate (Na_2HAsO_4-$7H_2O$) in a 100-mL volumetric flask to prepare a 1.5g As/L solution. Arsenite stock solution was prepared by pipetting 40 mL of 0.1 N sodium arsenite ($NaAsO_2$) solution in a 100-mL volumentric fask to prepare a 1.5gAs/L solution. Spex CertiPrep™ plasma grade arsenic 1000 ppm standard solution was used to prepare the standards used to calibrate the Atomic Absorption Spectrophotometer (AAS) for arsenic analysis.

Nickel nitrate solution (5%) was prepared by dissolving 24.780 g of Fisher Scientific™ $Ni(NO_3)_2 \cdot 6H_2O$ in 100 mL DI water. This solution was used as a matrix modifier in arsenic analysis.

Arsenic speciation was performed by ion exchange cartridges. A 50% Water-Methanol solution used for cartridge conditioning was prepared by diluting 50 mL of of Fisher Scientific™ Methanol in 50 mL DI water.A 0.5M HCl solution used for cartridge regeneration and elution was prepared by diluting 1.82 ml of Fisher Scientific™ HCl in 100mL DI water.

The culture media used for microbiological analysis was prepared by dissolving 23.5 g of Difco plate count agar in 1 L of DI water; the mixture was placed on a hot plate and continuously agitated with a magnetic stirring bar and pH was adjusted to neutral while stirring. Previously cleaned tubes were filled with approximately 10 mL of the warm media and covered with cotton balls. The tubes were autoclaved for 30 minutes in a Market Forge Sterilmatic Autoclave at 120°C and 15psi, and finally transferred into sterile BD Falcon™ Standard Style Petri dishes that were stored at 4°C.

The antibiotic combination used to inhibit biological activity in the root zone was prepared by dissolving the following antibiotics in DI sterile water in the corresponding concentrations: 100 mg/L Ampicillin Trihydrate, 100mg/L Carbenicillin Disodium salt, 100 mg/L Kanamycin Sulfate, 100 mg/L Streptomycin Sulfate salt, and 20 mg/L Chloramphenicol, all chemicals were purchased form Sigma-Aldrich™.

Phosphate buffered saline solution used for preservation of root samples was prepared by dissolving 8g of Mallinckrodt NaCl, 0.2g of Mallinckrodt KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4, in 800mL of DI water. pH was adjusted to 7.4 and additional DI waster was added to adjust volume to 1L.

A 4 % Formalin in Phosphate buffered saline solution was used in the fixation of root tissues and prepared by adding 40 ml of Formalin Sigma™ to a phosphate buffered saline solution. pH was adjusted to 7.4 and additional DI waster was added to adjust volume to 1L.

3.2 PLANT PREPARATION

Pteris cretica cv Mayii and *Nephrolepis exaltata* (Boston fern) ferns purchased form Milestone Agriculture™ (Apopka, Florida) in pots were transplanted into Ball® Quart Regular Mason Jars containing aqueous nutrient solution, coated with aluminum foil to provide a dark environment similar to soil and avoid algal bloom. Nutrient solutions were prepared using All Purpose Plant Food (Peter's Professional®), diluted with tap water to a 20-20-20 (Nitrogen-Phosphate-Potassium) concentration according to package instructions. Nutrient and experimental solutions were aerated by air lines that were fed into each jar by a 1/8" external diameter Tygon tubing.

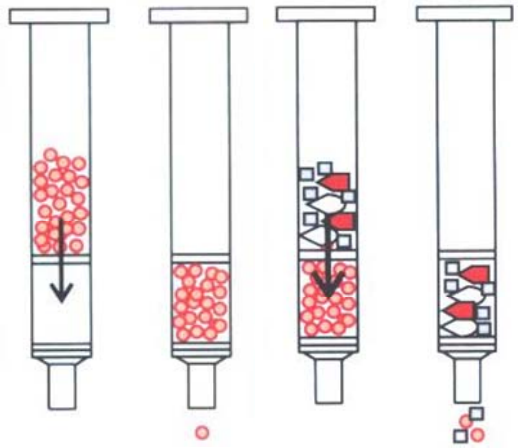
Figure 3.1 *Pteris cretica cv Mayii* and *Nephrolepis exaltata* transferred to hydroponic system

Plants were placed under halogen lights for 16-hr day lighting with a luminescence of 1200 FC measured using a Digital Light Meter (Amprobe®, LM-80). Temperature was maintained between 22-25^{o}C and 60-70% humidity was provided to the lab environment using a Warm Mist Humidifier (Bionaire™ CP-0470).

To guarantee constant nutrient availability for the plants, the nutrient solution was changed on a weekly basis and replenished every other day or when necessary to restore the volume losses caused by transpiration and plant uptake. The fronds were daily misted with tap water to prevent drying. Occasional trimming was carried out to remove necrotic and old fronds and to promote new growth.

3.3 ARSENIC SPECIATION

Arsenate and arsenite were separated using a silica based anion-exchange cartridges (Sigma-Aldrich Supelco® SAX 3mL/500mg) that retain arsenate. The packing material consists of an aliphatic quaternary amine group bonded to the silica surface. The quaternary amine is a strong base and it acts as a positively charged cation that attracts anionic species in the solution. A schematic representation of the ion exchange cartridges is shown in Figure 3.2.



a) Conditioning b) Sample addition

Figure 3.2 Ion exchange cartridges, conditioning and sample addition steps. [34]

As seen in figure 3.2a, the arrows represent the direction of the flow and the circles represent the conditioning solution. The ion-exchange cartridges were preconditioned with a 50% methanol and DI water solution [18] to wet the surface of the sorbent which will allow the water to wet the silica surface in a more effective way. After the conditioning step the aqueous solution containing the sample is added as shown in Figure 3.2 b. Arsenite, represented by squares, passes throughout the packing material, while arsenate, represented by pentagons, is retained by the resin.

Prior to the experimental phase, mass balances were conducted with both arsenic species to check the reliability of the arsenic speciation cartridges. After the samples was passed through, the cartridges were eluted with 0.5 M HCl [18] to recover the arsenate fraction. Each fraction was analyzed separately obtaining a mass balance closure within a 10% variation for the first two uses of a cartridge.

3.4 ARSENIC ANALYSIS

Total arsenic was determined using Perkin Elmer™ model 4100ZL Zeeman graphite furnace atomic absorption spectrometer equipped with a graphite tube atomizer and programmable sample dispenser shown in Figure 3.3. Argon gas of ultra high purity was used to purge the volatilized matrix materials and protect the heated graphite tube from air oxidation. Arsenic hollow-cathode lamp was used at a wavelength of 193.7 nm with a slit width of 0.7nm.

Figure 3.3 Graphite Furnace atomic absorption spectrometer (Perkin Elmer™4100ZL).

Four steps are typically utilized during the graphite furnace operation. A drying step is performed to remove solvent residues from the sample by keeping the temperature of the graphite tube above the solvent boiling point temperature. During pyrolisis, the second step, inorganic and organic matrix components are volatilized from the sample. The atomization step is used to produce an atomic vapor of the analyte, allowing the quantification of atomic absorption. The last step, clean out, is conducted to remove sample residues from the furnace. A six-step program was used for arsenic analysis in this study as shown in Table 3.1.

Table 3.1 Furnace Heating Program

Step	Temperature [° C]	Ramp Time [sec]	Hold Time [sec]	Internal flow [mL/min]
1	110	7	18	250
2	140	5	10	250
3	200	5	10	250
4	900	15	20	250
5	2200	0	5	0
6	2450	1	3	250

According to U.S. EPA method 7060, nickel nitrate was selected as the matrix modifier, for arsenic analysis to minimize volatilization losses during the drying and pyrolysis steps.[9] Concentrated nitric acid was used to acidify the samples and prevent analyte losses during the pyrolysis step as recommended by U.S. EPA method 3050.[8]

GF-AAS was calibrated for arsenic analysis in the linear range of 10 μg/L to 100 μg/L. Sample analysis was conducted in triplicate by GF-AAS and samples with relative standard deviation between the triplicates higher than 10% were re-analyzed.

3.4.1 Sample handling

Samples were extracted from the experimental solution at different time intervals using 2 mL volumetric pipettes, avoiding solid residues and root fragments. The samples were not filtered before the solid phase extraction procedure since the filtering step may reduce its efficiency if the analyte bounds to solid particles. Additionally, the ion exchange cartridge manufacturer recommends avoiding sample filtration.

Total arsenic and arsenite samples were acidified with 2% v/v nitric acid, according to the sample volume, to allow the metal ions to remain dissolved and avoid precipitation. Samples were refrigerated and diluted to attain a concentration in the linear calibration range. Prior to analysis, 1% v/v nitric Acid and 2% v/v nickel nitrate were added to each sample in accordance to U.S. EPA method 7060 for arsenic analysis[9].

3.5 MICROBIOLOGICAL ANALYSIS

Trimmed root samples of the plants were divided into four sets; one subset was placed on nutrient agar plates and incubated for three days at 35^{o}C in a Fisher Isotemp Standard-Capacity Refrigerated Incubator. One of the remaining subsets was transferred into to test tubes containing 4% formalin in phosphate buffered saline solution, one into test tubes containing phosphate buffered saline solution, and the last one was dried with filter paper and placed into empty test tubes, and refrigerated. The purpose of the different samples was to include different preservation methods to ultimately establish one that was more compatible with the staining techniques.

The agar plates were used as a visual control to observe the efficiency of root sterilization methods but no colony isolation was carried out.

A live/dead BacLight bacterial viability kit (Invitrogen Molecular Probes, Eugene, OR) was used as staining technique for the root samples. The samples were analyzed with a Scanning Laser Confocal Microscope (Leica™ TCS SP2) was used with a 20x objective and 1x or 2 x zoom. The microscopy analysis was performed by Dr. Stolz and Jonathan Franks at Duquesne University.

4.0 RESULTS AND DISCUSSION

This project intends to investigate the possibility of effectively using arsenic hyper-accumulating plants to remove arsenite, considering the predominance of trivalent arsenic species in Bangladesh groundwater, and that most of the approaches for arsenic control using phytoremediation as a treatment technology have been conducted using arsenate as arsenic source[26].

The scope of this project was to determine the role arsenic speciation in uptake by *Pteris* fern species, and make use of this knowledge to make the treatment process suitable for applications in the developing world where arsenic in water exceeds the Maximum Contaminant Level. In doing so, both the hyper-accumulating and arsenic sensitive ferns were exposed to different experimental conditions to determine the role arsenic speciation in uptake by *Pteris* fern and the factors influencing arsenic speciation.

4.1 ARSENIC OXIDATION IN AQUEOUS SOLUTION

In order to better understand the arsenic species involved in the arsenic uptake process, a 300µg/l (ppb) arsenite solution was aerated, and arsenic speciation analysis of the aqueous solution was conducted over 72 hours. Three replicates (A, B, C) of this experiment were carried out to have

more reproducibility. During the sampling interval it was observed that total arsenic concentration remained constant while the percentage of arsenite oxidized corresponded to about 50% of the initial addition, results are summarized in Table 4.1.

Table 4.1 Arsenite oxidation over 72 hour period. Trials A, B, C.

Time [Hour]	As III oxidized Trial A	As III oxidized Trial B	As III oxidized Trial C
4	45.7%	49.8%	40.8%
8	44.5%	48.5%	45.5%
24	54.7%	47.5%	44.7%
48	46.6%	45.8%	43.9%
72	45.7%	45.5%	37.1%

Kim and Nriagu purged a groundwater containing arsenite with air and pure oxygen and observed that about 54%-57% was oxidized within 5 days; the resulting oxidation was described by a first order kinetic model[15]. Although the obtained results are consistent with the literature, these initial trials did not provide sufficient information about arsenite oxidation over time. Thus, a similar test was conducted with a contracted sampling interval and a 200μg/L arsenite solution with two replicates (A and B) and arsenite oxidation was monitored for 10 hours.

Though the results obtained were similar to those obtained in the first trial, the oxidation trend was easier to visualize. It was observed that during the first 15 minutes after sample preparation, about 35% of arsenite was being oxidized in both replicates. Furthermore, about 50% of arsenite was oxidized within 2 hours and no further changes in the oxidation rate were observed during the following 8 hours as shown in Figures 4.1 and 4.2.

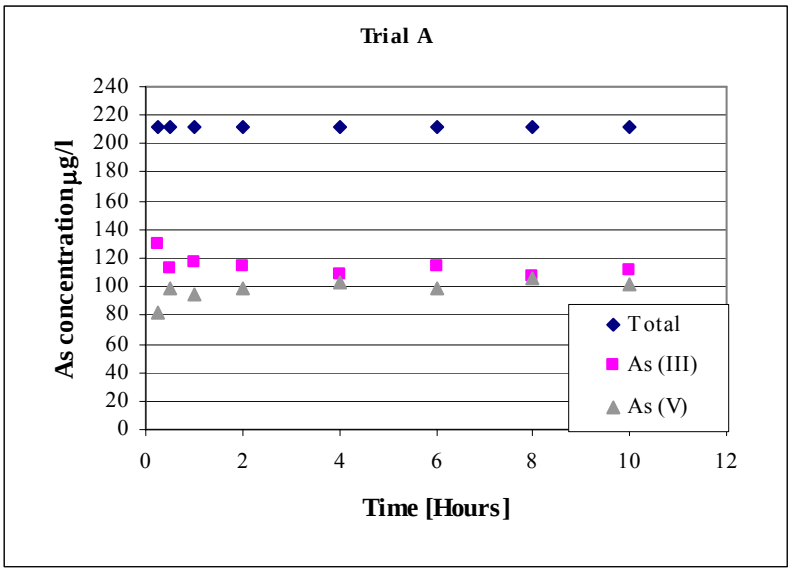


Figure 4.1 Arsenic oxidation in aqueous solution (Trial A); Co= 200 µg/l

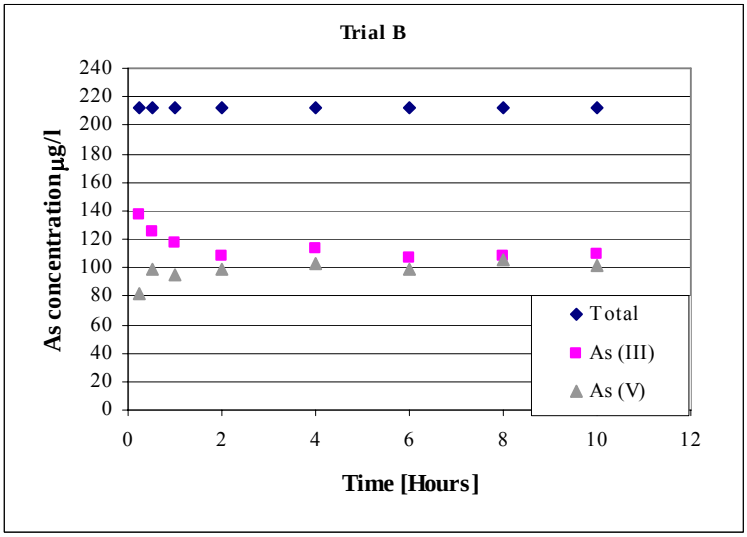


Figure 4.2 Arsenic oxidation in aqueous solution (Trial B); Co= 200 µg/l

Three different kinetic models (zero, first and second order reaction) were applied to the experimental data. The kinetic model that provided the best fit was the first-order depletion kinetics, which is described as:

$$\frac{\partial C}{\partial t} = -KC$$

$$\int_{C0}^{C} \frac{\partial C}{C} = -K \int_{t0}^{t} \partial t$$

$$\ln C - \ln Co = -Kt + const$$

$$\ln \frac{C}{Co} = -Kt + const$$

Where, C is the concentration at time t, C*o* is the initial concentrations, K is the kinetics rate constant and t is time. The data fit to the first-order depletion model is presented in Figure 4.3, and the model parameters in Table 4.2.

Table 4.2 First-order kinetic model parameters for arsenite oxidation (Co=200 ppb)

Trial Id.	K	Constant	R^2
A	0.031	-0.064	0.525
B	0.041	-0.047	0.772
Average	0.036	-0.055	0.649
St. Deviation	0.007	0.013	0.175
RSD %	18.58%		

It was found that despite the data scatter, the kinetic rate constant values for both trials were similar with an average of 0.036 h^{-1}, with a relative standard deviation of 18.58%.

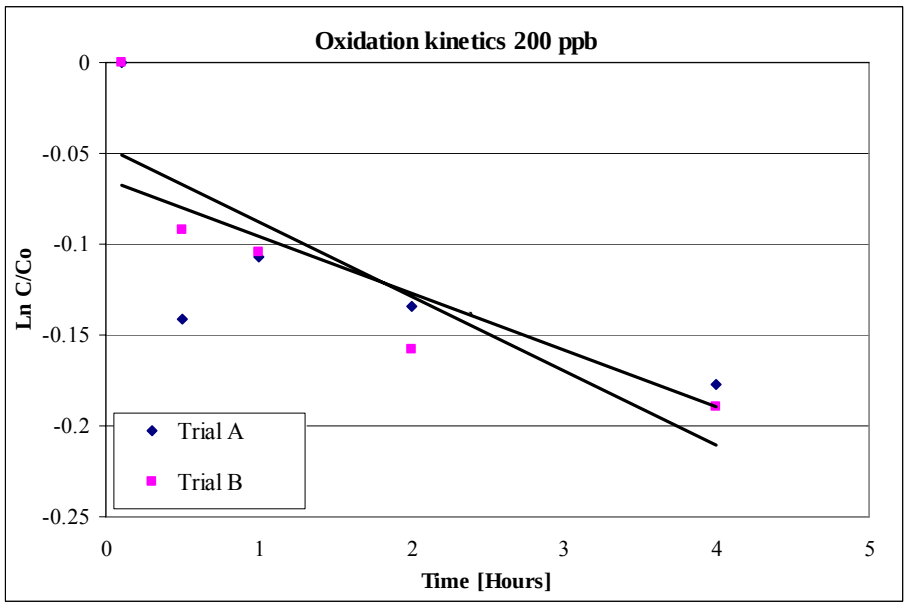


Figure 4.3 First-order kinetics model for Arsenite oxidation (Co=200 ppb)

To verify the accuracy of the results, an experiment was conducted to evaluate arsenite oxidation in a 10 hour period in a solution containing 150µg/L (ppb) of arsenite; a comparable trend was observed as shown in Figure 4.4, where about 55% of arsenite was oxidized to arsenate.

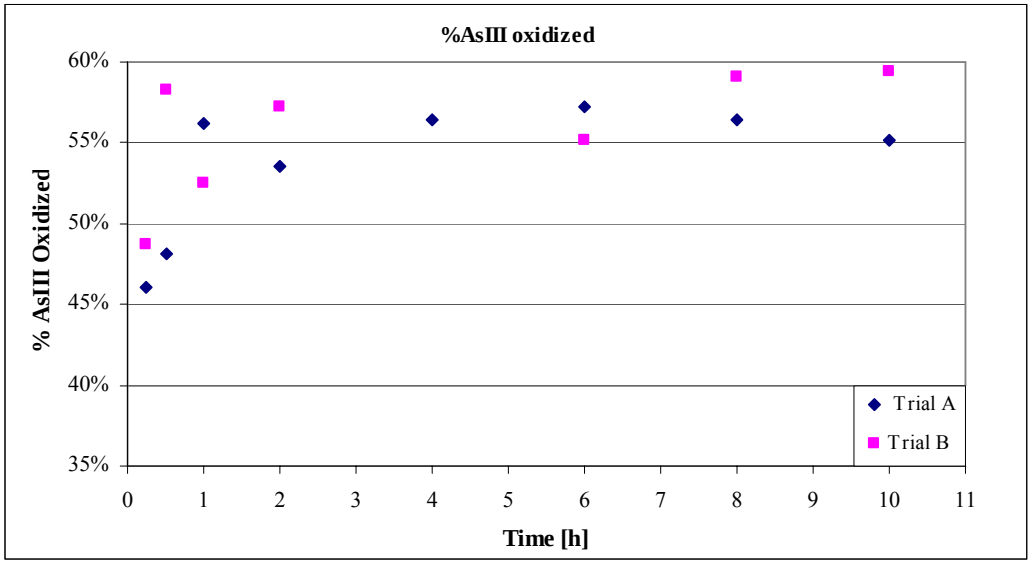


Figure 4.4 As (III) Oxidation (Co= 150 ppb)

As a result of these experiments it was confirmed that arsenite oxidation in the presence of air reaches 50% stability within 2 hours regardless of the initial concentration. Furthermore, the initial experiments demonstrated that the stability was not altered over a 72 hour interval. These data were fitted to the first-order kinetics depletion model presented in Figure 4.5 with the parameters in Table 4.3.

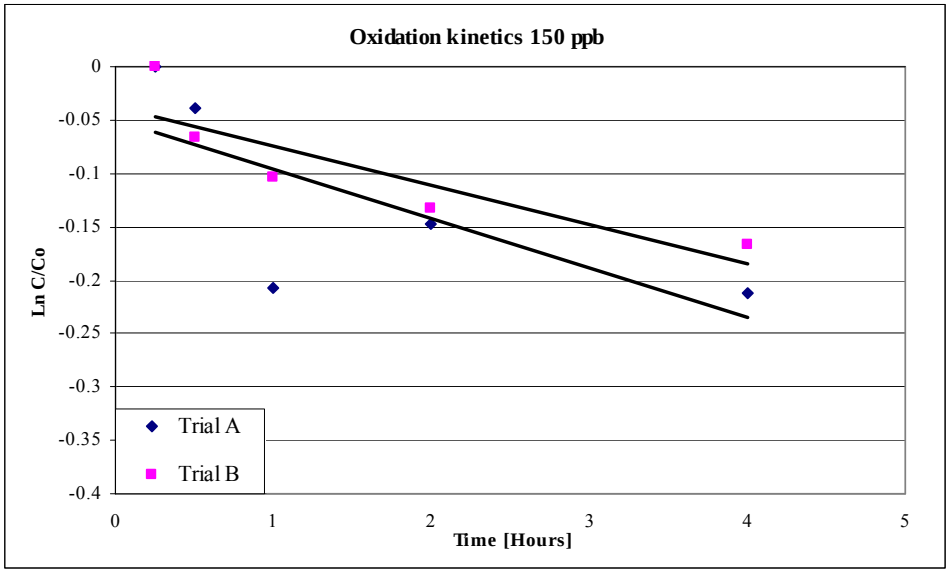


Figure 4.5 First-order kinetics model for Arsenite oxidation (Co=150 ppb)

Table 4.3 First-order depletion kinetics model parameters for Arsenite oxidation at 150 ppb

Trial Id.	K	Constant	R^2
A	0.046	-0.049	0.528
B	0.037	-0.037	0.760
Average	0.042	-0.043	0.644
St. Deviation	0.007	0.009	0.164
RSD %	16.17%		

Although some data scatter in the graph is observed, the kinetic rate constants for arsenite oxidation at initial concentrations of 150 ppb and 200 ppb have the same order and magnitude.

Thermodynamic equilibrium predicts arsenate predominance in all but strongly reducing conditions. It this case, the presence of air diffusers create an oxidizing environment for the redox system As(III)/As(V). The theoretical behavior was not accomplished possibly due to the reported stability of the redox couple and the slow oxidation rates. Bissen et al. 2003 reported that the reaction:

$$H_3AsO_4 + 2\,H^+ + 2e^- \rightarrow H_3AsO_3 + H_2O$$

is very slow compared to oxidation of iron in the presence of air[3]. Furthermore laboratory studies have reported that the half-live for arsenite in natural waters can be up to 1 to 3 years [31]. This indicates that arsenic speciation in solution can undertake gradual changes over longer timescales than those examined in this study.

4.2 ARSENIC OXIDATION IN PRESENCE OF BOSTON FERN

Arsenic oxidation in aqueous solution was observed to reach 50% stability in the presence of air diffusers over 10 and 72 hour periods. Different sets of experiments were conducted to evaluate the effect on arsenic oxidation produced by the presence of *Nephrolepis exaltata* (Boston fern), a non hyper-accumulating species that has been used in previous studies [26, 39].

Three different *Nephrolepis exaltata* plants B1, B4 and B5 were placed in a mason jar containing 800mL of a 300μg/L (ppb) arsenite solution. Total arsenic and arsenite concentrations were analyzed over a 72 hour period. It was observed that total arsenic concentration did not decrease over time while arsenite concentration exponentially decreased until it was completely oxidized. Figure 4.6 shows the average concentrations of total, trivalent and pentavalent arsenic observed during the experiment for the three plants. Figure 4.7 presents the measured concentration of total arsenic and arsenite for each plant.

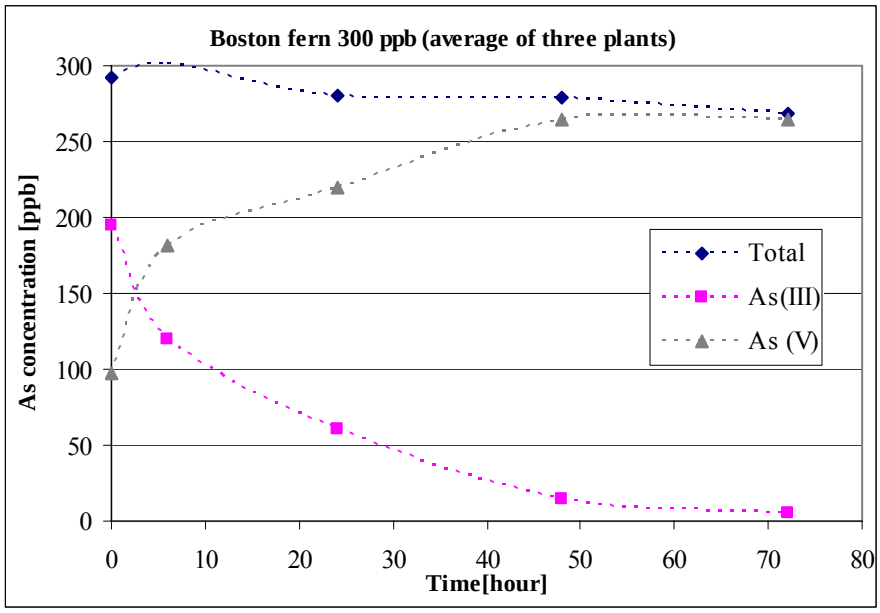


Figure 4.6 Average arsenic concentrations in the presence of Boston fern (Co= 300 ppb)

As can be seen in Figure 4.7, the three plants behave consistently. Total arsenic concentration remained constant over time, which indicates that no arsenic was removed and no arsenic absorption occurred on the roots in the presence of this species. On the other hand, arsenite oxidation was observed to reach 100% stability during the first 48 hours of contact with the roots.

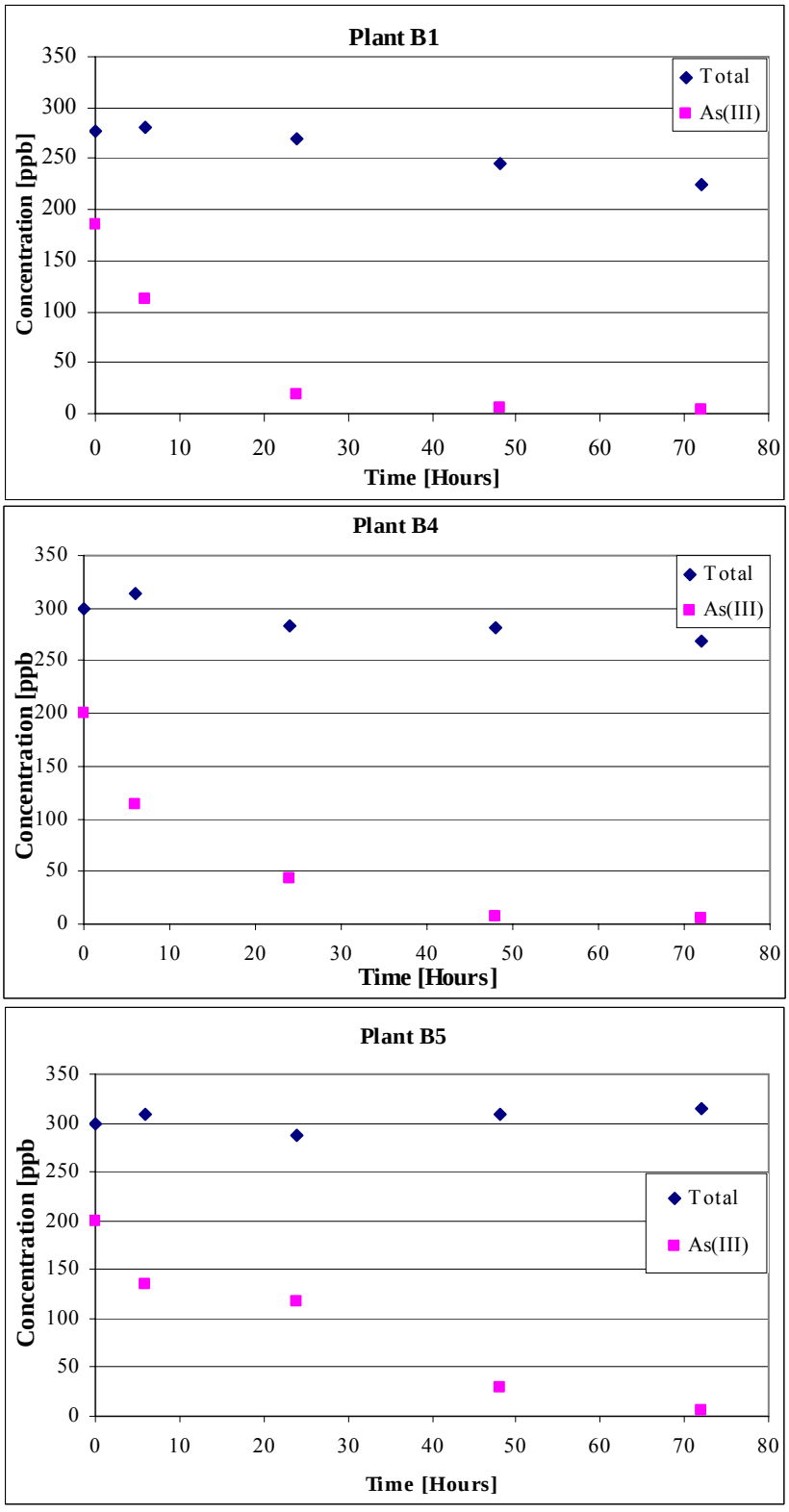


Figure 4.7Arsenic speciation in the presence of Boston fern (Co= 300 ppb)

The first-order depletion model was applied to the arsenite oxidation data to compare the oxidation kinetics in the presence of the Boston fern with previous experiments. Figure 4.8 illustrates the data fit to the model with the parameters summarized in Table 4.4.

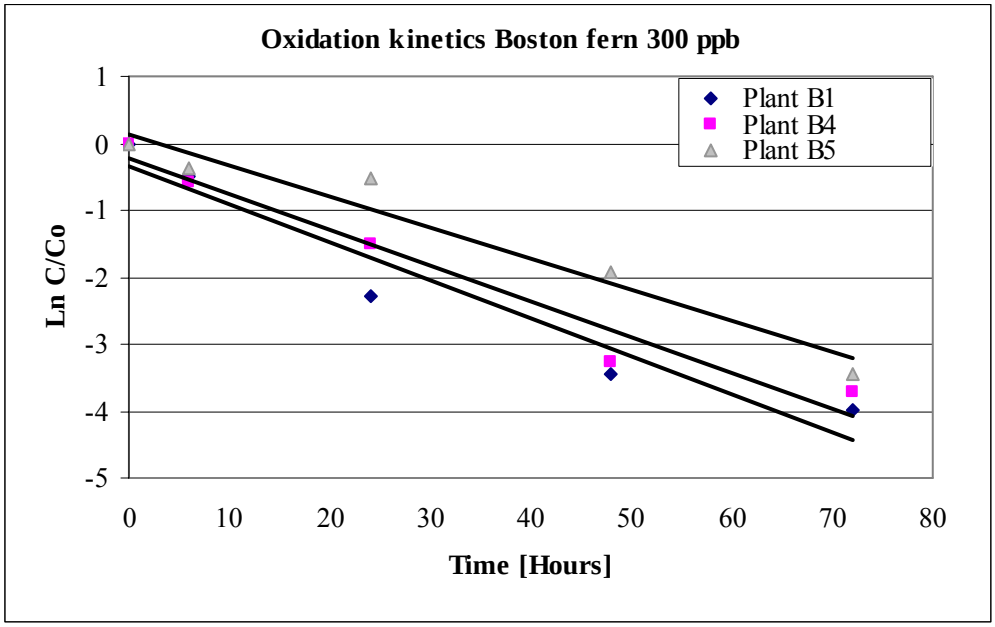


Figure 4.8 First-order kinetics model for arsenite oxidation by Boston fern (Co=300 ppb)

In the presence of Boston fern, with an initial concentration of 300 ppb, the kinetic rate constants for arsenite oxidation vary with an average value of 0.052 ± 0.005 $h^{-1}$and a relative standard deviation of 10.19%.

Table 4.4 First-order depletion model parameters for Arsenite oxidation by Boston fern (Co=300 ppb)

Plant Id	K	Constant	R^2
B1	0.057	-0.347	0.932
B4	0.054	-0.213	0.962
B5	0.046	0.137	0.954
Average	0.052	-0.141	0.949
St. Deviation	0.005	0.250	0.015
RSD %	10.19%		

The results obtained in the presence of the Boston fern revealed that arsenite oxidation was consistently taking place within the first 48 hours with an increase in the kinetic rate constant of 1.25 to 1.44 with respect to oxidation in aqueous solution.

The increase in the oxidation rates indicates that the presence of the non hyper-accumulating Boston fern mediates arsenite oxidation through a mechanism associated with the root zone. However these experiments did not provide any conclusive information about this mechanism.

4.3 ARSENIC OXIDATION IN PRESENCE OF *PTERIS CRETICA*

Different sets of experiments were conducted to determine the impact of the presence of the hyper-accumulating *Pteris cretica cv Mayii* fern on arsenic speciation and to determine if it produced similar effects to the behavior observed in the presence of the Boston fern. To monitor arsenite oxidation in the presence of the hyper accumulating fern, three single *Pteris* ferns were placed in a mason jar containing 800mL of a 300 μg/L (ppb) arsenite solution. Total arsenic and arsenite concentrations were analyzed over a 96 hour period. Figure 4.9 shows the average concentrations of total, trivalent and pentavalent arsenic observed during the experiment for the three plants.

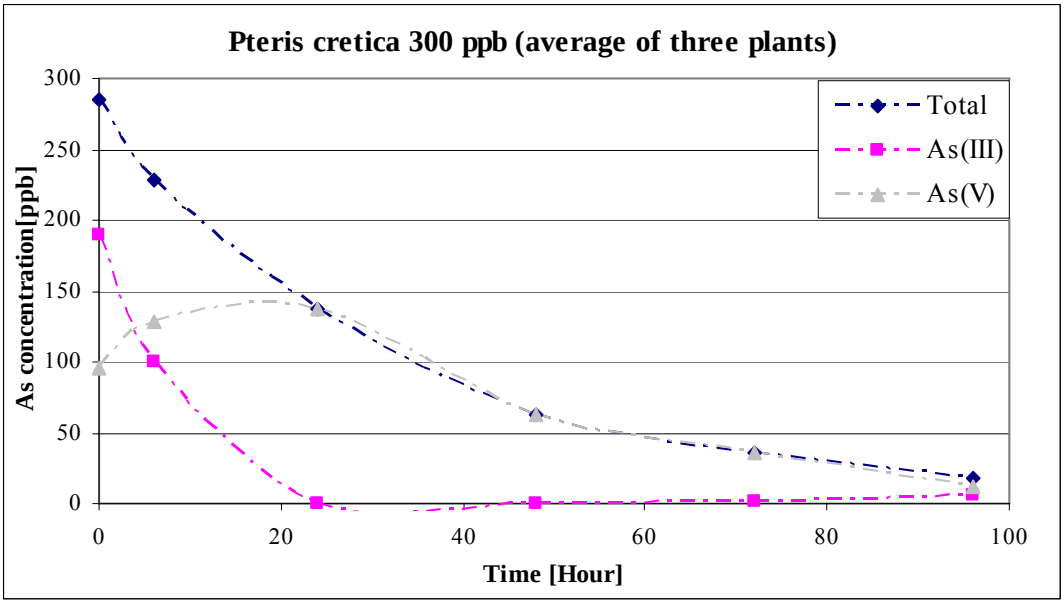


Figure 4.9 Average arsenic concentrations in the presence of *Pteris cretica* (Co= 300 ppb)

It was observed that as the total concentration decreased, the arsenite concentration exponentially decreased until it was completely oxidized. Figure 4.10 presents the results for all three plants.

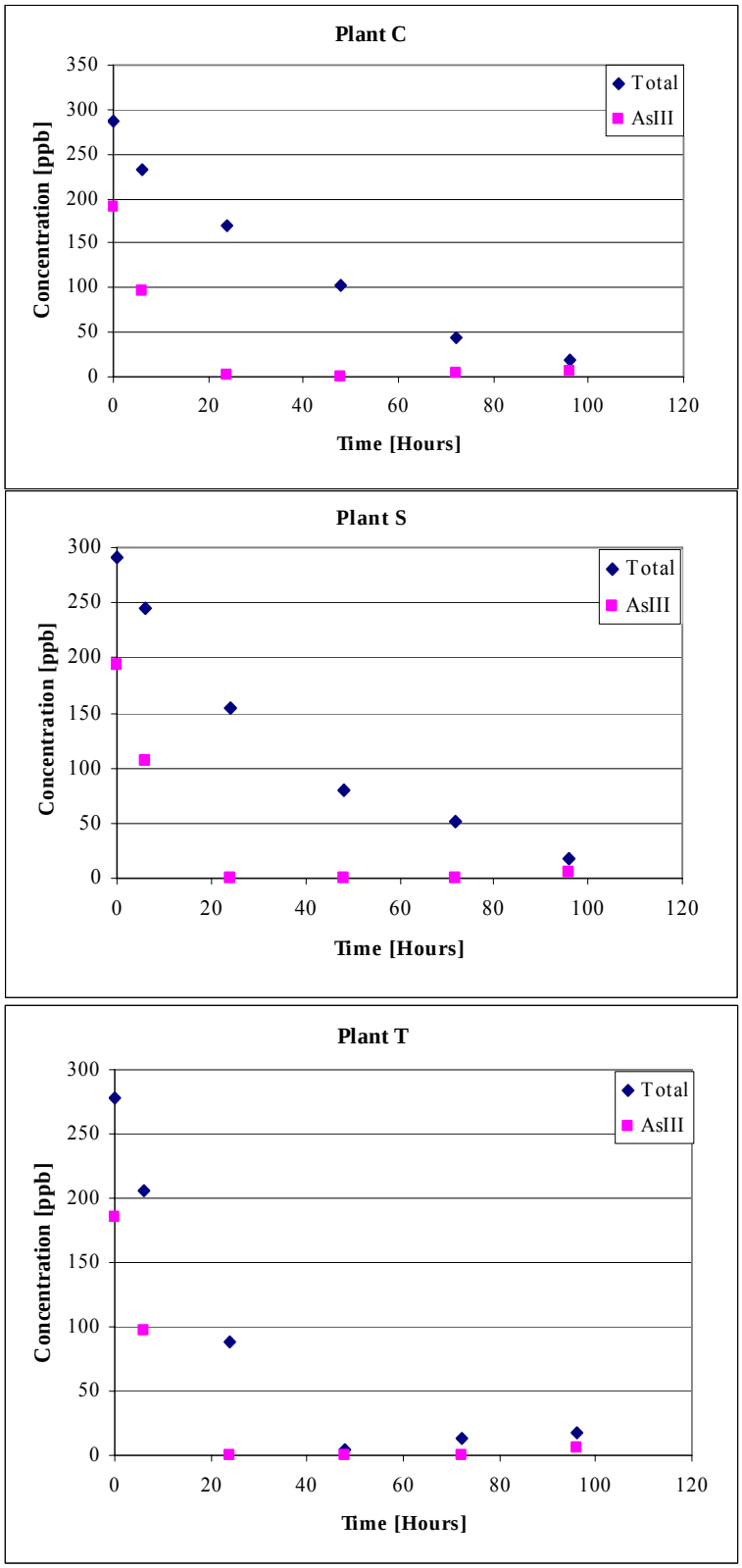


Figure 4.10 Arsenic speciation in the presence of *Pteris cretica* (Co= 300 ppb)

To analyze arsenic removal results presented in Figures 4.9 and 4.10, the arsenic removal data was fit to a first-order kinetics model in order to obtain a rate constant which will be used for the data analysis. Figure 4.11 depicts the data fit to the first-order depletion model with the parameters displayed in Table 4.5.

Table 4.5 First-order kinetic model parameters for arsenic uptake by *Pteris cretica* (Co=300 ppb)

Plant Id	K	Constant	R^2
C	0.028	0.074	0.982
S	0.028	0.026	0.985
T	0.034	-0.494	0.622
Average	0.030	-0.131	0.863
St. Deviation	0.003	0.315	0.209
RSD %	11.68%		

It was observed that the three plants behave similarly and efficiently removed arsenic from solutions; the kinetic rate constants had an average value of 0.030 $h^{-1}$and a relative standard deviation of 11.68%.

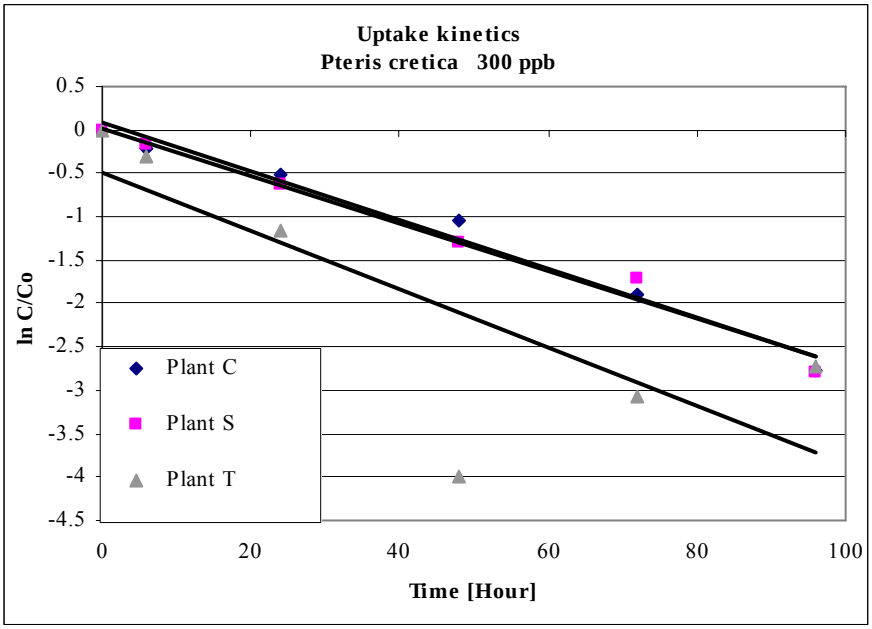


Figure 4.11 First-order kinetics model for arsenitc uptake by *Pteris cretica* (Co=300 ppb)

To obtain information on the impact of initial concentration on arsenic uptake in the presence of the plants, a new experiment was conducted. Three different *Pteris cretica cv Mayii* plants H, K, W were placed in a mason jar containing 800mL of a 150µg/L (ppb) arsenite solution. Total arsenic and arsenite concentrations were analyzed over a 96 hour period, and it was observed that the arsenite concentration decreased exponentially until it was completely oxidized as in the previous experiment while the total arsenic concentration decreased. Figure 4.12 presents the average concentrations of total, trivalent and pentavalent arsenic observed during the experiment for the three plants, and Figure 4.13 presents the measured concentration of total arsenic and arsenite for each plant.

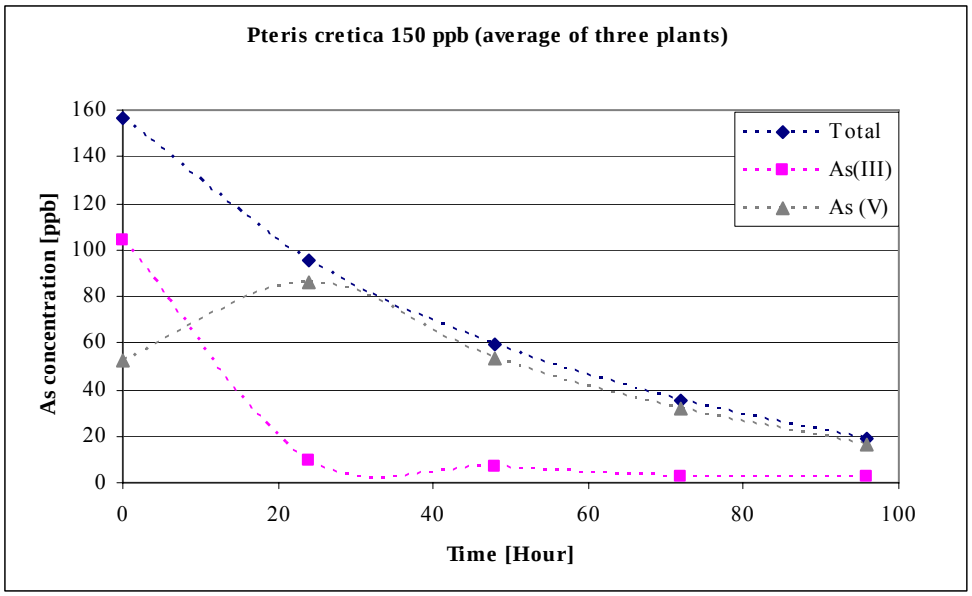


Figure 4.12 Average arsenic concentrations in the presence of *Pteris cretica* (Co= 150 ppb)

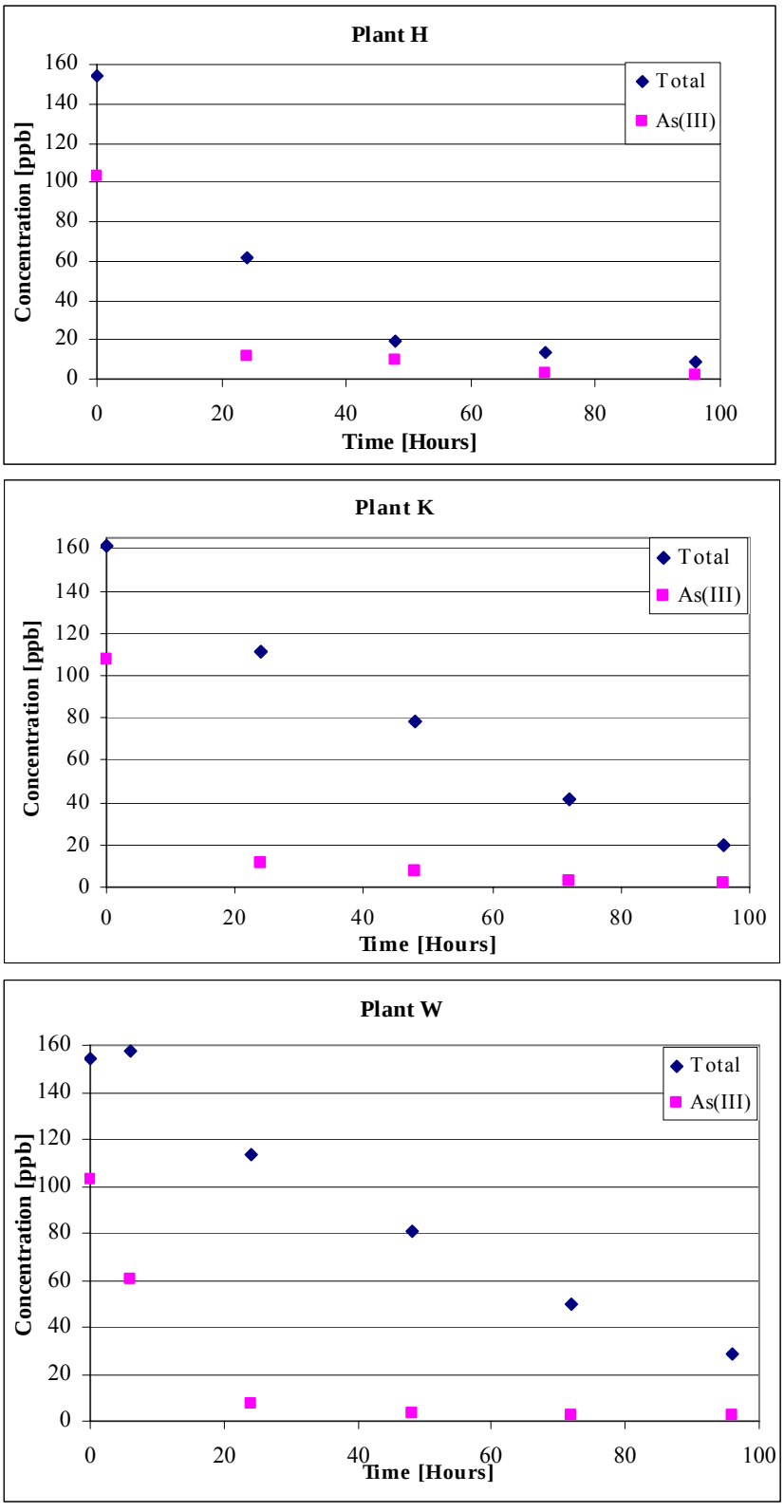


Figure 4.13Arsenic speciation in the presence of *Pteris cretica* (Co= 150 ppb)

Regardless of the difference in the removal efficiency for this set of plants, arsenite oxidation was completed to 100% in all three cases. These data support the assumption that arsenic oxidation is enhanced by the presence of the *Pteris* plant, the oxidation apparently does not depend on the uptake efficiency.

First-order depletion kinetics were applied to this set of data to establish a comparison between arsenic removal rates. Figure 4.14 illustrates the data fit to the model with the parameters presented in Table 4.6.

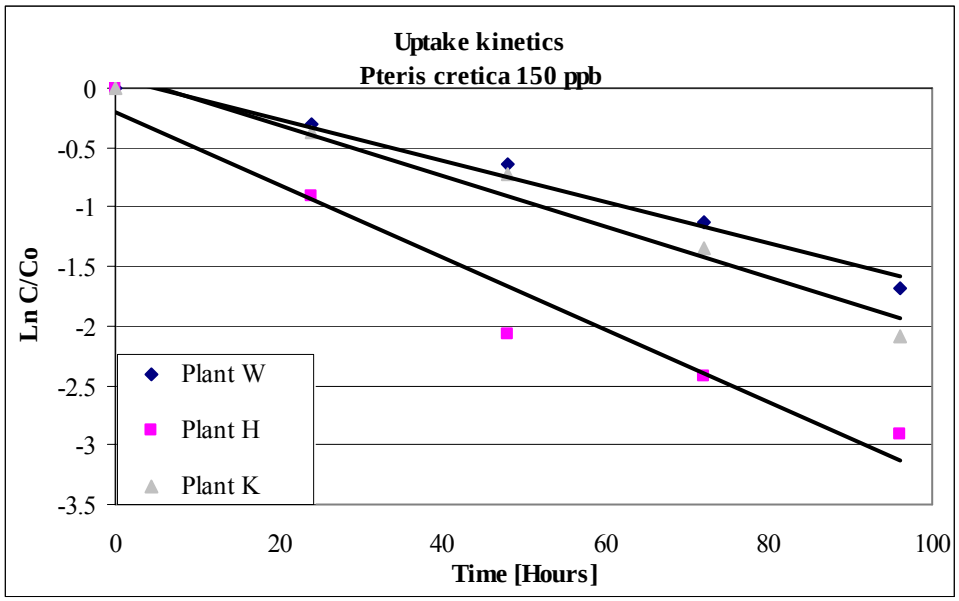


Figure 4.14 First-order kinetics model for arsenic uptake by *Pteris cretica* (Co=300 ppb)

The kinetics rate constants for arsenic uptake vary with an average value of 0.023 ± 0.007 $h^{-1}$and a relative standard deviation of 29.16%.

Table 4.6 First-order kinetic model parameters for arsenic uptake by *Pteris cretica* (Co=150 ppb)

Plant Id	K	Constant	R^2
W	0.017	0.084	0.983
H	0.031	-0.196	0.956
K	0.022	0.123	0.973
Average	0.023	0.004	0.971
St. Deviation	0.007	0.174	0.014
RSD %	29.16%		

Figure 4.13 indicates that plants H and K operate in a similar manner, and achieve different removal degrees 94% and 87% respectively, although the difference on the removal percentages between plants H and K does not seem considerable (7%), the first-order kinetic model reveals that the difference can be observed in Figure 4.14 is significant and may explain the variation between the rate constants. Arsenic removal kinetic constant are described by the slope of the straight line. Through data points shown in Figure 4.14 the slopes of the lines corresponding to plants W and K are more positive than the one corresponding to plant H, indicating that the less positive the slope, the more efficient the uptake process.

The initial arsenic concentration did not have a significant effect on the arsenic removal rates; Figure 4.15 shows the values of the uptake kinetic constants at initial concentrations of 300 ppb and 150 ppb with the relative standard deviations 11.68% and 29.16% respectively. Since the standard deviation at initial concentration of 150 ppb is larger than at 300 ppb, it is possible to affirm that the values do not differ from each other.

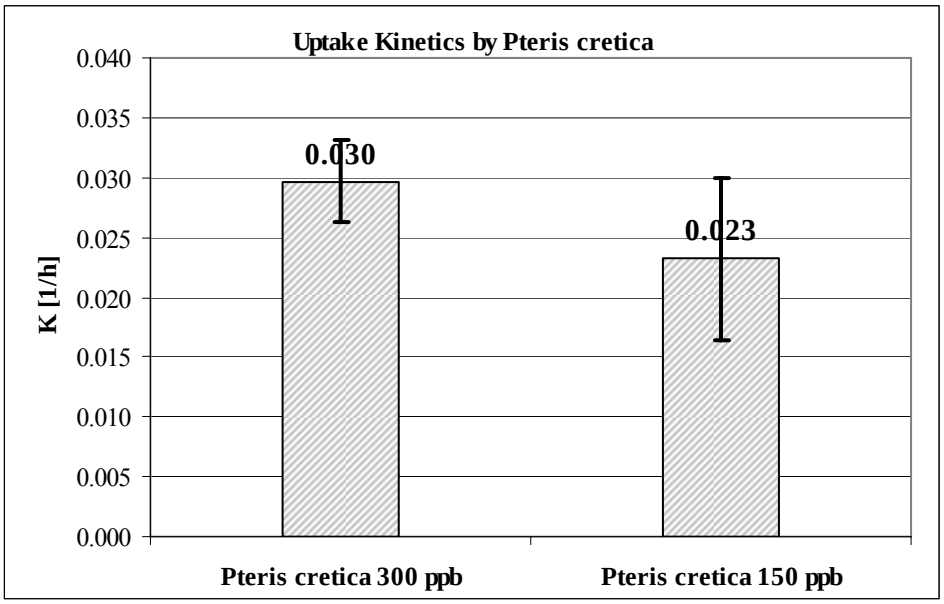


Figure 4.15 Uptake kinetic constants at different initial concentrations in the presence of *P. cretica*

Furthermore, by calculating the average kinetic parameters using the data at 300 ppb and 150 ppb shown in Table 4.7; it was obtained that the kinetic rate constant vary with an average value of 0.026 ± 0.006 $h^{-1}$and a relative standard deviation of 22.66 %, and falls within the range of the two independent relative standard deviations.

Table 4.7 First-order kinetic model parameters for arsenic uptake by *Pteris cretica* at two different initial concentrations

Plant Id	K	Constant	R^2
W	0.017	0.084	0.983
H	0.031	-0.196	0.956
K	0.022	0.123	0.973
C	0.028	0.074	0.982
S	0.028	0.026	0.985
T	0.034	-0.494	0.622
Average	0.026	-0.064	0.917
St. Deviation	0.006	0.240	0.145
RSD %	22.66%		

It has been hypothesized that arsenite is oxidized to arsenate before being taken up by the fern [20]. Despite the fact that arsenite uptake mechanisms have not been elucidated, the results presented herein validate the assumption since all the experimental solutions initially only

contained arsenite, which was oxidized, and in most of the cases removed by the plant to a significant degree.

The results obtained in the presence of the *Pteris* fern revealed that arsenite oxidation was consistently taking place within the first 24 hours and that the mechanism did not depend on the initial arsenic concentration. Moreover the experiments conducted in the presence of Boston fern suggest that oxidation does not depend on arsenic uptake rates since the Boston fern dos not remove arsenic from solution.

Since arsenite oxidation took place in a similar way in the presence of Boston ferns and *Pteris* ferns, it is possible that arsenite oxidation is mediated by the same mechanism in both plant species.

4.4 REMOVAL OF ROOT TISSUE FROM THE ARSENIC SOLUTION

Previous experiments demonstrated that arsenite oxidation took place in both the hyper accumulating fern and the non hyper accumulating plant species. It is known that plants use root exudates to affect metal speciation and facilitate their uptake[28]. An experiment was carried out to asses the significance of root exudates in the arsenite oxidation process.

The experiment consisted of exposing three *Pteris* ferns to arsenic for one week; Table 4.8 displays total arsenic concentration over time during the first week of arsenic exposure for each of the three plants. After this period the remaining aqueous solution was divided into two equal 250 mL volumes, one of which was filtered through a 0.2 μm Millipore™ filter to remove root fragments and most of the particulates present in the solution. 250 ml of a freshly prepared

150µg/l (ppb) arsenite solution was added to the filtered volume and to the one that was not filtered to observe the impact of root tissue and particulate material on arsenite speciation.

Table 4.8 Total arsenic concentration observed for 192 hours

Time [Hour]	As concentration Plant E	As concentration Plant O	As concentration Plant Y
0	140.4	140	143.4
24	80.4	34.4	133.8
48	30.2	2.6	129.4
72	12.2	1.6	117.4
96	2	3.2	109
192	6.7	0.7	103.9

The expected initial concentration on each replicate after the addition of the freshly prepared arsenite solution was calculated using the following equation:

$$\frac{\mu g}{L} afteroneweek \times 250ml \frac{1L}{1000ml} + 150 \frac{\mu g}{L} \times 250ml \frac{1L}{1000ml} = Final \frac{\mu g}{L} \times 500ml \frac{1L}{1000ml}$$

Table 4.9 summarizes the calculated and obtained arsenic concentrations after the addition of a fresh arsenite solution for each replicate.

Table 4.9 Expected and obtained arsenic concentrations after addition of 150ppb arsenite

	Expected As concentration [ppb]	Obtained As concentration[ppb]
E filtered	78.35	80.90
O filtered	75.35	78.70
Y filtered	126.95	145.80
	Expected As concentration [ppb]	**Obtained As concentration[ppb]**
E non-filtered	78.35	81.50
O non-filtered	75.35	80.50
Y non-filtered	126.95	149.50

Arsenic speciation was monitored for 48 hours after the addition of the freshly prepared arsenite solution and it was observed that arsenite was oxidized to about 50% of the initial concentration regardless if the solution was filtered or not. Figure 4.16 shows the total arsenic

concentration for the filtered and unfiltered solutions and Figure 4.17 presents arsenite concentration in the different solutions.

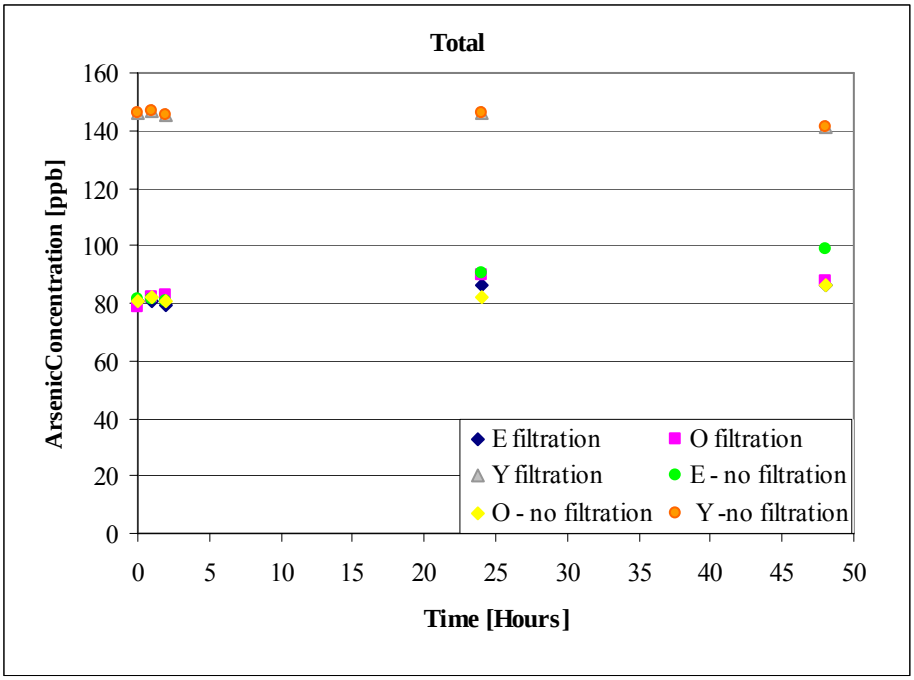


Figure 4.16Total arsenic concentration in filtered and unfiltered solutions

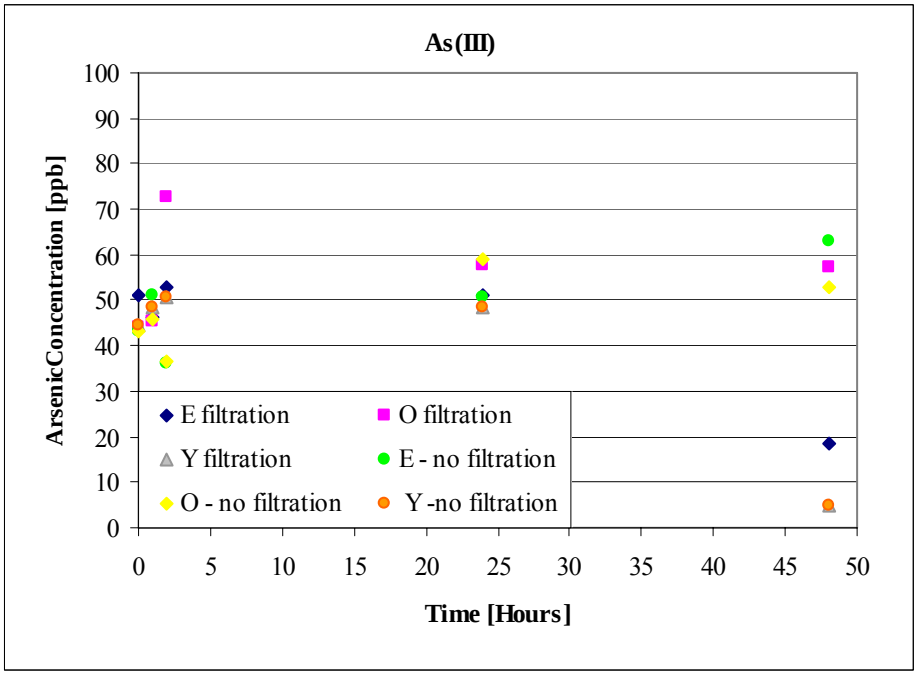


Figure 4.17 Arsenite concentration in filtered and unfiltered solutions

It was observed that arsenite concentration decreased to about 40-50 ppb in all solutions. Oxidation kinetics parameters were not calculated for these experiments because the sampling interval was in the order of days, and recalling the experiments conducted in aqueous solution the time required to reach 50% stability was in the order of minutes, therefore the first order kinetics is not applicable to these data.

Even though oxidation kinetics constants were not obtained under these conditions, the visual inspection of the arsenite oxidation data resembles the ones obtained form the aqueous phase oxidation experiments. This behavior indicates that 100% arsenite oxidation in presence of the plants is not likely related to root exudation but probably mediated by a microbial community associated to the plants.

4.5 ROOT STERILIZATION

One of the documented factors influencing phytofiltration is the presence of microorganisms in the root zone. Although each plant species has specific microbial associations, the possibility of a symbiotic relationship between arsenic metabolizing microorganisms and arsenic hyper-accumulator might be important to arsenic uptake. It is believed that arsenate was the arsenic form taken up by the hyper-accumulating plants. However the plant does not have the ability to oxidize arsenic by itself. In view of the results obtained in this study, the hypothesis is that the microbial community in the rizosphere is responsible for arsenite oxidation to facilitate arsenate uptake by the plant, which has never been evaluated or considered in other research studies. Different sterilization techniques were tested in order to

inactivate the bacterial community in the rizosphere and document its effect on arsenic speciation and mobility.

An initial approach was to use ethanol and chlorine to sterilize the roots of a Boston fern; the plant was exposed for 30 minutes to a 70% ethanol-sterile water solution and then for 30 minutes to a 50% commercial bleach-sterile water solution. Root fragments were cultured in agar plates as a visual control to observe the efficiency of root sterilization. It was observed that after one hour of treatment the plant started loosing its fronds and the texture and morphology of root changed almost immediately after the roots were exposed to the chlorine solution as can be observed in Figure 4.18.

Figure 4.18 Boston fern roots after exposure to Chlorine solution for 30 minutes

Despite the adverse effects observed in the root zone, this sterilization technique proved to be effective as demonstrated by the cell cultures. As seen in Figure 4.19, a significant number of colonies was observed in the agar plate before the treatment and no colonies developed on the agar plate after one hour treatment.

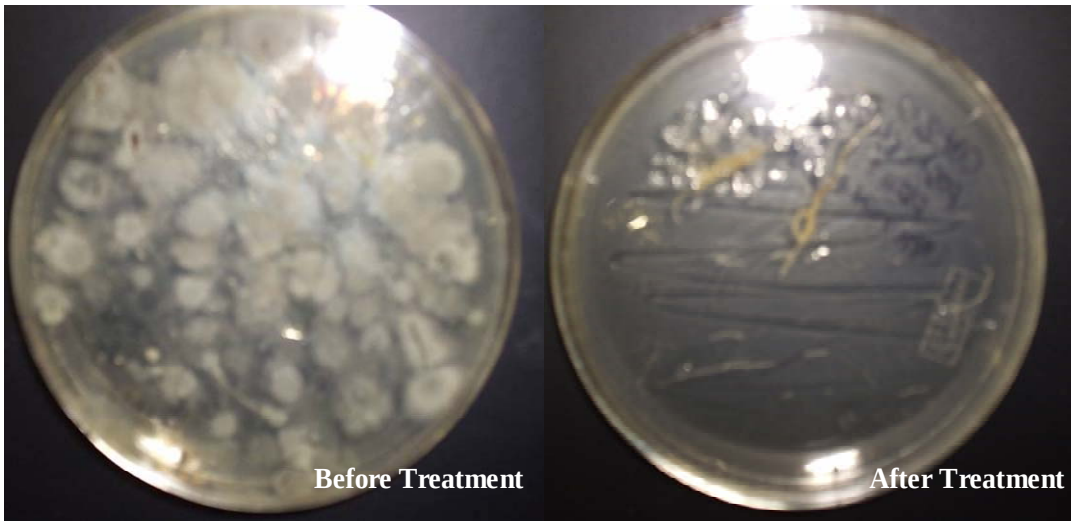


Figure 4.19 Cell cultures before and after root treatment with ethanol and chlorine solutions.

The method was determined to be inadequate for this research purposes because of the obvious adverse effect on the root zone and the plant as well as possible interference of chlorine with arsenic speciation.

A different sterilization technique based on the work done by Simon et al. 2005 [30] was tested in this study. The procedure consisted of preparing the experimental arsenic solution with sterile water containing antibiotic mixture, including 100 mg/L Ampicillin Trihydrate, 100mg/L Carbenicillin Disodium salt, 100 mg/L Kanamycin Sulfate, 100 mg/L Streptomycin Sulfate salt, and 20 mg/L Chloramphenicol. Prior to the use of antibiotics, two experiments were conducted to evaluate the possibility of interference of the antibiotics with arsenic speciation and solid phase extraction process.

To evaluate the effect of antibiotics on arsenite oxidation, a 130 μg/l (ppb) arsenite solution was divided into two equal volumes and one was combined with the antibiotic mixture. Total arsenic and arsenite concentrations were analyzed over a 48 hour period. It was observed that total arsenic concentration remained constant while the percentage of oxidized species corresponded to about 50-60% of the initial addition regardless the presence of antibiotics in

solution. These results, summarized in Table 4.10, verify that antibiotics do not affect arsenite oxidation in aqueous solution since arsenic speciation does not change in the presence of antibiotics.

Table 4.10 Arsenic oxidation in absence and presence of antibiotics.

Time	%AsIII oxidized		
	Non antibiotic	Antibiotic	RSD %
0	60.7%	54.2%	8.0%
24	56.6%	56.0%	0.7%
48	53.6%	59.2%	7.1%

To determine the possibility of antibiotic molecules binding arsenate and interfering with the ion exchange process, a 150 μg/l (ppb) arsenate solution was divided into two equal volumes and one was combined with the antibiotic mixture. Each solution was passed through two sets of ion exchange cartridges (A and B) to verify that arsenate was effectively being retained by the resin. It was observed that arsenate was consistently retained by the ion exchange cartridges regardless of the presence of antibiotics in solution. The results shown in Table 4.11 confirm that antibiotic binding to arsenate does not affect or interfere with ion exchange mechanisms.

Table 4.11 Arsenate retention on ion exchange cartridges in absence and presence of antibiotics.[1]

		Cartridge		
		A	B	RSD%
Total As concentration [ppb]	No antibiotic	165.4	167.6	
	With antibiotic	175.8	173.4	3.4%
		A	B	RSD%
As concentration after cartridge [ppb]	No antibiotic	-8.2	-4.2	
	With antibiotic	-1.4	-10.5	2.9%

[1] Note that a negative reading obtained from GF-AAS is considered to be zero, actual results are shown to calculate the relative standard deviation, but for practical purposes is should be taken as a zero.

4.5.1 Antibiotic use, preliminary study *Nephrolepis exaltata*

A preliminary experiment was carried out using the non hyper-accumulating fern to evaluate antibiotic treatment as a sterilization technique. Three plants (B2, B3, and B5) were exposed to a solution containing antibiotics and 150µg/l (ppb) of arsenite. Three other plants (B6, B8, and B12) were exposed to arsenite solution without antibiotics as a control. Total arsenic and arsenite concentrations were analyzed over a 96 hour period.

Figure 4.20 shows that the average arsenic oxidation carried out by the plants exposed to antibiotics was less pronounced when compared to arsenic oxidation in the absence of antibiotics.

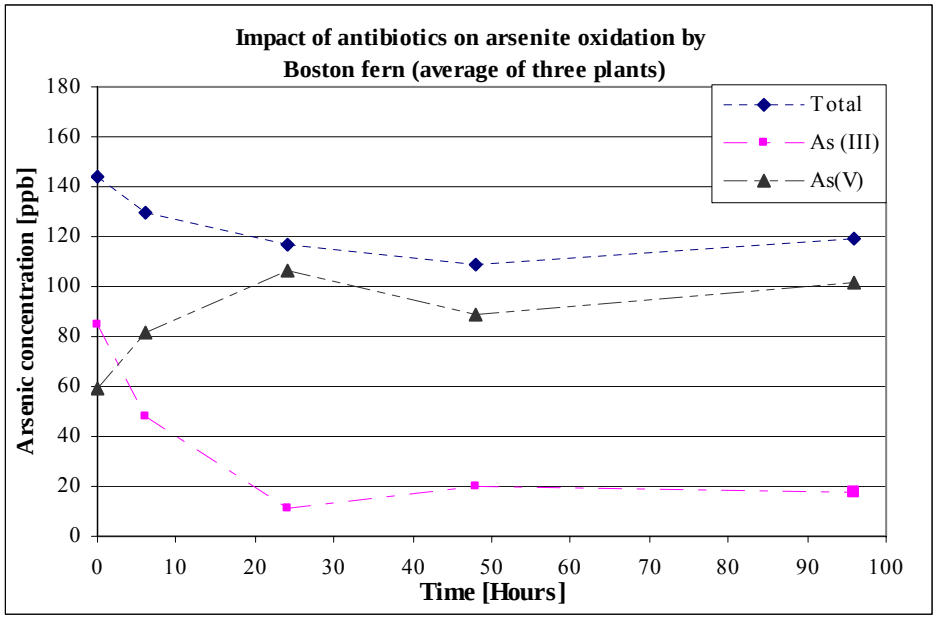


Figure 4.20 Impact of antibiotics on arsenite oxidation by Boston fern (Co= 150 ppb)

As shown in Figure 4.20, arsenite was not completely oxidized by the plants treated with antibiotics and about 13% of the initially added arsenite remained as arsenite. Such behavior indicates that the suppression of microbial activity in the root system has an impact on arsenite oxidation. The first-order depletion model was applied to the arsenite oxidation data to compare

the oxidation kinetics of the antibiotic treated plants with previous experiments and the control plants. Figure 4.21 illustrates the data fit to the model with the parameters summarized in Table 4.12.

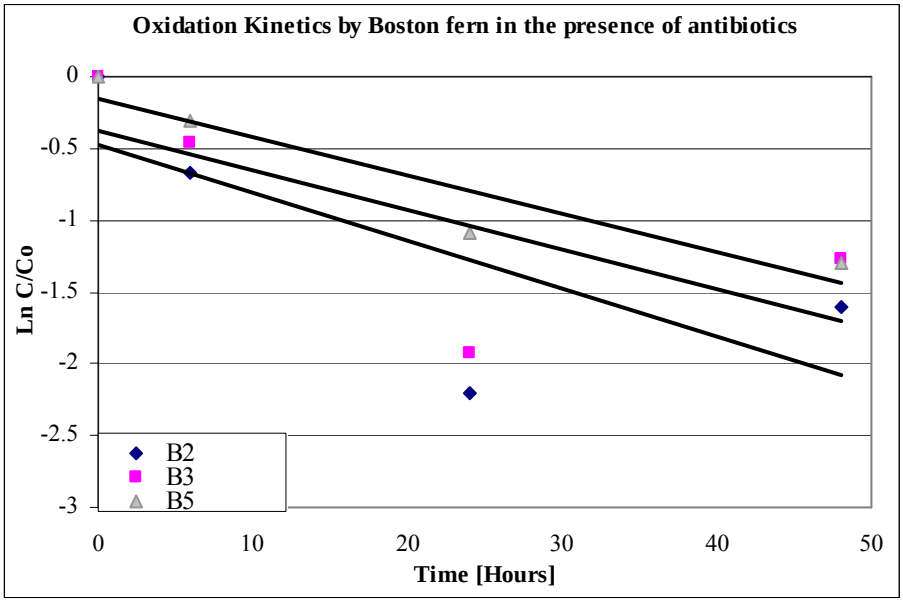


Figure 4.21 First-order kinetics model for arsenite oxidation by Boston fern treated with antibiotics (Co=150 ppb)

In the presence of Boston fern, with an initial arsenite concentration of 150 ppb and in presence of antibiotics, the kinetic rate constants for arsenite oxidation vary with an average value of 0.029 ± 0.004 $h^{-1}$and a relative standard deviation of 11.99%.

Table 4.12 First-order depletion model parameters for Arsenite oxidation by Boston fern in the presence of antibiotics (Co=150 ppb)

Plant Id	K	Constant	R^2
B2	0.033	-0.470	0.544
B3	0.028	-0.373	0.491
B5	0.027	-0.148	0.883
Average	0.029	-0.330	0.640
St. Deviation	0.004	0.165	0.213
RSD %	11.99%		

The significance of these data can only be observed by comparing rate constants of arsenic oxidation with the control plants. Figure 4.22 shows the average concentrations of arsenic species in the presence of Boston fern. It was observed that arsenite was completely oxidized within 48 hours.

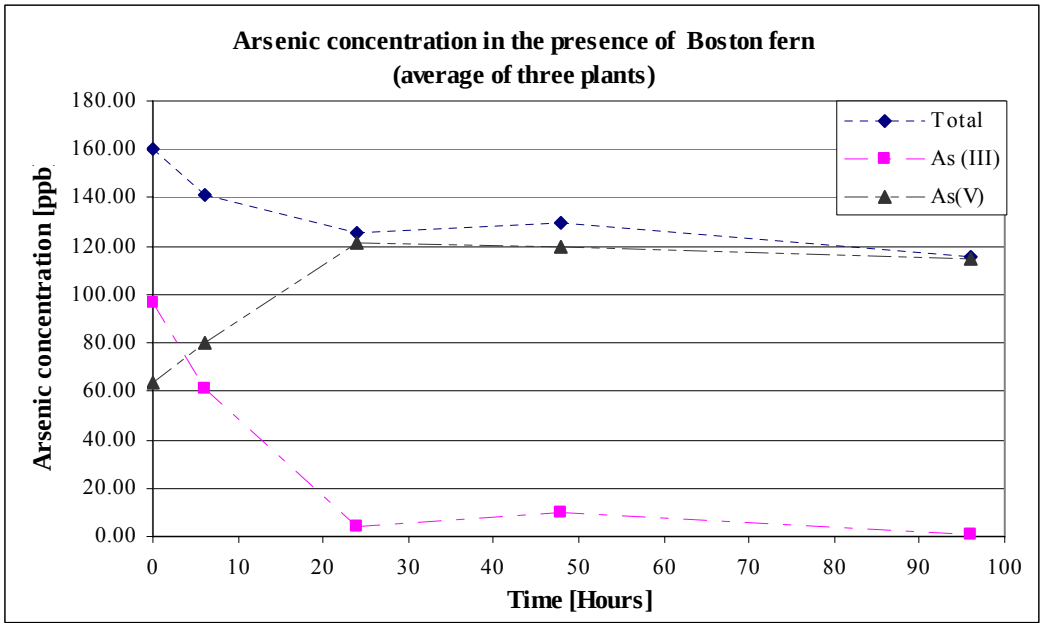


Figure 4.22 Average arsenic concentrations in the presence of Boston fern with no antibiotic exposure (Co= 150 ppb)

Figure 4.23 shows the arsenite oxidation data fit to the first order kinetic model in the absence of antibiotics with the parameters summarized in Table 4.13.

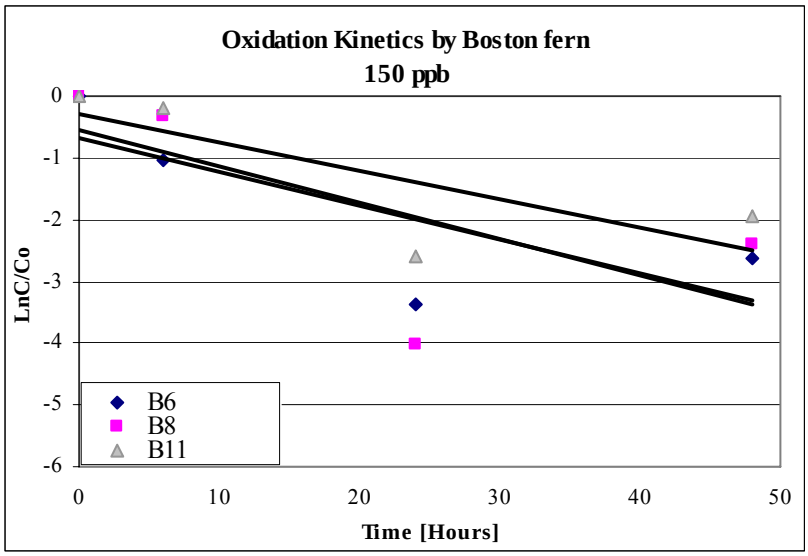


Figure 4.23 First-order kinetics model for arsenite oxidation by Boston fern with no antibiotic exposure (Co=150 ppb)

In the absence of antibiotics, the kinetic rate constant for arsenite oxidation with Boston fern has an average value of 0.053 ± 0.006 $h^{-1}$and a relative standard deviation of 12.05%.

Table 4.13 First-order depletion model parameters for Arsenite oxidation by Boston fern with no antibiotic exposure (Co=150 ppb)

Plant Id	K	Constant	R^2
B6	0.055	-0.693	0.591
B8	0.059	-0.545	0.454
B11	0.046	-0.279	0.596
Average	0.053	-0.506	0.547
St. Deviation	0.006	0.210	0.081
RSD %	12.05%		

It was observed that the oxidation rate is reduced to almost a half in the presence of antibiotics, indicating that the microbial activity of the root zone influences the fate of arsenic in solution by completing arsenite oxidation. However an initial attempt to visualize the microbial community using acridine orange staining revealed that the eradication of the microbial community was not achieved to a considerable degree as seen in Figure 4.24.

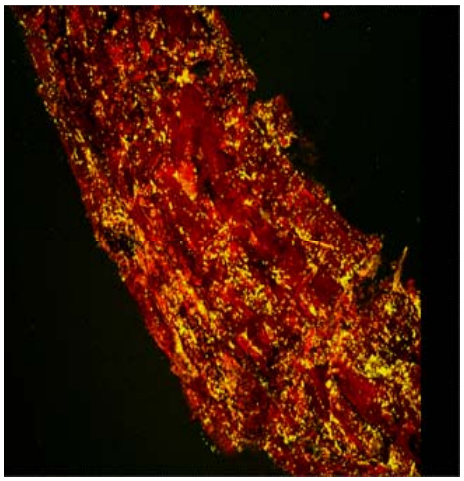


Figure 4.24 Confocal micrograph of the Boston fern rizosphere after antibiotic treatment

The confocal micrograph of roots stained with acridine orange revealed that the Boston fern rizosphere was colonized by microorganisms, visualized in yellow, indicating that either the selection of antibiotics or the method of application was not successful in eliminating microbiological activity.

4.5.2 Antibiotic use and pretreatment *Nephrolepis exaltata*

There are two factors that must be considered when applying a disinfection agent: the contact time and the concentration of the disinfectant. Based on the results obtained with the use of antibiotics to suppress biological activity in the root zone, it was presumed that in order to observe a more significant impact on arsenite oxidation, the antibiotic effect had to be enhanced. For the purpose of this study, an improved suppression of microbiological activity in the root zone was attempted by increasing the time of contact between the roots and antibiotic solution before exposing the plants to arsenic.

Three Boston ferns (B5, B6, and B12) were exposed to the antibiotic solution for three days to allow the antibiotics to act and achieve some reduction in the microbial population before

exposing the plants to arsenic. After three days, those plants were exposed to a solution containing antibiotics and arsenite, each plant was placed in a mason jar containing 800mL of a 140µg/l (ppb) arsenite solution and the antibiotic mixture. Three different plants (B2, B3, and B8) were only exposed to the arsenite solution as a control. Total arsenic and arsenite concentrations were analyzed over a 96 hour period. A decreased oxidation was observed in the plants treated with antibiotics when compared with the control plants. Figure 4.25 shows the average of the oxidation carried out by the plants exposed to arsenic and antibiotics after three days of antibiotic pretreatment.

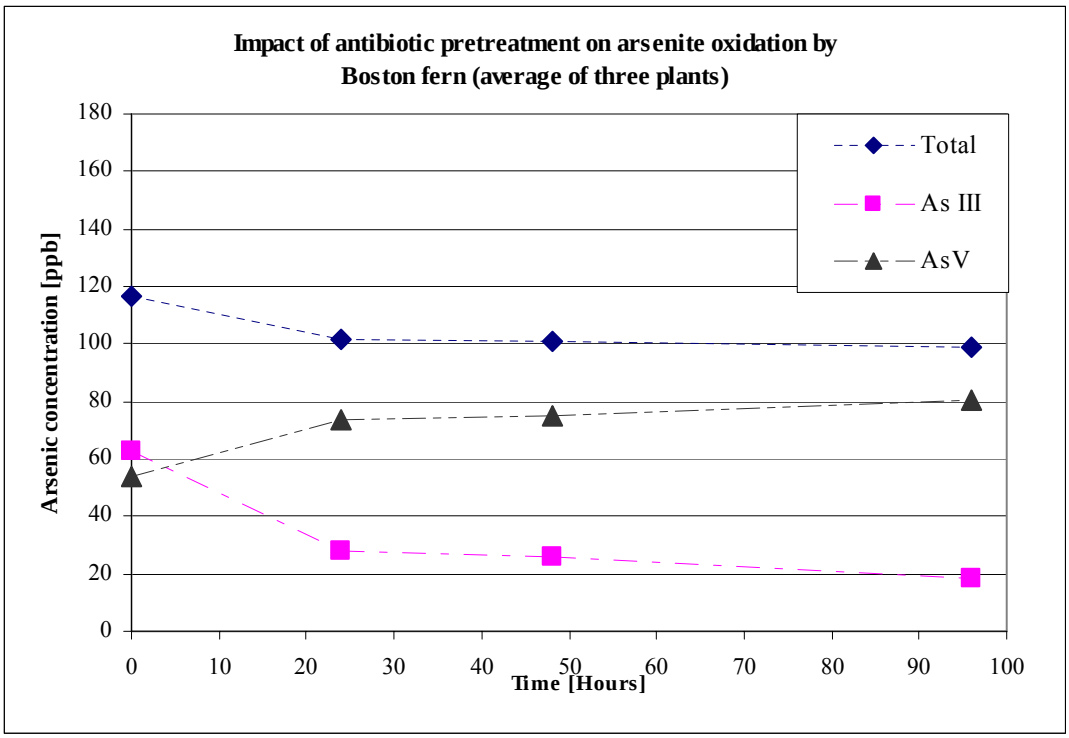


Figure 4.25 Impact of antibiotic pretreatment on arsenite oxidation in the presence of Boston fern (Co= 140 ppb)

As shown in Figure 4.25, arsenite was not completely oxidized by the plants after pre-treatment with antibiotics with about 16% of the initially added arsenite remaining as arsenite. However, the difference in the oxidation carried out by the plants that were not subjected to

pretreatment with antibiotics in the previous experiment is not significant, only 3%. The first-order kinetic model applied to the arsenite oxidation data using pretreatment with antibiotics is shown in Figure 4.26 and parameters are summarized in Table 4.14.

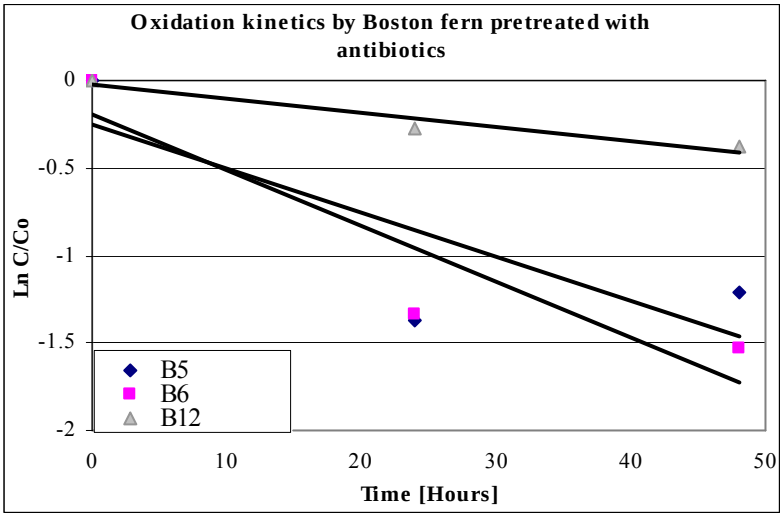


Figure 4.26 First-order kinetics model for arsenite oxidation by Boston fern pretreated with antibiotics (Co=140 ppb)

In the presence of Boston fern, after three days of antibiotic pre-treatment with an initial arsenite concentration of 140 ppb and in presence of antibiotics, the kinetic rate constants for arsenite oxidation averaged at 0.022 ± 0.012 h^{-1} with a relative standard deviation of 57.28%.

Table 4.14 First-order depletion model parameters for Arsenite oxidation by Boston fern pretreated with antibiotics (Co=140 ppb)

Plant Id	K	Constant	R^2
B5	0.025	-0.257	0.648
B6	0.032	-0.190	0.845
B12	0.008	-0.028	0.941
Average	0.022	-0.158	0.811
St. Deviation	0.012	0.118	0.149
RSD %	57.28%		

These data were compared with the control plants; Figure 4.27 shows the average concentrations of arsenic species in the presence of Boston fern. It was observed that arsenite was completely oxidized within 48 hours.

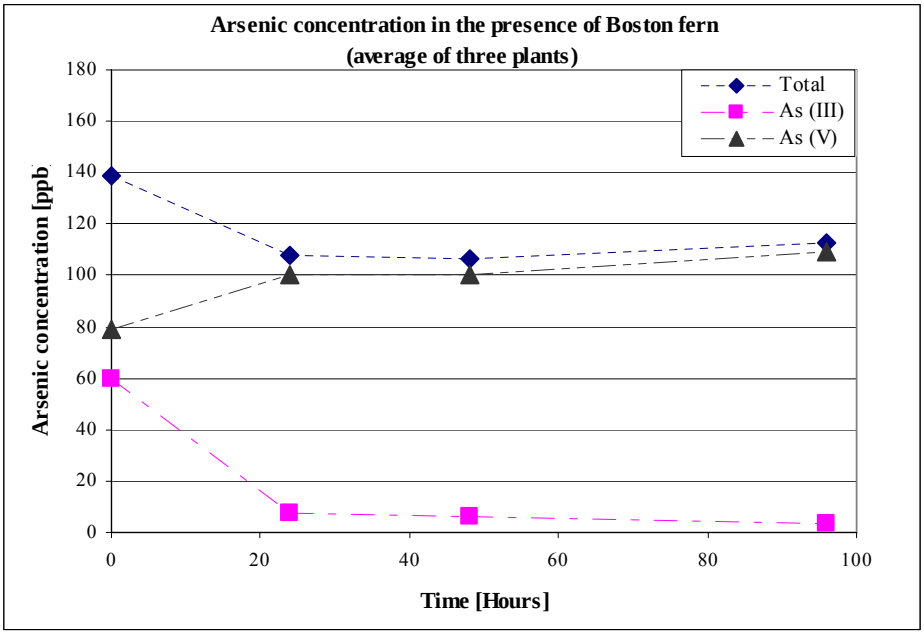


Figure 4.27 Average arsenic concentrations in the presence of Boston fern with no antibiotic exposure (Co= 140 ppb)

Figure 4.28 illustrates arsenite oxidation data fit to the first order kinetic model in the absence of antibiotics and the model parameters are summarized in Table 4.15.

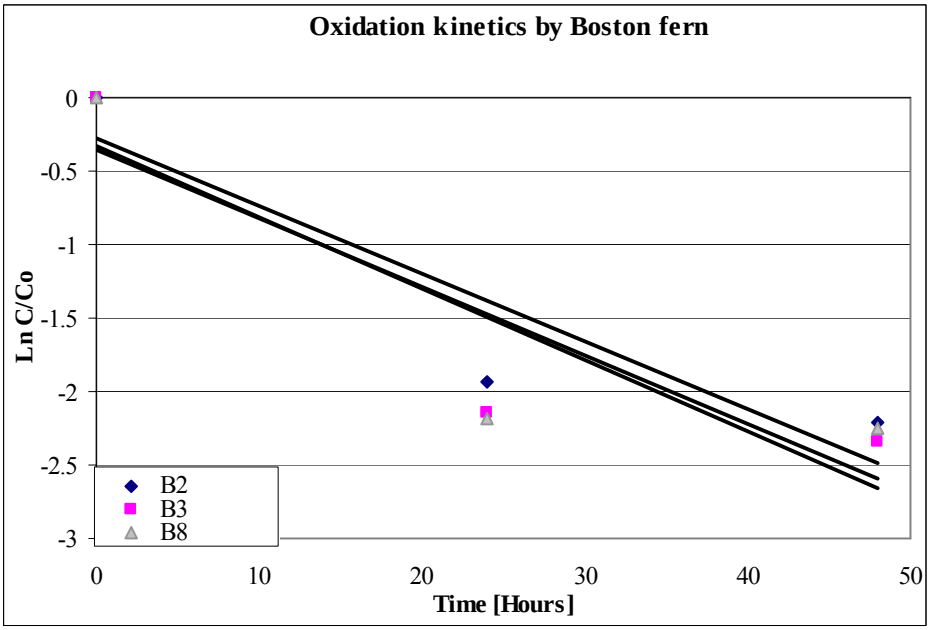


Figure 4.28 First-order kinetics model for arsenite oxidation by Boston fern with no antibiotic exposure (Co=140 ppb)

In the absence of antibiotics and pre-treatment, with an initial concentration of 140 ppb, the kinetic rate constants for arsenite oxidation with Boston fern has average value of 0.047 ± 0.001 $h^{-1}$and a relative standard deviation of 3.06%.

Table 4.15 First-order depletion model parameters for Arsenite oxidation by Boston fern with no antibiotic exposure (Co=140 ppb)

Plant Id	K	Constant	R^2
B2	0.046	-0.277	0.841
B3	0.049	-0.323	0.814
B8	0.047	-0.353	0.771
Average	0.047	-0.318	0.809
St. Deviation	0.001	0.038	0.035
RSD %	3.06%		

It was observed that an attenuation of the microbial activity in the root zone of Boston ferns reduced arsenite oxidation rates, providing evidence of a symbiotic association between arsenic metabolizing organisms and arsenic sensitive plants to oxidize arsenic.

4.5.3 Antibiotic use and pretreatment *Pteris cretica*

Experiments conducted with the non hyper-accumulating Boston fern support the hypothesis of the existence of a microbial community linked to arsenic oxidation. The use of antibiotics proved to be partially effective in suppressing microbial activity in the root zone without producing visible adverse effects to the plant. The same hypothesis was tested in the presence of the hyper-accumulating ferns and identical antibiotic treatment was carried out in an attempt to suppress microbiological activity.

Three *Pteris cretica* ferns (O, P, and AA) were first exposed to the antibiotic solution for three days. The plants were then exposed to a solution containing antibiotics and arsenite in a

mason jar containing 800mL of a 140µg/l (ppb) arsenite solution and the antibiotic mixture. Three different plants (T, V, and W) were only exposed to arsenite solution as a control. Total arsenic and arsenite concentrations were analyzed over a 96 hour period. Figure 4.29 shows the average concentration of total, trivalent and pentavalent arsenic observed during the experiment with the three plants exposed to arsenic and antibiotics after three days of antibiotic pre-treatment. Figure 4.30 presents the average concentration of total, trivalent and pentavalent arsenic observed during the experiment with the three control plants. It was observed that oxidation was reduced in the plants treated with antibiotics when compared to the control plants.

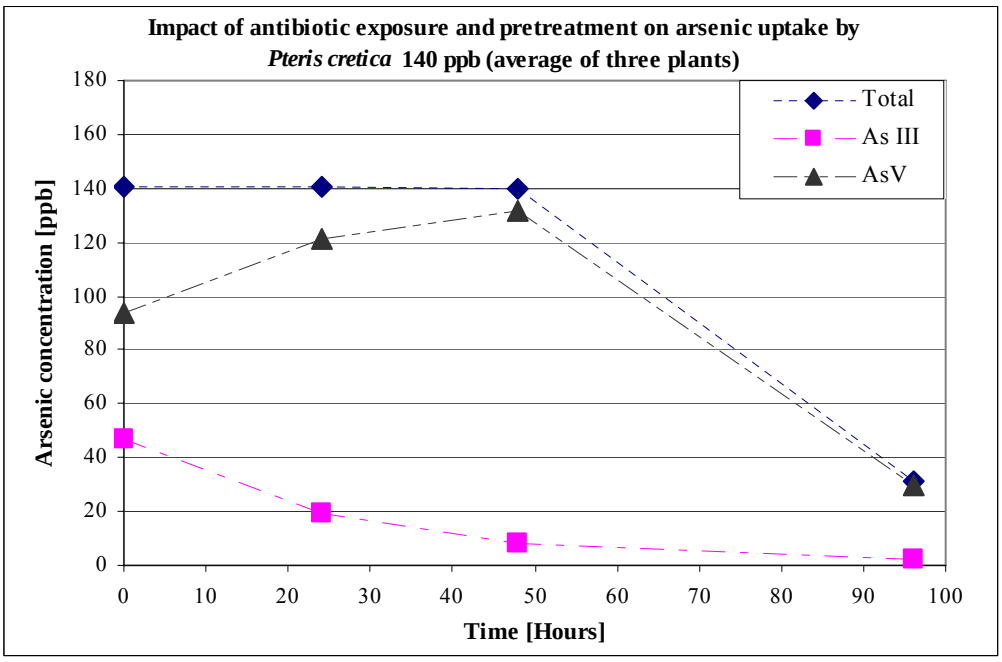


Figure 4.29 Impact of antibiotic pretreatment and treatment on arsenic uptake by *Pteris cretica* (Co= 140 ppb)

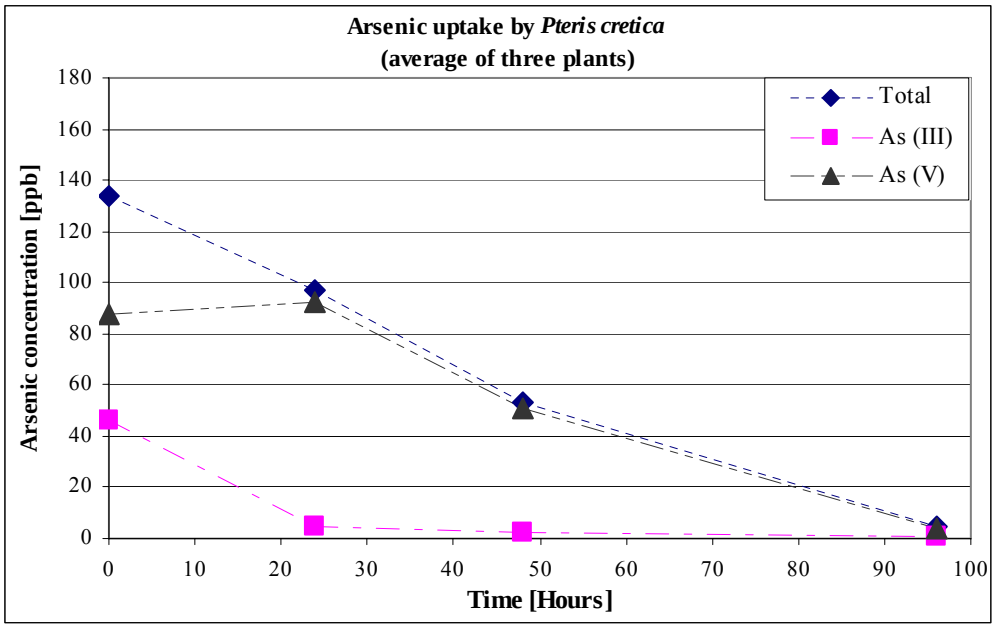


Figure 4.30Arsenic uptake by *Pteris cretica* (Co= 140 ppb)

As shown in Figure 4.29, arsenite oxidation became significant after the first 24 hours while the total arsenic concentration remained constant, after 48 hours total arsenic concentration decreased considerably and arsenite concentration decreased until it was almost completely oxidized. Figure 4.30 shows that arsenite was oxidized within the first 24 hours as it was removed from solution by the plants.

It has been documented that chloramphenicol may inhibit ion uptake and growth on ryegrass and alfalfa plants at concentrations above 20 μg/cm^3, additionally, the presence of chloramphenicol produced a reduction in the chlorophyll contents of the plants observable after 24 hours of the application [32]. Inhibition of phosphate uptake in corn plants by antibiotics has been documented to take place within 2 hours in the presence of Oligomycin [5].

It was observed that the plants exposed to antibiotics did not remove arsenic from solution after 48 hours, which might have occurred because of an inhibition on arsenic uptake produced by the mixture of antibiotics used during the experiment; ultimately when the

antibiotics are consumed, the plants recover their uptake capabilities and start removing arsenic from solution. The inhibition observed in arsenic uptake indicates that the antibiotic selection was probably not suitable for the hyper-accumulating species.

The slight decrease observed in arsenic oxidation rates provides evidence of the presence of a population or sub-population of arsenic metabolizing microorganisms in the root zone of the arsenic hyper-accumulating ferns that was temporarily suppressed by the antibiotic action. This indicates that the microbial activity of the root zone influences the fate of arsenic in solution by carrying out arsenite oxidation to ultimately facilitate arsenic uptake.

5.0 SUMMARY AND CONCLUSIONS

Pteris ferns as well as Boston ferns were examined under different conditions to provide useful information regarding arsenic oxidation and uptake. The results of this work provide significant information on the mechanisms involved in arsenic uptake in the presence of hyper-accumulating species *Pteris cretica* since it was observed that arsenite oxidation has a fundamental role in arsenic fate and speciation during the uptake process.

Arsenic oxidation rates obtained in the course of this study results are summarized in Table 5.1and presented as a bar diagram in Figure 5.1

Table 5.1 Summary of arsenite oxidation rates at different conditions

Conditions	Initial Arsenite Concentration [ppb]	Average kinetic K	RSD %
Aqueous solution 150 ppb	150	0.042	16.17%
Aqueous solution 200 ppb	200	0.036	18.58%
Boston 300ppb	300	0.052	10.19%
Boston No antibiotic treatment 150 ppb	150	0.053	12.05%
Boston with antibiotic treatment 150 ppb	150	0.029	11.99%
Boston No antibiotic treatment No pretreatment 140ppb	140	0.047	3.06%
Boston antibiotic treatment and pre-treatment 140 ppb	140	0.022	57.28%

- It was observed that the presence of Boston fern alters arsenic speciation in solution, and increases arsenite oxidation rates about 1.257-1.445 times the oxidation rates in aqueous solution, regardless of the initial arsenite concentration.

- Antibiotic treatment to attenuate microbial activity in the root zone of Boston ferns produced an effective reduction of 1.810 in the oxidation rates when compared to the untreated plants.
- Pretreatment with antibiotics proved to have a further impact on the microbial activity in the rizosphere of Boston ferns by reducing about 2.178 times the oxidation rates observed in the plants that were not exposed to antibiotics.

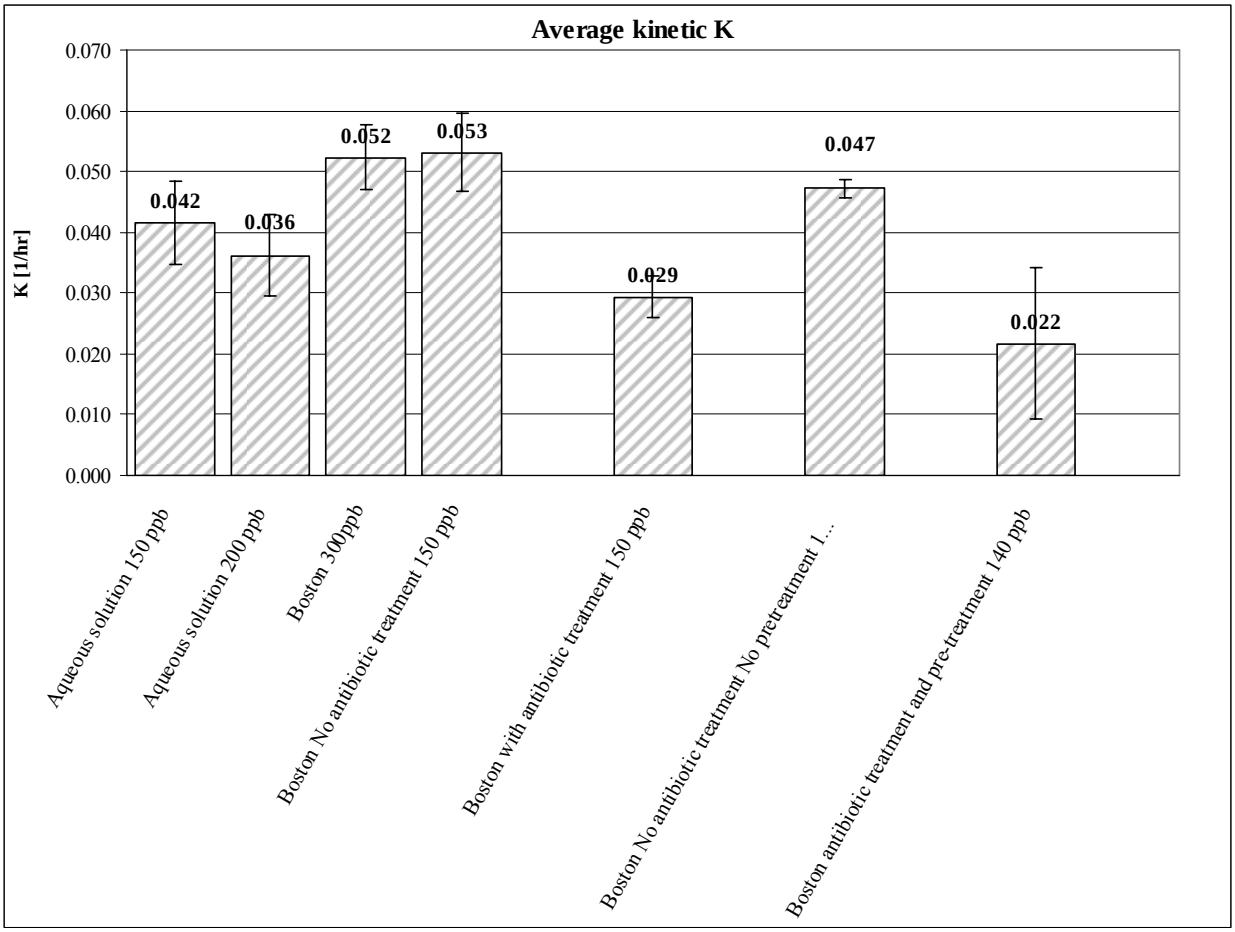


Figure 5.1Arsenite oxidation kinetic rate constants under different conditions

- During the course of this study, the hyper-accumulating plant species were able to efficiently remove arsenite from solution by oxidizing it to arsenate and demonstrated the

ability to withstand repeated exposure to arsenic with no visible adverse effects in to the plant.

- Arsenic uptake rates in the presence of hyper-accumulating species, summarized in Table 5.2, do not depend on the initial concentration since the kinetic rate constant values fall within the values of the calculated standard deviations.

Table 5.2 Summary of arsenic uptake rates at different initial concentrations

Conditions	Initial Arsenite	Average kinetic K	RSD %
Pteris cretica 300 ppb	300	0.030	11.68%
Pteris cretica 150 ppb	150	0.023	29.16%

- Arsenite oxidation consistently took place faster that arsenic uptake, which suggests that arsenic removal process is governed by arsenic uptake rather than arsenite oxidation.
- The results obtained in this study reveal that the oxidation mechanism is not carried out by the plant but more likely by an association with arsenic metabolizing microorganisms given that the effort to reduce the biological activity with the use of antibiotics had an impact on arsenite oxidation rates.
- The observed reduction on arsenic oxidation rates produced by the effect of antibiotics, provides evidence of a symbiotic relationship between arsenic oxidizing microorganisms and arsenic hyper-accumulating plants, and suggests that the oxidation step is crucial on the arsenic uptake process when arsenic is present as arsenite.
- The hypothesis that arsenite is oxidized to arsenate before is taken up by the hyper-accumulating fern [20] was validated by the fact that the kinetics rate constants for arsenite uptake presented herein are analogous to the ones obtained at an early stage of this project where arsenic was present as arsenate (0.021 ± 0.0167 hr^{-1}, 0.014 ± 0.011, at 200pbb and 300 ppb) [4].

6.0 RECOMMENDATIONS FOR FUTURE WORK

The results obtained in this study have offered insight on how the arsenic uptake mechanism of *Pteris cretica* ferns is affected by arsenic speciation and should be considered as a foundation for future work on how to enhance and optimize arsenic removal by hyper-accumulating species.

Since no colony counts or isolation were conducted during this study, it is recommended that future studies include colony counts as well as isolation and characterization of the microorganisms in the root system. Moreover, it should be determined if the presence of those microorganisms in the rizosphere represents a detrimental outcome on the quality of drinking water, if so, disinfection alternatives should be explored to be used in combination with the arsenic removal method.

However, it is recommended that future studies include an evaluation of the removal efficiency at different growth stages of the plants to evaluate the long term performance and efficiency of the hyper-accumulating species, which will provide useful information for a field application.

With the intention of strengthening the reliability of the experimental conclusions, future work should include the development of a model that takes into account arsenic oxidation and uptake kinetics since the ultimate removal efficiency depends on both steps.

Future work should also include an experimental design stage that takes into consideration all the variables involved in the process in order to design a protocol with enough replicates to produce a significant statistical analysis.

It is also recommended for future work the use or simulation of groundwater in the experimental stage to generate conditions similar to the field as well as evaluate the disposal options for arsenic rich biomass to generate a sustainable treatment alternative for arsenic treatment.

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