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Author

Matzen, Sarick

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Effects of soil biogeochemical and physical characteristics on arsenic hyperaccumulation in *Pteris vittata* L.

By

Sarick L Matzen

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of the

University of California, Berkeley

Committee in charge:

Professor Céline E. Pallud, Chair
Professor Mary K. Firestone
Professor Laura N. Lammers
Dr. Peter S. Nico

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Abstract

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Soil contamination with arsenic (As), a carcinogen, is a global problem that stems from irrigation with arsenic-contaminated water, use of arsenical pesticides, fossil fuel combustion, and industrial processes including mining, smelting, and glass production. Plant-based removal of arsenic from soil, or phytoextraction, with the fern *Pteris vittata* L. is an emerging *in situ* technology potentially suitable for moderately contaminated soils. While theoretically promising, field application of phytoextraction with *P. vittata* faces limitations due to variable and slow arsenic uptake rates and long remediation times. Fertilization with nitrogen or phosphorus, and inoculation with mycorrhizal fungi, have been shown to increase arsenic uptake rates under simplified hydroponic or pot experiment conditions. It is unclear whether those findings might apply to field conditions where plant roots are not confined to a limited volume of growth media and where soil texture and arsenic concentrations could be heterogeneously distributed.

In my dissertation I determined the effects of soil biogeochemical and physical characteristics, including 1) soil fertilization or inoculation with mycorrhizal fungus *Funneliformis mosseae*, 2) soil texture, and 3) soil arsenic concentration, on arsenic phytoextraction with *P. vittata* using a multi-scale approach. To test the feasibility of phytoextraction in fine-textured soils, I conducted a large-scale, 4-year field study at a prospective urban agriculture site (Berkeley, CA; 16-361 mg As/kg) working with community collaborators. To test whether leaching could limit the practical application of phytoextraction with *P. vittata*, I conducted a 1-year, high spatiotemporal resolution field study at a California superfund site (Richmond, CA; 25-114 mg As/kg). To determine mechanisms of arsenic mobilization and transport in soil during phytoextraction, I conducted a 0.4-year mesocosm study using novel unsaturated soil columns planted with *P. vittata*.

Across all studies, I found that *P. vittata* was best-suited to phytoextract arsenic from nutrient-poor soils. Nutrient application had either no, or a negative, effect on arsenic uptake rates in *P. vittata*. In the 4-year field and mesocosm studies, uptake rates were negatively correlated with percentage clay, which was associated with increased nutrient content. In the 1-year field and mesocosm studies, I showed that arsenic was mobilized for uptake in *P. vittata* through ion exchange processes also known to mobilize phosphorus, and therefore, according to the phosphorus starvation framework, arsenic uptake could be a byproduct of nutrient scavenging processes most active in nutrient-poor soils. My results suggest that mycorrhizal fungi could increase arsenic tolerance through mechanisms other than nutrient transport. In the 1-year field

study, inoculation with *F. mosseae* led to higher fern biomass, slightly higher uptake rates and significantly lower remediation times.

P. vittata removed a mean of 3.6% of initial soil arsenic in the 1-year field study. Across the 1- and 4-year field studies, *P. vittata* arsenic removal rates ranged from 0.02±NA to 28.9±11.1 kg As/ha/yr, leading to highly variable and long remediation time estimates (12 to >1000 years). In the 1-year field study, soil arsenic concentrations were positively correlated with arsenic uptake rates but did not affect remediation times, indicating that a given soil was remediated equally efficiently regardless of arsenic concentration. Across the 1- and 4-year field studies, changes in soil arsenic concentrations were not consistent with fern arsenic accumulation and indicated complex cycling of arsenic during phytoextraction. In the 4-year field study, surface soil arsenic concentrations significantly increased in the coarse-textured soil, where greater fern growth and arsenic accumulation was observed, suggesting phytoenrichment of arsenic in surface soils over longer (>1 year) time periods. However, in the 4-year field study in the fine-textured soil, where fern growth was lower and mortality higher, arsenic concentrations were greater in the lower than surface depth interval, suggesting arsenic leaching. Furthermore, in the 1-year field study, soil arsenic concentrations significantly decreased, with a greater decrease in the lower (10-20 cm) than surface depth interval (0-10 cm).

I quantified the first (to my knowledge) mass balance equating *P. vittata* arsenic uptake to significant decreases in soil arsenic, and found that arsenic uptake in *P. vittata* could explain only 38.5% of total arsenic depletion from soil in the 1-year field study. This discrepancy in the arsenic mass balance suggests arsenic leached in the 1-year field study, a finding I confirmed under mesocosm conditions. In the 1-year field and mesocosm studies, I showed that arsenic could leach as a result of rhizosphere processes mobilizing arsenic, processes which appeared primarily responsible for arsenic uptake in *P. vittata*. In both studies I found arsenic chemistry in the rhizosphere was distinct from in bulk soil, suggesting the rhizosphere was a unique reactive zone for arsenic mobilization. Furthermore, I found under mesocosm conditions that arsenic transported from bulk soil to the fern *via* transpiration flux of porewater accounted for <30% of arsenic content in *P. vittata*, indicating most uptake comes directly from rhizosphere soil. My results suggest the geochemical processes promoting arsenic mobilization in the rhizosphere could lead to arsenic leaching from both rhizosphere soil and bulk soil in proximity to, and below, the root zone. Indeed, arsenic budgets in the mesocosm study showed that arsenic leached at higher concentrations in the presence compared to absence of *P. vittata*. In the mesocosm study, smaller biomass, which appeared to be the metabolic cost of tolerating highly phytoavailable arsenic, led to greater leaching.

Overall, nutrient-rich soils, presence of clay, and arsenic leaching limited arsenic phytoextraction. Across all studies, I showed that over short time periods in simple systems, arsenic accumulation in *P. vittata* exceeds loss to leaching, but that over time periods of 1 to 4 years, leaching can exceed fern arsenic accumulation, and phytoenrichment of arsenic from aboveground biomass back to surface soils can increase surface soil concentrations. My work emphasizes the importance of rhizosphere processes to bulk geochemical cycles.

Dedication

I dedicate this dissertation to my grandmother, Iverna Phelps Morgan,
and to Emma Deboncoeur and Finley Coyl.

May their light shine on.

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Introduction

Urban agriculture and soil contamination intersect in the effort to reuse moderately contaminated land for urban agriculture (Wortman and Lovell, 2013). Indeed, the soils accessible to communities most in need of food sovereignty, whether post-industrial brownfields or residential soils, are often marginal and enriched in meta(loid)s (Sharma et al., 2015). In the absence of state initiative to remediate such contamination, yet in, ideally, contestation of such neoliberal policies (Crossan et al., 2016), community-based urban agriculture movements seek to remediate these soils themselves in order to grow food safely (Kuhl, 2010; Penniman, 2018).

Thus emerges a several-decades long interest in phytoremediation in the urban agriculture community. Phytoremediation, especially phytoextraction, or plant-based removal of contaminants from soil, is viewed with intense optimism by members of the urban agriculture community. In contrast to conventional remediation technologies which create large volumes of waste soil and are expensive (Tack and Meers, 2010), phytoremediation is viewed as consistent with urban agriculture movement values of sustainability, respect for land, and reliance on natural processes (Matzen, 2009a, 2009b; Kuhl, 2010; Penniman, 2018). Much interest centers on use of *Brassica juncea* and *Helianthus annuus* to remove lead (Pb) from soil, as I and others active in urban agriculture movements have documented (Matzen, 2009a, 2009b; Kuhl, 2010; Penniman, 2018).

Here, a community-university partnership formed to support community interest in using an arsenic hyperaccumulator, *Pteris vittata*, to remove arsenic from urban agricultural soils. In contrast to lead phytoextraction, arsenic phytoextraction appeared more promising due to the discovery of an arsenic hyperaccumulating fern. *P. vittata,* the Chinese brake fern, takes up arsenic from the soil and accumulate it in its fronds at up to ~100 times soil concentrations (Ma et al., 2001). Successively harvesting the fronds removes arsenic from the soil with limited disturbance. However, challenges, especially slow and variable remediation rates under field conditions (Kertulis-Tartar et al., 2006; Niazi et al., 2012; Robinson et al., 2015), hamper the practical application of arsenic phytoextraction (Xie et al., 2009). This dissertation began in support of community efforts to answer to this challenge, investigate methods to increase remediation rates under field conditions, and simultaneously remediate soil for urban agriculture. With community collaborators, we worked to remove arsenic from soil at a prospective community orchard site in Berkeley, CA, USA. When in the course of dissertation research it became clear that arsenic phytoextraction was not a feasible method to clean up urban agricultural soils, the community-university partnership turned to soil testing support, while research pivoted to quantify unanswered parts of the arsenic cycle, particularly arsenic leaching, during phytoextraction with *P. vittata*.

The overarching goal of this dissertation is to determine soil chemical and physical factors limiting arsenic phytoextraction with *P. vittata*. Although the context here is urban agriculture, the results pertain broadly to remediation applications, hyperaccumulator-based ecosystems, and the application of multi-scale research methods connecting rhizosphere processes to bulk geochemical cycles.

In Chapter 1, I review the soil chemical and physical factors limiting arsenic phytoextraction with *P. vittata* and evaluate the potential effectiveness of *P. vittata* for remediation use. In Chapter 2, I determine the effects of soil nutrient application and soil texture on arsenic phytoextraction with *P. vittata* over 4 years of *in situ* cultivation in an urban agriculture setting. In Chapter 3, I take a high spatiotemporal resolution approach to determine the effects of soil arsenic concentrations, fertilizer application, and mycorrhizal fungi inoculation on arsenic uptake rates, soil arsenic

depletion, and arsenic mass balances under field conditions. In Chapter 4, I examine themes emerging in Chapters 2 and 3, the leaching of arsenic during *P. vittata* cultivation. I use a mesocosm setup to compare arsenic uptake in *P. vittata* to arsenic leaching and determine importance of arsenic mobilization in rhizosphere and bulk soils to phytoextraction. Across chapters, and especially within Chapters 3 and 4, I work at multiple scales from field to micron to quantify arsenic cycling in the *P. vittata* plant-soil-water system and determine mechanisms of arsenic release from soil for uptake and leaching.

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Chapter 1. Critical perspectives on soil chemical properties limiting arsenic phytoextraction with hyperaccumulator *Pteris vittata*

Abstract

Arsenic is a metalloid, widely distributed in the environment, and of global concern for human health. In a promising breakthrough for arsenic soil remediation, a fern, *Pteris vittata* L., was discovered to take up arsenic from the soil and accumulate it in its fronds at up to ~100 times soil concentrations, in a process called phytoextraction. Successively harvesting the fronds removes arsenic from the soil with minimal disturbance. However, practical use of *P. vittata* for soil remediation faces challenges largely stemming from the complex nature of the soil. Here, we review soil geochemical processes governing transport of arsenic from soil to the roots of arsenic hyperaccumulating ferns, to provide a critical perspective on factors limiting arsenic phytoextraction. We conclude that rhizosphere processes play a crucial role in arsenic phytoextraction and that nutrient management is most successful with sparingly-soluble nutrient forms or ecologically-based approaches. Our synthesis suggests that phytoextraction as currently practiced is limited to soils with low arsenic concentrations, and that, though *P. vittata* is hardy in some niches to the point of being invasive, it is not conducive to widespread cultivation for remediation.

Introduction

Arsenic is a metalloid, widely distributed in the environment, and of global concern for human health. The chemical forms and oxidation states of arsenic control its toxicity (Mandal and Suzuki, 2002). In aqueous environments and soils, arsenic occurs mainly as its oxidized forms, arsenic(V) and arsenic(III), more specifically as the oxyanions arsenate (present as $H_2AsO_4^-$ and $HAsO_4^{2-}$ in pH range 2-11) in oxidizing conditions, and arsenite (present as H_3AsO_3 for pH below 9) (Bissen and Frimmel, 2003) in moderately reducing conditions. Even in oxic soils, arsenite can be present (Smith et al., 2006) due to reducing conditions in soil microsites (Masue-Slowey et al., 2013; Amend et al., 2014).

Additionally, the type and amount of sorbents control the mobility and bioavailability of arsenic. In soils and sediments, arsenic sorbs readily to iron, aluminum and manganese (oxy)hydroxides as well as to calcium compounds (O'Day, 2006), with a stronger sorption potential for arsenate than arsenite in acidic conditions, and the opposite in alkaline conditions (Dixit and Hering, 2003; Morin and Calas, 2006). In oxic soils arsenic is typically found sorbed to iron(III) oxides, with aluminum oxides a secondary sink (Jacobs et al., 1970; Lombi et al., 2000; Bissen and Frimmel, 2003). The form of iron oxides affects arsenic sorption, with more arsenic sorbed to amorphous rather than crystalline phases due to surface area, although sorption constants to crystalline phases are slightly higher (indicating sorption is stronger) (Dixit and Hering, 2003). Manganese oxides can control arsenic cycling in soils (Deschamps et al., 2003), oxidizing arsenic, which then can resorb to iron oxides (Ying et al., 2012). Arsenic can also be associated with clay minerals in soil (Manning and Goldberg, 1996b), and with organic matter (Buschmann et al., 2006), although these sinks are considered less important than soil (oxy)hydroxides, due to surface charge effects. The presence of phosphate and organic matter limits sorption of both arsenate and arsenite in soils and consequently increases arsenic mobility and leachability (Xu et al., 1988; Redman et al., 2002; Smith et al., 2002; Bauer and Blodau, 2006; Huang et al., 2010).

The global average concentration of arsenic in uncontaminated soils is 5-6 mg/kg (Morin and Calas, 2006), but background arsenic concentrations in soils vary by several orders of magnitude depending on the parent material from which they were formed (Chen et al., 2002a), making it difficult to establish typical values (Cullen and Reimer, 1989). Arsenic enrichment in soils originates from agriculture, through the use of arsenical pesticides, manure, and irrigation with arsenic-contaminated water; use of arsenic-containing wood preservatives, such as chromated copper arsenate (CCA); and mining and smelting of metals (Bissen and Frimmel, 2003). Arsenic contamination persists in soils for decades or centuries (Matschullat, 2000). Soil cleanup criteria for arsenic are not universal but vary greatly between and within countries (Davis et al., 2001; Teaf et al., 2010).

Arsenic can cause acute and chronic poisoning through exposure routes including inhalation, as well as ingestion of water, food and soil material. Arsenic causes numerous adverse health effects to humans, which Mitchell (2014) reviewed extensively. Globally, the major concern to human health due to arsenic comes from contamination of drinking water from natural geological sources (Oremland and Stolz, 2003; Ratnaike, 2003). However, health risks from arsenic contaminated soil should not be underestimated. Arsenic tops the Priority List of pollutants created by the Agency for Toxic Substances and Disease Registry/US EPA, which ranks pollutants at Superfund sites based on toxicity and potential for exposure (ASTDR, 2019).

In a promising breakthrough for arsenic soil remediation, a fern, *Pteris vittata* L., was discovered to take up arsenic from the soil and accumulate it in its fronds at up to ~100 times soil concentrations (Ma et al., 2001) in a process called phytoextraction. Successively harvesting the fronds removes arsenic from the soil with limited disturbance. However, many challenges still lie ahead for arsenic phytoextraction with *P. vittata* (Xie et al., 2009). These challenges largely stem from the complex nature of the soil. While *P. vittata* is efficient in removing arsenic from contaminated soils in greenhouse and hydroponic experiments, the understanding of its performance under more complex field conditions is still limited. Remediation time estimates derived from the few existing field studies (Kertulis-Tartar et al., 2006; Niazi et al., 2012; Lessl and Ma, 2013) range from 10 to 78 years to remove 390 kg As/ha (100 mg As/kg) from soils. Application of soil amendments has been investigated in efforts to increase arsenic uptake rates. Frond arsenic concentrations and biomass were higher in *P. vittata* supplied with sparingly soluble compared to soluble phosphorus (Lessl and Ma, 2013; da Silva et al., 2018), though in other cases phosphorus application did not affect arsenic uptake in *P. vittata* regardless of solubility (Chen et al., 2002b; Cao et al., 2003; Tu and Ma, 2003b; Fayiga and Ma, 2006), and soluble phosphorus increased arsenic concentrations in porewater but not in *P. vittata* fronds (Caille et al., 2004). Furthermore, some studies have shown that the presence of other soil contaminants will negatively affect arsenic uptake by *P. vittata* (Caille et al., 2004; Shelmerdine et al., 2009). Finally, it is not well understood how arsenic depletion from soil relates to arsenic accumulation in *P. vittata* fronds (Kertulis-Tartar et al., 2006; Niazi et al., 2012; Lei et al., 2018), especially under field conditions where fern roots have access to a larger volume of soil than in pot experiments (Neugschwandtner et al., 2008; Gerhardt et al., 2016).

Here, we review soil geochemical processes governing transport of arsenic from soil to the roots of arsenic hyperaccumulating ferns, to provide a critical perspective on factors limiting arsenic phytoextraction. Biological aspects of arsenic phytoextraction have been reviewed extensively (Rathinasabapathi et al., 2006; Xie et al., 2009; Danh et al., 2014; Fayiga and Saha, 2016). Fayiga and Saha (2016) briefly reviewed effects of soil properties including arsenic availability, nutrient addition, and root exudation on arsenic uptake in *P. vittata*. Here, we discuss

chemical stabilization to give context to work on arsenic phytoextraction. We then review the effects of soil characteristics on arsenic phytoextraction by the fern, including soil texture and minerology, arsenic and metal concentrations, and mechanisms for arsenic release from soil. We discuss efforts to increase phytoextraction efficiency, including the use of fertilizers, compost, chelating agents, and mycorrhizal fungi inoculants. We consider remediation rates, estimates of remediation times, and the need for mass balances in phytoextraction studies. Finally, we discuss implications of existing research for the widespread application of arsenic phytoextraction with *P. vittata*, and suggest avenues for future work based on a consideration of the soil-plant system.

Methods to remediate arsenic-contaminated soils

Chemical stabilization

Chemical stabilization, which involves applying a sorptive amendment to the soil to lower the soluble fraction of contaminants and decrease plant uptake (Warren and Alloway, 2003), could be a less destructive alternative to conventional remediation approaches (Komárek et al., 2013). Chemical stabilization of arsenic involves applying precursors of iron-oxides (ferrous ($FeSO_4$) or ferric sulfates ($Fe_2(SO_4)_3$)) and zerovalent iron (iron(0)), or directly applying poorly crystalline (ferrihydrite (iron$(OH)_3$) or crystalline iron oxides goethite α-FeOOH).

Amending soil with iron compounds decreases arsenic mobility and therefore soil and porewater toxicity in *ex situ* studies. Amendment with iron-sulfates, together with lime to avoid soil acidification, decreased the concentration of water soluble arsenic (Moore et al., 2000) and of arsenic in leachates (Hartley et al., 2004; Hartley and Lepp, 2008a), as well as arsenic uptake by crops (Warren and Alloway, 2003; Warren et al., 2003). The addition of zerovalent iron decreased the concentration of arsenic in soil porewater, in leachates, and in plant tissues, as well as of extractable and bioaccessible arsenic (Bleeker et al., 2002; Hartley et al., 2004; Kumpiene et al., 2006; Mench et al., 2006; Hartley and Lepp, 2008a; Ascher et al., 2009). Goethite or ferrihydrite amendments decreased arsenic concentrations in porewater (García- Sanchez et al., 2002; Nielsen et al., 2011; González et al., 2012), in leachates (Hartley et al., 2004; Hartley and Lepp, 2008a; Nielsen et al., 2011), and arsenic uptake by plants and bioavailability (Hartley and Lepp, 2008b).

However, there are limitations to using these methods in more complex field conditions (Puschenreiter et al., 2005; Mench et al., 2006; Kumpiene et al., 2008; Komárek et al., 2013). For example, amendments with iron-sulfates plus lime or zerovalent iron led to increased concentrations of metals such as cadmium, copper, zinc, lead in leachates or in plant tissues compared to untreated soils (Hartley et al., 2004; Kumpiene et al., 2006; Hartley and Lepp, 2008a). Additionally, when used *in situ*, arsenic stabilization methods were much less efficient than when used in batch or pot experiments (Bleeker et al., 2002; Warren et al., 2003). Finally, even if amendments effectively decrease arsenic mobility under current geochemical conditions, changing conditions, especially changing redox potential due to microbial activity (Öncü et al., 2012) could lead to arsenic release. On-going site monitoring is required to ensure effective remediation over time, akin to monitored natural attenuation (Reisinger et al., 2005).

Phytoextraction using *Pteris vittata* and other arsenic-hyperaccumulating plants

Phytoextraction with hyperaccumulators

Phytoextraction involves the use of plants to extract contaminants from the soil and concentrate them in their aboveground biomass, which is then harvested, thus removing the contaminants from the soil with relatively little disturbance.

Brooks et al. (1977) established the term hyperaccumulator in a study focusing on nickel to describe plants able to accumulate nickel at concentrations >1,000 mg/kg (ppm) in dry leaf tissue. Since then, the term hyperaccumulator has been extended to other metals and metalloids with element-specific thresholds. Other criteria include extreme metal tolerance (Ent and Baker, 2013), a shoot to root metal(loid) concentration ratio (or translocation ratio) typically >1 (Baker, 1981), and a ratio of metal(loid) concentrations in plant biomass to those in soils (or bioconcentration factor) typically >1. Hyperaccumulators tolerate high concentrations of metal in soils, but hyperaccumulation is a genetically distinct trait from tolerance (Maestri et al., 2010). Natural hyperaccumulation depends on both constitutive up-regulation of transporters to move metal(loid)s across membranes (Chaney et al., 2007), and mechanisms that confer tolerance of the high concentrations of metal(loid)s thus taken up (McGrath et al., 2001).

A number of theories have been proposed to explain hyperaccumulation, including the defense theory, where high metal(loid) concentrations kill or deter pests (Pollard and Baker, 1997; Jiang et al., 2005); allelopathy, where litter fall locally increases soil metal(loid) concentrations (Morris et al., 2009); and the phosphorus starvation theory, where metal(loid) uptake is a byproduct of nutrient acquisition (Meharg and Hartley-Whitaker, 2002).

Arsenic-hyperaccumulators

As early as 1975, many plants accumulating >1,000 mg/kg arsenic in their leaves and with a translocation ratio >1 were found growing in soils enriched in arsenic, mainly around mining sites. Among them *Jasione montana* L., *Calluna vulgaris* (L.) Hull, *Agrostis tenuis* Sibth. and *Agrostis stolonifera* L. were found in the UK (Porter and Peterson, 1975), *Agrostis castellana* in Portugal (De Koe, 1994) and *Paspalum racemosum* and *Bidens cynapiifolia* in Peru (Bech et al., 1997). However, since those plants grew on soils extremely enriched in arsenic, the bioconcentration factor was <1, despite high concentrations in plant tissues, and those plants are consequently not hyperaccumulators, according to the bioconcentration criteria presented earlier.

The first plant to be labeled an arsenic hyperaccumulator was the Chinese brake fern *Pteris vittata* L. *P. vittata*, an invasive fern in Florida, USA (Langeland et al., 2008), was identified in 1998 as an arsenic hyperaccumulator during a survey of plants growing on a site contaminated with chromated copper arsenate (CCA) (Komar et al., 1998; Komar, 1999). There, *P. vittata* accumulated between 1,442 and 7,526 mg/kg arsenic in its fronds with no observed toxicity effects (Ma et al., 2001). In addition, in a pot experiment with a soil spiked with 1,500 mg/kg arsenic, *P. vittata* was able to accumulate as high as 22,630 mg/kg arsenic (Ma et al., 2001). The fronds to roots arsenic concentration ratio in *P. vittata* was >20 and the bioconcentration factor ranged between 5 and >100 (Ma et al., 2001). Simultaneously, arsenic hyperaccumulation in *P. vittata* was discovered separately in populations growing in an arsenic sulfide mine in Hunan Province, China (Chen et al., 2002c).

Since then, other arsenic-hyperaccumulating plants have been identified, most of them belonging to the fern family, and more specifically to the *Pteris* genus (Xie et al., 2009). However, not all *Pteris* species hyperaccumulate arsenic (Meharg, 2003; Wang et al., 2007). Known arsenic-

hyperaccumulating ferns include many varieties of the Cretan brake fern *Pteris cretica* (Wei et al., 2001, 2007; Zhao et al., 2002; Meharg, 2003; Srivastava et al., 2006; Wang et al., 2007; Feng et al., 2011), the silver fern *Pityrogramma calomelanos* (Francesconi et al., 2002; Visoottiviseth et al., 2002), *Pteris longifolia* (Zhao et al., 2002), *Pteris umbrosa* (Zhao et al., 2002; Meharg, 2003; Koller et al., 2007), many varieties of *Pteris multifida* (Wang et al., 2007; Wei et al., 2007), *Pteris biaurita L.*, *Pteris quadriaurita* Retz and *Pteris ryukyuensis* Tagawa (Srivastava et al., 2006), *Pteris aspericaulis*, *Pteris fauriei*, and *Pteris oshimensis* (Wang et al., 2007).

P. vittata is the most studied of the arsenic-hyperaccumulating plants (Singh and Ma, 2007), although other species could be better suited for arsenic phytoextraction under certain circumstances. *Pityrogramma calomelanos* showed a greater ability to accumulate arsenic than *Pteris vittata* (Visoottiviseth et al., 2002; Zhao et al., 2002; Niazi et al., 2012). Furthermore, native species, like *Pteris umbrosa* in Australia, could be better candidates for phytoextraction than non-native and potentially invasive species like *P. vittata* (Koller et al., 2007).

Zhao et al. (2002) suggested that arsenic hyperaccumulation is a constitutive property in *P. vittata*, (i.e., it is expressed in all members of the species regardless of soil arsenic level) similarly to what was proposed for hyperaccumulation of metals generally (Pollard et al., 2000), and of both zinc and cadmium in *Arabidopsis halleri* and *Thlaspi caerulescens* more specifically (Baker and Whiting, 2002). The constitutive property of arsenic hyperaccumulation was later revealed in populations of *P. multifida*, *P. oshimensis* and *P. cretica* var. *nervosa* and confirmed in *P. vittata* (Wang et al., 2007).

Arsenic hyperaccumulation in P. vittata

The mechanisms of arsenic tolerance, translocation and transformation in *P. vittata* have been extensively reviewed (Rathinasabapathi et al., 2006; Xie et al., 2009; Danh et al., 2014) and will be discussed here only briefly. *P. vittata* primarily takes up arsenate and only limited arsenite, with the rate of arsenate uptake 10 times that of arsenite uptake (Wang et al., 2002). Like many plants, *P. vittata* takes up arsenate, a chemical analogue of phosphate, through the phosphate intake pathway, specifically through phosphate transporters in the root plasmalemma (Meharg and Hartley-Whitaker, 2002; Wang et al., 2002). Arsenite uptake is also efficient and occurs through an active transport process (Wang et al., 2011). *P. vittata* translocates and sequesters most accumulated arsenic in its pinnae (Lombi et al., 2002), though the rhizome can be a secondary storage organ when soil arsenic is highly available (Liao et al., 2004), and arsenic translocation decreases under metal stress (An et al., 2006). In pinnae, arsenic is compartmentalized in the vacuoles of pinnae epidermal cells (Lombi et al., 2002), located on pinnae surfaces as crystalline deposits (Datta et al., 2017), and in trichomes on pinnae surfaces (Li et al., 2005). Between 47 and 94% of the arsenic in *P. vittata* fronds is present as arsenite, the rest being present as arsenate (Ma et al., 2001; Lombi et al., 2002; Zhang et al., 2002; Tu et al., 2003). Indeed, during the course of arsenic translocation in the fern, arsenate is reduced to arsenite (Ma et al., 2001), although there is disagreement about whether reduction occurs in roots (Vetterlein et al., 2009), stipes (Webb et al., 2003) and/or pinnae (Pickering et al., 2006). Arsenite reoxidation was found in older fronds 18 weeks of age (Tu et al., 2003).

Combining simple and complex experimental systems

Processes including release of arsenic and nutrients from soil and transport of released constituents to *P. vittata* roots; arsenic uptake, translocation, and sequestration in *P. vittata* fronds; and *P. vittata* frond and root biomass production, affect arsenic accumulation in *P. vittata* and

therefore arsenic phytoextraction rates. Simple systems allowing detailed investigation of the mechanisms underlying the above processes must be combined with work approximating real-life phytoextraction conditions (i.e., work in historically contaminated soils under *in situ* conditions) to a) develop a mechanistic understanding of phytoextraction and b) apply this understanding to develop viable remediation methods. We acknowledge the inherent limitations to synthesizing the results of a wide body of work on arsenic phytoextraction that consists mainly of hydroponic and greenhouse/pot studies with few *in situ* studies, and to extrapolating the results of controlled conditions to field performance, characterized by inherent heterogeneity.

Hydroponic studies provide mechanistic information on *P. vittata* arsenic uptake into roots, but typically use much higher concentrations of arsenic (10^1-10^5 μg/L) than those found in the soil solution (1-4 μg/L) (Sadiq 1997; Wenzel et al. 2002; Fitz & Wenzel 2002, van der Ent et al., 2013). Extrapolating hydroponic studies to field conditions is challenging because soil properties influence (and likely dramatically decrease) the solubility and therefore the supply of nutrients and arsenic to the fern (Fitz and Wenzel, 2002).

Pot experiments with arsenic-spiked soils also likely overestimate arsenic accumulation due to high arsenic availability and ideal greenhouse conditions. Metal(loid)s added to soil typically as soluble salts (e.g., NaH_2AsO_4) under laboratory conditions are more plant-available and less heterogeneously distributed than metal(loid)s in contact with soil for longer times (i.e., years-centuries) (Basta et al., 2005), as is the case with most geogenic and anthropogenic soil arsenic. For example, total arsenic accumulation *per* plant was 4 times larger in *P. vittata* grown in soil spiked with arsenic, than in historically contaminated soil with similar arsenic concentrations (Cao et al., 2003).

Surveys of wild *P. vittata* populations (Liao et al., 2004; Wei and Chen, 2006; Wei et al., 2007) provide invaluable insight into behavior of well-established populations growing in historically contaminated soils, but comparisons of ecotypes is complicated by the uncertain phylogeny of *Pteris*, as *Pteris* could be more than one genus and hybridizing is common among fern species (Chao et al., 2014).

In situ conditions, characterized by heterogeneous distribution of soil arsenic, low and varied arsenic and nutrient phytoavailability, and climate stress, most closely approximate *P. vittata* cultivation for phytoextraction and therefore provide best estimates of *P. vittata* phytoextraction rates. Promisingly, the number of field studies is increasing (Salido et al., 2003; Kertulis-Tartar et al., 2006; Chen et al., 2010; Niazi et al., 2012; Yang et al., 2012, 2019; Lei et al., 2018; Ma et al., 2018). However, the complexity of *in situ* systems, especially high variability in arsenic concentrations, can mask treatment effects and changes in soil arsenic during phytoextraction (Kertulis-Tartar et al., 2006; Niazi et al., 2012). In some cases, limited replication (Salido et al., 2003; Kertulis-Tartar et al., 2006; Niazi et al., 2012) decreases the impact of results. Finally, only a few field studies investigate *P. vittata* field performance outside of the humid subtropics (Salido et al., 2003; Niazi et al., 2012). Rigorous *in situ* studies coupled to process-based experiments under controlled conditions are required to determine if arsenic phytoextraction can fully "emerge" as a viable remediation technology (Robinson et al., 2015).

Effects of soil conditions on phytoextraction with *P. vittata*

Effect of soil texture and mineralogy

Soil texture affects *P. vittata* frond arsenic concentrations and biomass. Arsenic is less mobile, and consequently less plant available in fine- than in coarse-textured soils (Jacobs and Keeney,

1970; Woolson et al., 1973) because it strongly associates with the clay particle size fraction, including iron oxides (Lombi et al., 2000; Smith et al., 2006). Although *P. vittata* can hyperaccumulate arsenic from soil textures ranging from loamy sand (2 to 4.5% clay) to silty clay (59% clay) (Cao et al., 2003; Fayiga et al., 2004; Lessl and Ma, 2013; Fitz et al., 2003; Caille et al., 2004b; Mandal et al., 2012; Wei et al., 2006), increasing clay content decreases *P. vittata* frond arsenic concentrations (Chen et al., 2002b; Tu and Ma, 2003a, 2005; Liao et al., 2004; Xu et al., 2010), biomass, and arsenic accumulation *per* plant (Liao et al., 2004). *P. vittata* removed arsenic associated with iron and/or aluminum oxides that decrease arsenic mobility (Gonzaga et al., 2008), but uptake of these forms was lower compared to more soluble forms of arsenic (Ma et al., 2001; Tu and Ma, 2002; Xu et al., 2010). While *P. vittata* serves as an indicator plant for calcareous soils and limestone (Chen et al., 2002c), it nonetheless hyperaccumulated arsenic from acidic soils (Xu et al., 2010), so soil pH could be less important than texture in limiting phytoextraction.

Effects of soil arsenic concentrations

Arsenic hyperaccumulation is defined as an aboveground arsenic concentration threshold, but the trait is also dependent on a soil arsenic threshold. In soils with historical arsenic concentrations lower than 70 mg/kg, hyperaccumulation has only rarely been reported (Mandal et al., 2012), with *P. vittata* frond arsenic concentrations typically lower than 600 mg/kg (Caille et al., 2004; Gonzaga et al., 2008; Lessl and Ma, 2013). Hyperaccumulation is well established in *P. vittata* grown in sandy soils with 105-130 mg arsenic/kg (e.g. Cao et al., 2003; Fayiga et al., 2004; Lessl and Ma, 2013). At low soil arsenic concentrations (<50 ppm), arsenic concentrations were higher in young fronds, suggesting some arsenic is beneficial for growth, whereas at concentrations >100 mg/kg, arsenic concentrations were higher in older fronds (Tu and Ma, 2002, 2005), suggesting a shift in tolerance mechanisms occurs between low and moderate soil arsenic concentrations.

P. vittata tolerates very high concentrations of arsenic in soil. *P. vittata* grown in soils spiked with arsenic tolerated 500 mg arsenic/kg with phytotoxic effects, but died when exposed to 1,000 mg arsenic/kg (Tu and Ma, 2002). The soil spiked with 500 mg arsenic/kg contained 78-89 mg/kg water soluble arsenic (Tu and Ma, 2002), about 4 times the soluble arsenic predicted for historically contaminated soils (Wenzel et al., 2002). Naturally occurring populations of *P. vittata* hyperaccumulated arsenic from soils with more than 38,000 mg arsenic/kg and up to 1,000 mg/kg available arsenic (Liao et al., 2004; Wan et al., 2013). Aboveground biomass was smaller than in populations growing at sites with lower total and available arsenic (Liao et al., 2004; Wan et al., 2013), suggesting phytotoxicity (Liao et al., 2004) or energy reallocation away from biomass production towards arsenic tolerance (Audet and Charest, 2008). Even when frond arsenic concentrations are high, lower biomass can lead to lower phytoextraction efficiency. For example, frond arsenic concentrations were the same in ferns grown in soils with high and dilute arsenic concentrations, but more arsenic was removed by larger ferns in the diluted soil (Wu et al., 2009).

P. vittata populations adapt to exclude arsenic from aboveground biomass in high-arsenic habitats (Wan et al., 2013). When exposed to the same arsenic level, translocation was greater in ferns from low-arsenic habitats (Wan et al., 2013). Translocation decreased and rhizome storage increased in some *P. vittata* populations growing in soils with moderate (10^2 to 10^3 mg/kg) as well as high (10^4 mg/kg) soil arsenic levels (Liao et al., 2004). Arsenic phytoextraction could be optimized using a population from an area with low arsenic concentrations in soil.

Effects of metals

P. vittata excludes metals (lead, copper, nickel, chromium(VI), cadmium and zinc) from aboveground biomass instead of hyperaccumulating them, such that metal concentrations are higher in roots than fronds (Baker, 1981, 1987; Nishizono et al., 1987; Fayiga et al., 2004; An et al., 2006; Kachenko et al., 2007; Wu et al., 2007; Xiao et al., 2008; De Oliveira et al., 2014; Wan et al., 2014). The fern's tolerance mechanisms for metals and arsenic function together up to certain metal concentrations, enabling phytoextraction to occur in the presence of moderate soil concentrations of metals (Caille et al., 2004), though frond arsenic concentrations decreased as soil metal concentrations increased (An et al., 2006; Fayiga et al., 2004; Fayiga and Ma, 2006; Xiao et al., 2008). However, high levels of metals in soil (arsenic, copper, zinc, manganese on the order of 10^3 mg/kg and lead up to 10^4 mg/kg) (Caille et al., 2004; Wu et al., 2007, 2009) inhibit arsenic hyperaccumulation in *P. vittata* (Caille et al., 2004; Wu et al., 2009), mainly due to decreases in root biomass (Caille et al., 2004). Both metal exclusion (Baker, 1987) and metal phytotoxicity (Caille et al., 2004; Fayiga et al., 2004; Ciurli et al., 2014) can lead to lower biomass, higher mortality (Ciurli et al., 2014), and lower phytoextraction efficiency.

Notably, lead inhibited arsenic hyperaccumulation in naturally-occurring populations of *P. vittata* growing on mine wastes containing higher levels of lead than arsenic (Zheng et al., 2003; Wu et al., 2007; Wan et al., 2014), but lead had no effect in studies with spiked soils and solution culture (Fayiga et al., 2004; Wan et al., 2014). Precipitation of arsenic-lead mineral phases over long equilibration times could cause low arsenic availability in *in situ* conditions (Zheng et al. (2003).

Metal tolerance and hyperaccumulation of arsenic in the presence of metals vary with fern population. Wu et al. (2009) found the ability to tolerate metals could be constitutive in *P. vittata*, since metallicolous fern populations were not more tolerant to metals than the nonmetallicolous ferns. However, arsenic uptake in the presence of metals was higher in ferns from nonmetallicolous than metallicolous ecotypes, suggesting low arsenic uptake could be interpreted as an adaptive tolerance mechanism and not interference of metals with uptake (Wu et al., 2009). Arsenic phytoextraction could be optimized using a nonmetallicolous *P. vittata* ecotype.

Mechanisms for arsenic release from soil

Arsenic uptake, and therefore hyperaccumulation, is likely linked to nutrient uptake in *P. vittata*. According to the phosphorus starvation theory, arsenic could be released from soil and taken up into the fern as a byproduct of nutrient scavenging processes (Meharg and Hartley-Whitaker, 2002; Audet, 2012). In response to nutrient deficits, *P. vittata* likely releases root exudates (Fitz et al., 2003; Tu et al., 2004; Gonzaga et al., 2006; Lessl and Ma, 2013) that release phosphorus, iron, and arsenic through ligand-enhanced dissolution of iron minerals (Furrer et al., 1986; Fitz et al., 2003; Reichard et al., 2007). *P. vittata* exudates include oxalic ($C_2H_2O_4$), malic ($C_4H_6O_5$), and phytic ($C_6H_{18}O_{24}P_6$) acids (Tu et al., 2004; Lessl and Ma, 2013; Liu et al., 2016). Oxalic and malic acids are common root exudates across plant communities (Jones, 1998). Phytic acid is less well known as a root exudate but is released by fern roots (Tu et al., 2004; Liu et al., 2016). Oxalic acid, in particular, is an effective metal complexer (Keiluweit et al., 2015; Chen et al., 2016a), as is phytic acid (Shang et al., 1992), such that iron-oxalate and -phytate complexes lead to dissolution of iron oxides (Liu et al., 2017a).

Both oxalic and phytic acid lead to release of arsenic from iron oxides and to higher *P. vittata* frond arsenic concentrations (Liu et al., 2016, 2017a). However, *P. vittata* produced 4 to 7 times

more phytic acid than oxalic acid (Tu et al., 2004), and phytic acid released 1.2 to 50 times more arsenic from $FeAsO_4$ than oxalic acid (Tu et al., 2004; Liu et al., 2016). Indeed, the ability to sustain phytic acid production in the presence of arsenic could be a characteristic of arsenic tolerance in *P. vittata*, as phytic acid release from non-hyperaccumulating ferns decreased in the presence of arsenic (Tu et al., 2004; Liu et al., 2016). Additionally, rhizosphere microbial activity, especially siderophore release, increased arsenic release in the presence of phytic acid (Reichard et al., 2007; Liu et al., 2017a).

But the link between exudate compounds and nutrient uptake in *P. vittata* is less clear. *P. vittata* frond phosphorus concentrations increased when ferns were supplied with higher (Liu et al., 2016, 2017b) but not lower (Liu et al., 2017a, 2017b) concentrations of phytic acid. It remains unclear how phytic acid release relates to *P. vittata* phosphorus budgets. *P. vittata* could use externally supplied phytic acid as a phosphorus source (Liu et al., 2016). Phytase present in *P. vittata* root exudates (Lessl et al., 2013; Liu et al., 2016) could help the fern recover released phytic acid (Brinch-Pedersen et al., 2002). Furthermore, *P. vittata* frond iron concentrations increased when ferns were supplied with phytic acid and $FeAsO_4$ (Liu et al., 2016), but not with oxalic acid and phytic acid and arsenic-goethite (Liu et al., 2017a). When ferns were supplied with oxalic acid, phytic acid, and/or siderophores, iron increased the most in siderophore-supplied ferns, but biomass increased the most in ferns supplied with the combination (Liu et al., 2017a).

In addition to acting through ligand-exchange mechanisms, root exudates could affect rhizosphere pH. *P. vittata* rhizosphere pH, a key factor in arsenic release from iron oxides, is not well understood. Compared to bulk soil, *P. vittata* rhizosphere pH has been found to be lower (Gonzaga et al., 2009), similar (Fitz et al., 2003), or higher (Gonzaga et al., 2006; Das et al., 2017). Counterintuitively, decreasing pH below neutral would increase iron oxyhydroxide solubility (Hem, 1972) but also increase the amount of arsenic sorbed (Dixit and Hering, 2003). pH also affects root exudate behavior. Oxalate has a higher affinity for iron at lower pH (Kraemer, 2004), so decreasing pH could increase ligand-enhanced dissolution of iron oxides, increasing arsenic release. Alternately, oxalic and acetic acid release was shown to increase soil pH adjacent to artificial roots, possibly due to mineral dissolution or dissimilatory metal reduction (Keiluweit et al., 2015), which would also increase arsenic release.

Soil treatments to increase phytoremediation efficiency

Because nutrient and arsenic uptake in *P. vittata* appear integrally related (Lessl and Ma, 2013), considerable research has explored use of soil amendments, including fertilizers, compost, chelating agents, and/or mycorrhizal fungi, to increase phytoextraction rates. Applying soil amendments to *P. vittata* is challenging because the fern appears to be well-adapted to low nutrient soils (Wei et al., 2006; Wang et al., 2019) and have low nutrient requirements (Han et al., 2005; Amatangelo and Vitousek, 2008). However, the goal of soil amendment is not necessarily to meet *P. vittata* nutritional needs, but also to increase arsenic availability in soil.

Fertilization with phosphorus

Phosphorus application increased arsenic phytoextraction from historically contaminated and arsenic-spiked soils with a range of textures from sandy to silty clay (Chen et al., 2002b; Cao et al., 2003; Tu and Ma, 2003a; Fayiga and Ma, 2006; Mandal et al., 2012; Lessl and Ma, 2013), though in other cases phosphorus application decreased (Shelmerdine et al., 2009) or had no effect on arsenic phytoextraction (Chen et al., 2002b; Cao et al., 2003; Tu and Ma, 2003a; Caille et al.,

2004; Fayiga and Ma, 2006). Sparingly soluble phosphorus more successfully promoted phytoextraction than soluble phosphorus (Cao et al., 2003; Lessl and Ma, 2013).

Beyond plant nutrition, phosphorus application in arsenic phytoextraction is interesting because arsenate and phosphate behave similarly in soil, so phosphate addition could stimulate arsenic uptake. In soil, phosphate can compete with arsenate for sorption sites and release arsenic to solution (Peryea, 1991). Because phosphate has a smaller ionic radius and more dense charge distribution (Wenzel et al., 2001), it could have a higher binding affinity for soil than arsenate (Parfitt, 1979). However, desorption of arsenic in the presence of phosphorus is incomplete (Smith and Naidu, 2009) and could be rate-limited due to diffusion of arsenic into the sorbent (Darland and Inskeep, 1997). In plants, phosphate can compete with arsenic for uptake at the phosphate intake pathway (Meharg and Hartley-Whitaker, 2002), though it is unclear whether the fern's phosphate transporter system has a higher affinity for phosphate or arsenate (Wang et al., 2002; Poynton et al., 2004). Consequently, it is possible that high levels of phosphate in soil could competitively desorb arsenic from mineral surfaces, increase arsenic availability for the fern, limit arsenic uptake by *P. vittata* due to competition for the transporters, and/or promote fern growth leading to increases in arsenic removal from soil.

Phosphorus increases the mobility of arsenate in the soil solution (Peryea, 1991; Manning and Goldberg, 1996a; Smith et al., 2002), which could make it more available for plant uptake. In hydroponic studies, phosphorus outcompeted arsenate, and to a lesser extent arsenite, for uptake in *P. vittata* over short timescales of hours to months (Wang et al., 2002; Tu and Ma, 2003b; Huang et al., 2007; Fayiga et al., 2008; Natarajan et al., 2009). This apparent competition was not observed over longer time scales or within field-relevant concentration ranges in artificial growth media, nor in soil (Natarajan et al., 2009; Vetterlein et al., 2009), suggesting the fern could express different phosphorus transporters to maintain phosphorus uptake even under competition from arsenic (Vetterlein et al., 2009). Additionally, *P. vittata* took up arsenic made available by phosphorus addition (Mandal et al., 2012), suggesting in some cases phosphorus competes more effectively at soil sorption sites than at the root intake pathway. In other cases the fern did not take up arsenic made available through phosphorus addition (Cao et al., 2003; Caille et al., 2004), indicating phosphorus competition in both soil and root uptake. Furthermore, in sludge-amended soil with very high available phosphorus, phosphorus successfully competed with arsenic for uptake (Shelmerdine et al., 2009).

P. vittata is sensitive to phosphorus availability and requires only a small amount of phosphorus, though demand could increase with stress. In coarse-textured soil, soluble phosphorus application kept *P. vittata* alive but did not promote growth or arsenic accumulation to the extent sparingly soluble phosphorus did (Lessl and Ma, 2013). In fine-textured soil, soluble phosphorus appeared to exchange for arsenic on sorption sites, mobilizing arsenic to increase *P. vittata* uptake, but did not affect biomass, suggesting phosphorus itself was not available for uptake (Chen et al., 2002b; Mandal et al., 2012). Under conditions of arsenic phytotoxicity, increasing soluble phosphorus supply alleviates stress and promotes growth (Tu and Ma, 2003a). In other cases, supplying *P. vittata* with phosphorus did not affect arsenic accumulation (Chen et al., 2002b; Cao et al., 2003; Tu and Ma, 2003a; Fayiga and Ma, 2006), perhaps due to high arsenic availability or a large soil sorption capacity for phosphorus and arsenic.

Fertilization with nitrogen, sulfur, potassium, and calcium

Nitrogen is well known to increase above and below ground plant biomass, and affect soil pH (Marschner, 1995). Surprisingly, contrary to expected anion-balancing effects, nitrate (NO_3^-) more

effectively promoted frond biomass and arsenate uptake from solution culture than ammonium (NH_4^+) (Fayiga et al., 2008). However, in sandy soil, both NH_4^+-N and NO_3^--N equally increased arsenic accumulation in *P. vittata* through an increase in frond biomass but not root biomass nor frond arsenic concentrations, possibly due to a dilution effect (Liao et al., 2007).

Sulfur is a component of the anti-oxidant glutathione (GSH), which helps neutralize arsenic toxicity in plants (Meharg and Hartley-Whitaker, 2002), and consequently sulfur application could increase the fern's ability to withstand arsenic stress. Positive effects of sulfur application on arsenic phytoextraction in simple systems, including increases in GSH (Wei et al., 2010) and arsenic (Vetterlein et al., 2009; Wei et al., 2010; De Oliveira et al., 2014) concentrations in *P. vittata*, suggests sulfur application should be further investigated in soils. Like phosphate, sulfate can decrease arsenic sorption in soil (Xu et al., 1988) and could increase arsenic availability to *P. vittata* in the soil solution.

Few studies have investigated the effects of potassium, a major plant nutrient, on arsenic uptake in *P. vittata*. Potassium presence in solution culture increased *P. vittata* biomass and sometimes arsenic uptake (Fayiga et al., 2008). Frond potassium and arsenic were co-located in *P. vittata* pinnae (Lombi et al., 2002) and concentrations were positively correlated in *P. vittata* grown under greenhouse conditions in a sandy soil (Tu and Ma, 2005) and under natural conditions in a clay soil (Wei et al., 2006). Many plants use potassium to maintain charge balance during anion transport and storage in vacuoles (Marschner, 1995). The correlation between potassium and arsenic in *P. vittata* pinnae suggests that at least some arsenic is still present as the arsenate anion during xylem transport and when taken into pinnae cell vacuoles, and only later reduced to the neutral arsenite species.

Effects of calcium on arsenic uptake in *P. vittata* are inconsistent. Although like potassium, calcium is associated with maintaining charge balance in vacuoles (White and Broadley, 2003), this does not appear to be the case in *P. vittata*, as calcium was negatively correlated with frond arsenic concentrations in a naturally-occurring population (Wei et al., 2006). Calcium promoted arsenic uptake in *P. vittata* when supplied hydroponically (Fayiga et al., 2008). In soils, solubility might explain effects of calcium on *P. vittata*, as solubility of calcium arsenates varies widely (Bothe and Brown, 1999a). *P. vittata* took up arsenic as calcium-arsenate more effectively than other forms of arsenic (Tu and Ma, 2002). Addition of calcium-phosphate increased frond arsenic and calcium concentrations, indicating calcium-phosphate is solubilized during *P. vittata* growth (Fayiga and Ma, 2006). However, calcium supplied as lime did not increase *P. vittata* arsenic uptake and instead decreased arsenic availability, perhaps due to formation of sparingly soluble calcium arsenate phases (Bothe and Brown, 1999b; Caille et al., 2004).

Compost addition

Compost could be an important amendment for promoting *P. vittata* growth in very low-fertility matrices like some mine wastes. Under harsh field conditions, compost application increase *P. vittata* survival from 50% to 85% (Ciurli et al., 2014). However, care should be taken when working with a negatively charged substrate like compost to avoid arsenic leaching. Compost addition increased arsenic mobilization in the soil solution (Cao et al. 2003; Yan et al. 2012; Fleming et al. 2013), likely because compost increases anionic organic compounds in soil, which compete with anionic arsenate for soil sorption sites on iron and Al oxides/hydroxides (Xu et al., 1988). Under greenhouse conditions *P. vittata* appears to not take up this mobilized arsenic, perhaps because phosphorus in compost competes with arsenic for uptake. Treating soil with compost did not increase frond arsenic concentrations and total arsenic accumulation in *P. vittata*

(Cao et al., 2003; Yan et al., 2012), and had no effect on *P. vittata* biomass, leaving labile arsenic higher in compost-treated soil than in untreated controls after fern growth (Yan et al., 2012).

Chelating agents

Although chelating agents have been explored extensively to increase efficiency in phytoextraction of metals (i.e., lead) (Chaney et al., 2007), few studies have explored treating soil with chelating agents to increase arsenic (and phosphorus) availability to *P. vittata* (Yan et al., 2012). Chelating agents could increase arsenic availability *via* surface complexation with iron- and aluminum-oxides, releasing inorganic arsenic to solution. The abiotic chelating agent sodium polyacrylate ($(C_3H_3NaO_2)_n$) increased arsenic availability in soil and accumulation in *P. vittata*, though EDTA ($C_{10}H_{16}N_2O_8$) only increased arsenic availability (Yan et al., 2012). Some siderophores (biological chelating agents) were highly effective at facilitating arsenic uptake in the fern, while others led to higher soluble arsenic concentrations but did not facilitate arsenic uptake. Siderophores can increase iron oxyhydroxide dissolution rates in partnership with organic acids (Reichard et al., 2007). Arsenic accumulation increased dramatically to 34 times the control in *P. vittata* supplied with phytate, a siderophore (PG-12) and arsenic-goethite, while iron increased 1.5 times (Liu et al., 2017a), indicating phytate and the siderophore together were very effective in releasing arsenic and iron through ligand-enhanced dissolution of goethite. However, in the same study, a different siderophore (DFO) led to increases only in soluble arsenic but not in *P. vittata* arsenic uptake, perhaps because the DFO affected arsenic speciation and reduced arsenic, limiting uptake (Liu et al., 2017a). Concern persists that amending soil with chelating agents promotes leaching over contaminant phytoextraction (Chaney et al. 2007, Yan et al., 2012).

Inoculation with mycorrhizal fungi

Mycorrhizal associations benefit host plants by improving access to nutrients including nitrogen (Ames et al., 1983) and phosphorus (Hattingh et al., 1973), and to water, by increasing the soil volume accessible to the plant, thus potentially increasing remediation efficiency. *P. vittata* forms associations with many mycorrhizal fungi (Leung et al., 2006; Wu et al., 2007), especially arbuscular mycorrhizal fungi species, primarily in the *Glomus* genus (Wu et al., 2007).

Inoculation with mycorrhizal fungi increased *P. vittata* biomass (Al Agely et al., 2005; Liu et al., 2005; Leung et al., 2006, 2010, 2013; Trotta et al., 2006), which could be a sign that the mycorrhizae were helping the plants access more nutrients. Species shown to increase *P. vittata* biomass include *Funneliformis mosseae* (previously *Glomus mosseae*) (Liu et al., 2005; Trotta et al., 2006; Leung et al., 2013), *Rhizophages intraradices* (previously *G. intraradices*) (Leung et al., 2013), *Gigaspora margarita* (Trotta et al., 2006), and indigenous mycorrhizal fungi (IM) associated with *P. vittata* growing at contaminated sites (Al Agely et al., 2005; Leung et al., 2006, 2010, 2013). *F. mosseae,* alone or mixed with other mycorrhizal fungi, was the most effective at promoting biomass production in *P. vittata* (Liu et al., 2005; Trotta et al., 2006; Leung et al., 2010, 2013). In particular, inoculation with a mixture of *F. mosseae*, IM, and phosphate rock was most effective at increasing biomass of ferns planted in a highly contaminated mine soil (9600 mg/kg arsenic) (Leung et al., 2010).

In ferns planted in soil spiked with arsenic, frond arsenic concentrations increased when ferns were inoculated with mining site IM (Leung et al., 2006, 2013), *G. intraradices*, *F. mosseae*, or mixtures of the IM with either *Glomus* species (Leung et al., 2013). Mixtures of mining site IM with either *F. mosseae* or *G. intraradices* were equally or more effective than the individual treatments at increasing frond arsenic concentrations and arsenic accumulation (Leung et al.,

2013), suggesting that different species fill different roles with the net effect of increased arsenic uptake in the fern. For example, mixtures of the IM with either *F. mosseae* or *G. intraradices* increased arsenic reductase activity in root extracts, more than the individual species or IM alone (Leung et al., 2013).

Effects of fungi on biomass and therefore arsenic uptake could be related to arsenic availability. Arbuscular mycorrhizal fungi increased arsenic uptake (total uptake and/or translocation) in *P. vittata* when the ferns were grown in media with potentially higher arsenic availability (solution culture, spiked soils, highly contaminated soils) (Al Agely et al., 2005; Liu et al., 2005; Leung et al., 2006, 2010, 2013; Trotta et al., 2006), though uptake was not affected when ferns were grown in a moderately contaminated soil where arsenic could have been less available (Liu et al., 2005) or at lower spiked concentrations (Al Agely et al., 2005). In cases where fungi inoculation did not lead to increases in arsenic uptake, fungi could have transported phosphorus instead of arsenic, and therefore contributed to increases in biomass (Liu 2005). Phosphorus and nitrogen content increased in *P. vittata* fronds and roots associated with mycorrhizal fungi (Liu et al., 2005; Trotta et al., 2006; Leung et al., 2013).

Because most studies have occurred in arsenic-spiked soils, more work is required to elucidate whether mycorrhizae preferentially transport phosphorus over arsenic to the fern under conditions of low arsenic and phosphorus availability in soil. Furthermore, as with other fertilizers, the interesting results obtained with mixtures of mycorrhizal fungal species, especially with phosphate rock, suggests that complex assemblages are required to optimize arsenic accumulation in *P. vittata*.

Remediation efficiency

Phytoextraction efficiency can be evaluated based on fern arsenic uptake rates and/or depletion of total or extractable arsenic from soil as a function of time. Linear or modeling-based estimates of changes in soil arsenic as a function of time can be used to predict time to achieve cleanup goals. Uncertainty around changes in arsenic availability as arsenic concentrations decrease, growth of *P. vittata* roots and aboveground biomass, and spatial heterogeneity of arsenic distribution in soil limit estimates of remediation rates and remediation time, and ultimately practical application of phytoextraction (Gerhardt et al., 2009; Robinson et al., 2015).

Fern arsenic uptake is the easiest phytoextraction metric to measure (Gerhardt et al., 2009; Niazi et al., 2012). In a long term container study where ferns were supplied with phosphate rock, arsenic uptake rates fluctuated over the study around an average of 147 mg/plant/year (36.9 kg/ha/yr at 15 cm spacing) with no clear trend of increases or decreases with time (da Silva et al., 2018). Based on average and maximum plant arsenic uptake in the first 2.5 years of this same study, Lessl and Ma (2013) calculated 6-7 years to remediate soil containing 125 mg/kg arsenic down to a local target cleanup level of 2.1 mg/kg (Lessl and Ma, 2013). But in an *in situ* field study, *P. vittata* arsenic uptake decreased from 57 to 7 mg/plant over 27 months due to decreases in frond arsenic concentrations and biomass (Niazi et al., 2012). Similarly, *P. calomelanos* arsenic accumulation decreased over 27 months from 124 to 40 mg/plant (2012). Based on fern arsenic uptake trends over 3 years, Niazi et al. (2012) estimated 55 and 143 years to remediate arsenic in surface soils from about 900 mg/kg to the threshold of 20 mg/kg, at 30 cm spacing.

Basing remediation rates and remediation time estimates on changes in soil arsenic can be more challenging due to *in situ* spatial heterogeneity in soil arsenic distribution. Soil arsenic decreased by 47% (from 129 to 69 mg/kg) over 5 years when *P. vittata* was grown outdoors in

large containers filled with CCA-contaminated soil and supplied with phosphate rock (da Silva et al., 2018). In *in situ* field studies, no significant change was observed in surface soil arsenic concentrations over 2 years of *P. vittata* growth (Kertulis-Tartar et al., 2006; Niazi et al., 2012), though concentrations significantly decreased over 2 years of *P. calomelanos* growth (Niazi et al., 2012). Soil arsenic heterogeneity could explain the differences in phytoextraction rates and times found in the container and *in situ* field studies. Distribution of arsenic in soil in the container study could have been less heterogeneous than in *in situ* conditions, such that changes in concentrations could have been significant in the container study but masked by variability in the *in situ* studies.

Furthermore, under *in situ* field conditions, density and depth distribution of roots are likely to be heterogenous, contributing to slower phytoextraction rates (Gerhardt et al 2009). It is not well understood from what distance in soil arsenic is transported to the root, though arsenic depletion could be limited to rhizospheric soil, as with phosphorus (Gahoonia et al., 1994), given the importance of rhizosphere processes mobilizing arsenic. The high phytoextraction rate found by Lessl and Ma (2013) and da Silva could be due to *P. vittata* being root-bound in the large containers by the end of the study (da Silva et al., 2018), such that roots accessed the entire 35 cm soil depth and most soil could have been under rhizosphere influence. In contrast, in natural *P. vittata* populations, root density was the highest in the 0-10 cm depth (Liao et al., 2004), limiting phytoextraction to surface soils. Furthermore, root turnover will affect the volume of extracted soil over time and deserves investigation for *P. vittata*, as for phytoremediation of petroleum-contaminated soils (Thoma et al., 2003), including whether arsenic, like nutrients, is taken up primarily from young roots (Yanai, 1994), especially the root tip (Marschner, 1995), or also from older roots (Clarkson et al., 1968; Marschner, 1995).

Soil arsenic decreases should be linked to fern arsenic uptake through rarely-calculated mass balances (Robinson et al., 2015). In one field study, the change in mean soil arsenic concentrations (404 kg/ha) grossly outweighed the uptake in *P. vittata* (9.7 kg/ha), with an even greater disparity for *P. calomelanos* (Niazi et al., 2012). The disparity could be due to high variability masking true changes in soil arsenic, and/or due to arsenic leaching from soil (Yang et al., 2012). Even in the container study where ferns were root bound and soil was presumably well-mixed (da Silva et al., 2018), we calculated that 29% of initial arsenic could not be accounted for at the end of the study. Inputs to soil arsenic stocks should also be assessed during phytoextraction. Arsenic inputs from air pollution explained the increase in soil arsenic concentrations during one phytoextraction study (Lei et al., 2018), while phytoenrichment of arsenic in surface soil due to arsenic leaching from fronds (Yan et al., 2009) or litter fall, as observed with other hyperaccumulators (Liu et al., 2020), could explain greater decreases in soil arsenic at depth than in surface soils (Kertulis-Tartar et al., 2006; Ma et al., 2018).

Because plant arsenic uptake is the easiest way to quantify remediation rates once phytoextraction has started, it would be useful to correlate plant arsenic accumulation and soil depletion and predict this in advance based on soil properties. Nonlabile arsenic, the pool from which labile and soluble arsenic are derived, could be better correlated with arsenic uptake during active growth (Lessl et al., 2014). A Free Ion Activity Model (Shelmerdine et al., 2009) that captures the ability of soil to resupply soluble arsenic from recalcitrant fractions in the absence of fern growth could better predict arsenic uptake in advance of fern growth.

From research to practical application

This review focused on soil geochemical processes governing transport of arsenic from soil to the roots of arsenic hyperaccumulating ferns. Evidence suggests root exudates, especially phytic acid, release arsenic from soil through ligand-enhanced dissolution of iron minerals. More research is needed to determine from what distance in soil arsenic travels to roots for uptake, and if arsenic released from soil might leach instead of being taken up into *P. vittata*. Efforts to increase remediation rates seem most successful when nutrients are supplied in sparingly-soluble forms or with an ecologically-based approach combining nutrient sources and biological inputs (for example, mycorrhizal fungi and phosphate rock), and more work is needed in this direction. Nonetheless, in the absence of easy solutions in to increase *in situ* uptake rates dramatically, phytoextraction appears limited to soils with low arsenic concentrations, according to the "gentle remediation" or "soil polishing" approach (Gerhardt et al., 2016).

Indeed, there is considerable debate whether phytoextraction can be successfully applied (Gerhardt et al., 2009, 2016; Robinson et al., 2015), with few known industrial-style test sites in the United States. In China, the jump from research to practice occurs through on the hectare scale in farmland trials (Chen et al., 2007b, 2010). We take from these reports that arsenic hyperaccumulators could grow best in their native habitat, but even then, fern arsenic uptake can decrease dramatically over 27 months (Niazi et al., 2012), presumably due to field stress exacerbating uptake already low due to root and soil arsenic heterogeneity. These results point to a paradox where arsenic hyperaccumulators are niche plants very hardy in certain conditions (e.g., growth on walls) but outside of these conditions, apparently are challenging to cultivate on the long timescales needed for remediation. Finally, the inherent heterogeneity of arsenic distributions in soil needs to be addressed to determine mass balances comparing fern accumulation to soil arsenic depletion, perhaps through use of smaller well-mixed plots within larger field applications. The gross discrepancy between fern arsenic uptake and soil arsenic depletion in long-term studies is concerning and suggests we need to further quantify parts of the arsenic cycle during phytoextraction, including leaching and phytoenrichment. To advance arsenic phytoextraction, we need systematic studies coupled at root-, plant-, and field-scales to integrate soil biogeochemical and plant physiological perspectives on arsenic cycling and inform practical cultivation methods.

Chapter 2. Poor *Pteris vittata* performance in a long-term field study indicates limits to cultivating hyperaccumulators for phytoextraction

Abstract

To apply arsenic phytoextraction, we must increase our understanding of how to optimize biomass production, arsenic accumulation, and survival in the arsenic-hyperaccumulating fern *Pteris vittata* under field conditions over time. In a 4-year field study, we investigated the effects of nutrient application (compost, inorganic and organic nitrogen, inorganic or organic phosphorus) and soil texture (13% and 35% clay) on arsenic phytoextraction with *P. vittata* in moderately contaminated soils. We found the highest remediation rates, 5.06±0.85 kg As/ha/yr, in coarse-textured unamended soil after 3 years of phytoextraction. Across soil textures, nitrogen or phosphorus application led to a 60% decrease in mean arsenic concentrations in fronds, leading to mean remediation rates 54% lower than in control ferns. In fine-textured soil, frond arsenic concentrations were 54% lower than in coarse-textured soil, ranging from 135.3 to 1283.1 mg/kg, compared to 359.2 to 2159.3 mg/kg in coarse-textured soil. Furthermore, in fine-textured soil, fern survival decreased from year 3 to 4 compared to coarse-textured soil. Across soil type, compost application increased fern survival, with 55 to 97% of compost-amended ferns surviving compared to 41 to 62% of control ferns. After 3 to 4 years of phytoextraction, total soil arsenic concentrations increased, but concentrations did not change in non-phytoextracted control soils. In phytoextracted coarse-textured soils, soil arsenic concentrations were 47% higher in the 0-15 cm depth than in the 15-30 cm depth, whereas in fine-textured phytoextracted soils the reverse was true with the bottom depth 20% higher than in the top depth. We attribute the increase in coarse-textured soil arsenic concentrations to arsenic relocation and enrichment due to leaching of arsenic from *P. vittata* aboveground biomass. Phytoextraction did not affect relative bioaccessibility of soil arsenic. Remediation time estimates ranged from 30 to 5,563 years to removal total soil arsenic to background levels, were higher in fine-textured soils, and increased with time.

Introduction

Widespread contamination of soil with arsenic, a carcinogen, presents health risks to human and ecological receptors globally (Crecelius et al., 1974; Colbourn et al., 1975; Wang and Mulligan, 2006; Singh et al., 2015). Remediation methods suitable for moderately contaminated soils (e.g. farmland) are urgently needed to supplement targeted use of conventional excavation-based methods for high risk areas (Tack and Meers, 2010). Arsenic phytoextraction, or plant-based removal of arsenic from soil, with the arsenic-hyperaccumulating fern *Pteris vittata,* is an emerging technology potentially suitable to remediate large, moderately contaminated areas (Ebbs et al., 2010).

Long term field studies are required to bridge the gap between research and practical application, constrain remediation rates, and predict remediation times (Robinson et al., 2015). Existing field studies found that slow remediation rates led to estimates of several decades to deplete 390 kg As/ha (100 mg As/kg) from soil (Kertulis-Tartar et al., 2006; Niazi et al., 2012; Lessl and Ma, 2013), indicating a need to optimize *P. vittata* biomass production, arsenic accumulation, and survival in field applications. Effects of nutrient application on arsenic accumulation in *P. vittata* have been extensively investigated in greenhouse and container studies. In such studies, *P. vittata* frond biomass increased with nitrogen application, leading to greater

mass of arsenic accumulated (Liao et al., 2007). Frond biomass and arsenic concentrations were greater with application of sparingly-soluble compared to soluble phosphorus (Lessl and Ma, 2013; da Silva et al., 2018), while soluble phosphorus application led to increases in arsenic solubility but not *P. vittata* biomass or arsenic concentrations (Caille et al., 2004). Likewise, arsenic solubility, but not arsenic mass accumulated *per* plant, increased with compost application (Cao et al., 2003; Yan et al., 2012). Furthermore, mycorrhizal associations can increase host plant uptake of nutrients including nitrogen (N) (Ames et al., 1983) and P (Hattingh et al., 1973), and *P. vittata* associates with mycorrhizal fungi (Leung et al., 2006; Wu et al., 2007). These findings from greenhouse and container studies might not apply to multi-season *P. vittata* production under *in situ* field conditions (Gerhardt et al., 2009), where root volume is unconstrained yet mass of soil *per* plant is much higher (Neugschwandtner et al., 2008) and plants face field stress (Ciurli et al., 2014).

Additionally, soil arsenic phytoavailability could affect *P. vittata* remediation rates. Arsenic is primarily found associated with iron and aluminum oxides in the clay fraction of soil (Lombi et al., 2000; Smith et al., 2006), such that arsenic phytoavailability could decrease in fine-textured soils (Jacobs and Keeney, 1970; Woolson et al., 1973). Arsenic hyperaccumulation in coarse-textured soils is well-established (e.g. Tu and Ma, 2003a; Silva Gonzaga et al., 2006; Lessl and Ma, 2013; da Silva et al., 2018). Few studies compare arsenic accumulation in *P. vittata* from soils of different textures, but a survey of a natural population of *P. vittata* found that biomass and frond arsenic concentrations were 2 to 3 times higher in loam soils than clay soils with similar arsenic concentrations (69 to 83 mg/kg arsenic) (Liao et al., 2004). Similarly, in a pot study, arsenic concentrations were lower in a soil with 59% compared to 10% clay, although aboveground biomass was similar in the two soils (Xu et al., 2010).

P. vittata arsenic uptake can affect soil arsenic availability. Depletion of phytoavailable arsenic could lead to decreases in remediation rates over time, but could also lead to shorter remediation times through the concept of bioavailable contaminant stripping, where remediation progress is measured against thresholds for bioavailable, not total, arsenic (Fitz et al., 2003). Bioavailable contaminant stripping theory suggests arsenic availability to plant, human, and environmental receptors is linked, such that plant uptake of arsenic decreases plant available and therefore human bioaccessible arsenic.

We present a four-year field study that determines effects of nitrogen, phosphorus, and compost application and soil texture on arsenic accumulation in *P. vittata*, and compares bioaccessibility of arsenic in phytoextracted to control soils. We hypothesized that nutrient application would increase arsenic accumulation due to increases in fern biomass; that arsenic would be more phytoavailable in coarse-textured soils; and that soil arsenic concentrations and relative arsenic bioaccessibility would decrease after four years of phytoextraction.

Methods

Study site and experimental design

A field site (84 m^2) was established at prospective urban agriculture site, an abandoned railroad right-of-way (Berkeley, CA, USA) in soils of two textures and with elevated arsenic concentrations (13% clay, 73.6 mg As/kg and 35% clay, 79.2 mg As/kg) (Table 1). Clay minerals identified with X-ray diffraction (PANalytical) following USGS protocols (Poppe et al., 2002) include nontronite, trioctahedral montmorillonite, and/or vermiculite.

Before starting experiments, the soil was moistened, tilled to a depth of 30 cm, and limed (3,022 kg/ha). We compared seven soil treatments: (i) fertilization with compost (1.28% N, 0.29% P, 151.6 kg N/ha, 34.0 kg P/ha), (ii-iii) fertilization with organic and inorganic nitrogen (blood meal, 12% N, 50 kg N/ha or $(NH_4)_2SO_4$, 50 kg N/ha) or (iv-v) fertilization with organic and inorganic phosphorus (bone meal, 6.6% available P, 85 kg P/ha or superphosphate, 8.7% available P, 85 kg P/ha), (vi) controls with ferns without amendments, and (vii) controls without ferns and without amendments. Each treatment was applied to individual plots (6.3 m^2), with 1 set of plots in coarse-textured soil (sandy loam) and 1 set of plots in fine-textured soil (silty clay loam), with 1 replicate *per* treatment *per* texture. Powdered fertilizers and compost were tilled into plots and were resupplied *via* top dressing (same rate) annually.

Seventy young ferns (30 cm spacing) were planted in each plot (except no-fern controls) in January-March 2013 and watered by hand or *via* drip irrigation as required. The study field was protected by hoophouses. Experiments lasted 4 years (2013-2016). Mortality surveys were conducted annually (April 2014, June 2015, September 2016). Over winter 2013-2014, 29% of ferns were lost to frost damage (data not shown) and were replaced in March 2014. Replanted ferns did not establish well and ferns were not replaced in subsequent years.

Soil and fern sample collection

Annually in September, the aboveground biomass of each fern was cut 20 cm above the rhizome. Fronds from 5 randomly selected ferns *per* plot were collected for analyses, with presenescent (living) fronds (hereafter, fronds) separated from senesced fronds. Samples were dried at 37°C (fronds) and ground (20 gauge mesh).

Soil samples were collected in triplicate from two depths, 0-15 cm and 15-30 cm. Soil was sampled following year 3 (2015) frond harvest, combining 3 cores 5 cm in diameter from each plot into a composite sample. Each plot was divided into 3 increments 60 cm by 80 cm. One core was sampled from each increment between fern crowns. Cores for second and third replicates were collected from locations in each increment approximately equidistant from other cores in a triangle pattern. Soil was sampled following the final fern harvest in year 4 (2016) by dividing each plot into an 8 by 6 grid and combining 48 subsamples, one from each square of the grid, into a composite sample of approximately 1 kg. Soil samples were air-dried and ground (2 mm) before analyses.

Fern and soil sample analyses

Dry biomass was measured on final frond and senesced frond samples. Plant tissue and soil samples were digested following a modified EPA 3050B protocol. Total arsenic, phosphorus, potassium, calcium, magnesium, zinc, copper, manganese, and iron concentrations of fern digests and total arsenic, phosphorus, potassium, calcium, magnesium, zinc, copper, manganese, iron, aluminum, sodium, sulfur, and boron concentrations of soil digests were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES).

Physiologically based extraction test (PBET)

Bioaccessible arsenic was extracted in synthetic gastric solution (Ruby et al., 1996; Defoe et al., 2014). Gastric solution (1.25 g pepsin, 0.50 g citrate, 0.50 g malate, 420 µL lactic acid, and 500 µL acetic acid in 1 L deionized water) was acidified to pH 2.5 with 12 M HCl (trace metal grade) and heated to 37°C on a hot plate before 100 mL was added to approximately 1 g dry soil (sieved to <250 µm) in a wide mouth high density polyethylene (HDPE) bottle. pH was adjusted

as needed with trace metal grade HCl after adding the solution to soil. The mixture was shaken for 1 hour (150 rpm) at 37 °C. Post-extraction, pH changes greater than 0.05 log units were adjusted with trace metal grade HCl before a 20 mL aliquot of the supernatant was removed, filtered (0.45 μm), and frozen until analysis. Total arsenic was analyzed using hydride-generation-ICP-OES after addition of 0.8 mL of 40% KI/8% ascorbic acid to 4 mL sample and 2.7 mL 1.1 M HCl.

Calculations

Arsenic accumulated *per* fern (mg/fern) was calculated by multiplying fern arsenic concentration by the dry fern aboveground biomass. Arsenic uptake rate (kg/ha/y) was calculated by dividing the arsenic accumulated *per* fern by the soil surface area *per* fern and study duration. Remediation time (y) to remove arsenic in the 0-20 cm depth interval was calculated by dividing the mass of soil arsenic *per* unit surface area by the arsenic uptake rate. Relative bioaccessibility was calculated by dividing the PBET-extractable arsenic concentration by the total soil arsenic concentration.

Statistical analysis

Statistical analysis was performed with R (R Core Development Team, 2013). Analyses of variance were performed on linear models to analyze effects of soil treatment on fern arsenic concentrations, biomass, arsenic accumulation, arsenic uptake rate, estimated remediation times, and changes in soil arsenic concentrations during phytoextraction. Models were selected based on AIC criteria using the MuMIn package (Barton, 2019). Two-way interactions were included when those models were most highly ranked. Regression summaries were used to compare effects of treatments to the control.

Results

Arsenic uptake

Frond arsenic concentrations were higher ($P<0.001$) in fronds presenescent at harvest (hereafter, fronds) (mean 688.7 mg As/kg) compared to in senesced fronds (mean 245.2 mg As/kg) (Figure 1A-B). Frond arsenic concentrations ($P<0.001$; Figure 1A and B), the mass of arsenic accumulated *per* fern ($P<0.01$; Figure 1D), and remediation rates ($P<0.01$; Table 2) decreased with time.

Soil nutrient application decreased frond arsenic concentrations ($P<0.001$), accumulated arsenic mass ($P<0.001$), and remediation rates ($P<0.001$) compared to unamended control ferns (Figure 1A and D, Table 2). Across soil textures, mean frond arsenic concentrations in control ferns were 1259.7 mg As/kg, significantly higher than in nitrogen- and phosphorus- treated ferns, where they were only 497.8 mg As/kg (Figure 1A). Frond arsenic concentrations were especially low in phosphorus-treated ferns, where values never surpassed the hyperaccumulation threshold (1000 mg As/kg) (Figure 1A). Additionally, the mass of accumulated arsenic in nitrogen- and phosphorus-treated ferns was only 45% of that in control ferns, a significant decrease (Figure 1D), while mean remediation rates were 1.25 kg As/ha/yr in nitrogen and phosphorus treated ferns, significantly lower than remediation rates in control ferns, where they were 2.7 kg As/ha/yr (Table 2).

Furthermore, soil texture affected frond arsenic concentration ($P<0.001$), biomass ($P<0.001$), and mass of accumulated arsenic ($P<0.001$). Frond arsenic concentrations in ferns growing in fine-textured soil, with a mean of 429.2 mg/kg, were significantly lower compared to those in coarse-

textured soil (mean 937.3 mg/kg; Figure 1A). A texture by treatment interaction ($P<0.01$) showed that in coarse-textured soil, frond concentrations were lower in ferns receiving compost, inorganic nitrogen, and both phosphorus treatments. Frond biomass was also lower ($P<0.001$) in fine-textured soil, with a mean only 55% of that in ferns growing in coarse-textured soil (Figure 1C). Therefore, ferns growing in fine-textured soil accumulated a mean of only 37% of the mass of arsenic accumulated in ferns growing in coarse-textured soil, a significant decrease ($P<0.001$; Figure 1D), leading to lower remediation rates ($P<0.001$) in ferns growing in fine-textured soil (Table 2). Mean remediation rates in fine-textured soils were 0.91 kg As/ha/yr, compared to 2.45 kg As/ha/yr in ferns growing in coarse-textured soil (Table 2). Correspondingly, remediation times were higher ($P<0.01$) in fine-textured soil, though remediation times varied widely from several decades to millennia (Table 2).

Fern survival

The number of surviving ferns decreased ($P<0.01$) from summer 2015 to summer 2016 (Figure 2A), although the percent survival did not change (Figure 2B). More ferns ($P<0.01$) and a higher percent ($P<0.01$) of ferns survived in coarse-textured soil compared to in fine-textured soil (Figure 2A-B). Across both years, percent survival ranged from 43% to 100% in coarse-textured soils, but only 11% to 90% in fine-textured soil (Figure 2A-B). Compost application increased the number ($P<0.05$) and percent ($P<0.05$) of surviving ferns, with 55% to 97% of compost-amended ferns surviving compared to 41% to 62% of control ferns (Figure 2A-B).

Nutrient uptake

Frond concentrations of plant macro and micro nutrients, specifically phosphorus, potassium, calcium, magnesium, copper, manganese, and iron, decreased with time ($P<0.01$-0.001; Table 3). Phosphorus concentrations in the organic ($P<0.01$) and inorganic ($P<0.05$) nitrogen-treated ferns (with a mean of 3,034 mg/kg) were lower than in the control ferns (with a mean of 3,729 mg/kg) across textures. In ferns growing in coarse-textured soil, frond potassium (mean 24,077 mg/kg; $P<0.01$) and manganese (mean 30.7 mg/kg; $P<0.001$) concentrations were higher, while calcium concentrations were lower (mean 2,864 mg/kg; $P<0.01$), compared to ferns growing in fine-textured soil (mean 21,440 mg K/kg, 20.4 mg Mn/kg, 3,173 mg Ca/kg). Frond zinc concentrations were higher in ferns receiving either of the two phosphorus treatments (organic phosphorus, mean 37.0 mg Zn/kg, $P<0.01$; inorganic phosphorus, mean 33.4 mg Zn/kg, $P<0.05$), compared to control ferns (mean 23.8 mg Zn/kg). A soil by treatment interaction showed that manganese concentrations were lower ($P<0.001$) in the phosphorus-treated ferns growing in the coarse-textured soil.

Soil arsenic and nutrient concentrations

We compared arsenic concentrations in soil phytoextracted for 3 or 4 years to control soil and found that time ($P<0.001$), soil texture ($P<0.01$), soil depth ($P<0.01$), and nutrient application ($P<0.01$) affected arsenic concentrations, whereas within the control soil alone, arsenic concentrations did not change significantly with time ($P=0.10$), texture ($P=0.20$), or depth ($P=0.34$) (Figure 3). Surprisingly, we found arsenic concentrations significantly increased with time from a mean of 59.8 mg As/kg in 2015 to 77.0 mg As/kg in 2016 across phytoextracted and control soils, textures, depths, and treatments (Figure 3). Furthermore, arsenic concentrations were significantly higher in the coarse-textured soil, with a mean of 73.2 mg As/kg, compared to the fine-textured soil, where mean concentrations were 63.5 mg As/kg (Figure 3). A texture by depth interaction ($P<0.001$) revealed that in phytoextracted coarse-textured soil, arsenic concentrations were 47%

higher in the top 0-15 cm than in the 15-30 cm depth interval, whereas the reverse was true for fine-textured phytoextracted soil, where concentrations were 20% higher in the bottom depth (Figure 3).

Across soil textures, the only significant change in soil arsenic concentrations in phytoextracted compared to control soils occurred in the inorganic phosphorus-treated soil ($P<0.05$), where concentrations decreased by 28% compared to the control soil (Figure 3). The effect of phosphorus differed with texture. In the fine-textured soil, arsenic concentrations in the presence of both organic and inorganic phosphorus significantly decreased to 63% of those in control soil ($P<0.01$) or 71% of the untreated control ($P<0.05$) (Figure 3). In contrast, in the coarse-textured soil, soil arsenic concentrations significantly increased by 23% in organic phosphorus-treated soil compared to nonphytoextracted soil ($P<0.05$) (Figure 3).

Relative arsenic bioavailability was higher ($P<0.01$) in coarse- (19.3%) compared to fine- (14.3%) textured soil (Figure 4).

Extractable phosphorus concentrations were higher ($P<0.001$) in phosphorus-treated soils, with means of 27.6 mg P/kg in soils treated with organic phosphorus and 42.4 mg P/kg in soils treated with inorganic phosphorus, compared to the control, with a mean of 13 mg P/kg (Figure 5). In contrast to arsenic concentrations, extractable phosphorus concentrations did not change significantly with time, were lower in coarse-textured soil, and were consistently higher in the top depth of soil, regardless of soil texture.

Discussion

Nutrient application does not accelerate phytoextraction

Remediation rates were slow compared to those found in other long-term field studies (Kertulis-Tartar et al., 2006; da Silva et al., 2018) and did not increase in response to fertilization. Our fastest remediation rate, 5.06 kg As/ha/year, was equivalent to that found in a 2-year *in situ* field study in Florida, USA, 4.36 kg As/ha/yr (Kertulis-Tartar et al., 2006), but only about a third of the rate (17.0 kg As/ha/yr) found in a 5-year outdoor large container study also in Florida, USA (da Silva et al., 2018) (with all rates adjusted to 30 fern spacing). Our slower rates were likely due to differences in climate (especially humidity) and soil texture.

In particular, both organic and inorganic phosphorus decreased arsenic accumulation compared to the control, especially in coarse-textured soil. Arsenate is taken up *via* the phosphate intake pathway in *P. vittata* (Sun et al., 2020) and other plants (Meharg and Hartley-Whitaker, 2002). Consequently phosphorus competes with arsenic for uptake, and decreases arsenic concentrations in *P. vittata* (Wang et al., 2002; Tu and Ma, 2003b; Huang et al., 2007; Fayiga et al., 2008; Natarajan et al., 2009). Competition for uptake was shown to increase with phosphorus availability (Lessl and Ma, 2013). We found phosphorus was especially available to compete for uptake in ferns grown in coarse-textured soil, which has less sorptive potential for phosphorus (Atalay, 2001).

Furthermore, our results suggest that phosphorus competition at soil sorption sites increases arsenic availability in soil, but that this arsenic is susceptible to leaching since it is not taken up by the fern, either due to phosphorus competition at the root intake pathway, or because broad phosphorus application mobilized arsenic in soil where fern roots were not present. We found that arsenic concentrations in phosphorus-treated soils were lower compared to non-phytoextracted control soils, but that this depleted arsenic was apparently not taken up into *P. vittata*. Our findings agree with others who found that phosphorus application increased arsenic solubility leading to

arsenic leaching (Peryea, 1991), especially when available arsenic is not taken up in *P. vittata* (Caille et al., 2004).

Nitrogen application led to lower frond phosphorus concentrations, arsenic concentrations, mass arsenic accumulated, and remediation rates, perhaps due to negative impacts of nitrogen application on soil mycorrhizal fungi communities supporting phosphorus, and perhaps therefore arsenic, uptake in *P. vittata*. Nitrogen application can decrease mycorrhizal fungi abundance, perhaps due to lower plant investment in mycorrhizal fungi when the plant is no longer under nitrogen stress (Treseder, 2004). Mycorrhizal fungi are known to increase phosphorus and arsenic uptake in *P. vittata* (Liu et al., 2005; Trotta et al., 2006; Leung et al., 2013), in keeping with the hypothesis that plant communities in infertile soils rely on mycorrhizal fungi for nutrient acquisition (Koide et al., 1988). Mycorrhizal fungi might transport arsenic to the plant (Chen et al., 2007a) or simply increase arsenic availability *via* enhanced geochemical processes that solubilize phosphorus, and therefore arsenic, from a greater volume of soil (Clark and Zeto, 2000; Villegas and Fortin, 2002; Tawaraya et al., 2006).

By extension, we would also expect phosphorus fertilization to decrease mycorrhizal fungi abundance (Treseder, 2004), which could similarly help explain the decreases in arsenic uptake we found in the phosphorus-treated ferns. Although we did not find lower phosphorus concentrations in phosphorus-treated ferns, we did find that phosphorus concentrations decreased over time, as did other macro and micro nutrient concentrations, across treatments. These decreases could indicate lower *P. vittata* investment in mycorrhizal fungi with time if mycorrhizal fungi support *P. vittata* uptake of other macro and micro nutrients in addition to phosphorus, as has been shown in other plants (Clark and Zeto, 2000).

Unlike phosphorus and nitrogen, we found compost application did not affect arsenic accumulation in *P. vittata*. Moreover, compost application increased fern survival, as others observed in harsh field conditions (Ciurli et al., 2014), which would increase fitness and lead to more efficient arsenic removal in field applications. Compost application increases soil microbial community diversity, biomass, and activity (Martínez-Blanco et al., 2013), which we hypothesize increased stress tolerance in *P. vittata*, leading to greater survival. Soil bacteria have been shown to facilitate arsenic accumulation in *P. vittata* (Yang et al., 2012, 2020; Tiwari et al., 2016; Zeng et al., 2019) and could also be important for *P. vittata* stress tolerance through release of plant growth promoting compounds (Tiwari et al., 2016). Mycorrhizal fungi also have been shown to increase arsenic reductase and therefore possibly stress tolerance in *P. vittata* (Leung et al., 2013).

Finally, we found very weak to nonexistent correlations between frond biomass, frond arsenic concentrations, and plant and soil macro and micro nutrients in *P. vittata* growing in coarse-textured soil, where we found greater fern arsenic accumulation. Our findings suggest that like other plants in communities found in infertile soils, *P. vittata* does not respond to nutrient application (Koide et al., 1988). *P. vittata* can outcompete other plant species in harsh conditions to the point of becoming invasive (Langeland et al., 2008), yet we found *P. vittata* did not survive well under cultivation or respond to nutrient application like agricultural/domesticated crops. We conclude that *P. vittata* nutrient needs, the relationships between *P. vittata* nutrient and arsenic access, and therefore the requirements for long-term cultivation of *P. vittata*, are complex and demand an ecosystem-based approach (e.g. polycultures (Ma et al., 2018; Zeng et al., 2019) instead of responses to individual environmental stress factors (Audet, 2012) like fertilization.

Arsenic phytoenrichment coupled to greater growth in coarse-textured soil

In coarse-textured soil, where ferns were larger and arsenic accumulation greater, we found that soil arsenic concentrations were negatively correlated with depth. Our results are in keeping with other multi-year arsenic phytoextraction field studies where a greater decrease in soil arsenic concentrations was observed in the 10-20 cm depth than 0-10 cm depth (Ma et al., 2018; Chapter 3) or 15-30 cm depth compared to 0-15 cm depth (Kertulis-Tartar et al., 2006). To explain the greater arsenic depletion in the lower than surface depth interval in the coarse-textured soil, we hypothesize a three-part cycle, including 1) mobilization of arsenic for root uptake, primarily from 0-10 cm depths, and subsequent frond sequestration; 2) leaching of mobilized arsenic from the root zone and below coupled to leaching of DOC; 3) return of arsenic from *P. vittata* biomass back to surface soil.

Trends of greater arsenic depletion at lower depths stand in contrast to expectations based on *P. vittata* root density. Root density is highest in the 0-10 cm depth interval, though roots are also present down to 30 cm (Liao et al., 2004; Ma et al., 2018). Leaching can explain loss of arsenic within and below the root zone. Here, in the fine-textured soil with higher sorption capacity, our results indicate arsenic could leach from surface soils and sorb in the 15-30 cm depth interval, whereas in the coarse-textured soil, with lower sorption capacity, arsenic could leach to depths below 30 cm. Others have found arsenic leaches from soil during phytoextraction (Yang et al., 2012; Chapter 4). In coarse- and medium-textured soil excavated from this same field site, *P. vittata* growth increased arsenic leaching below the 40 cm depth in soil (Chapter 4). While arsenic can leach from soil in the absence of *P. vittata* growth (Chapter 4), rhizosphere processes enhancing arsenic mobilization from soil (Fitz et al., 2003; Tu et al., 2004; Gonzaga et al., 2006; Lessl and Ma, 2013; Chapters 3 and 4) could explain increases in leaching with *P. vittata* growth.

Even as arsenic is lost to leaching, arsenic could be returned to surface soils through phytoenrichment. Hyperaccumulators phytoenrich metal(loids) in surface soils through excretion and leaching of soluble elements from leaf surfaces and through litter fall (Morris et al., 2009). Arsenic could leach from *P. vittata* fronds during growth and after senescence. Although arsenic has been shown to be stored in *P. vittata* pinnae vacuoles (Lombi et al., 2002), additional evidence suggests large quantities of exocellular arsenic are located on pinnae surfaces as crystalline deposits (Datta et al., 2017) and in trichomes (small hairs) (Li et al., 2005). Arsenic on pinnae surfaces could leach from living and senescent fronds. Arsenic has been shown to leach from *P. vittata* fronds during field cultivation at concentrations of 60 to 380 µg/L, for losses of 1.8 to 6.7% of the mass of arsenic accumulated in fronds (Yan et al., 2009). Furthermore, we found lower arsenic concentrations in senesced fronds compared to living fronds, as did a prior study (Kertulis-Tartar et al., 2006), suggesting that arsenic reoxidizes and leaches during drying to enrich soil (Tu et al., 2003).

Effluxing of arsenic from *P. vittata* roots could be a secondary process enriching arsenic concentrations in surface soil. As in other plants (Xu et al., 2007), effluxing of arsenic(III) from roots has been shown to occur in *P. vittata,* with 12% of arsenic(V) uptake effluxed as arsenic(III) under low-arsenic exposure conditions (Chen et al., 2016b). Especially if effluxed arsenic oxidizes and sorbs to soil, effluxing could help explain higher concentrations of arsenic in root zone soils compared to greater loss at lower depths.

Phytoextraction is more effective in coarse-textured soils

Phytoenrichment notwithstanding, we found phytoextraction was more effective when ferns were planted in coarse- compared to fine-textured soil, across metrics including arsenic

concentrations and accumulation, biomass production, remediation rates and times, and fern survival. The only other study (to our knowledge) investigating effects of soil texture on *P. vittata* arsenic accumulation under field conditions, a survey of a natural population of *P. vittata* growing in an arsenic sulfide mine, also showed that biomass and arsenic concentrations were negatively correlated with soil clay content (Liao et al., 2004). The negative correlation between frond arsenic concentrations and percent clay could be due to lower arsenic phytoavailability in fine-textured soils. Indeed, arsenic strongly associates with the clay particle size fraction, and the iron oxides therein (Lombi et al., 2000; Smith et al., 2006), leading to a lower phytoavailability in fine- than in coarse-textured soils (Jacobs and Keeney, 1970; Woolson et al., 1973).

High mortality in fine-textured soils

Beyond arsenic uptake, the decrease in biomass production and fern survival associated with increasing clay content suggests growth in fine-textured soils stresses *P. vittata* to the point of lowering fitness. We hypothesize that lower porosity, aeration, infiltration, and drainage stressed *P. vittata* to the point of mortality. We observed water ponding on the surface of plots in fine-textured soil for weeks to month annually during the winter rainy season. Although *P. vittata* has been shown to take up more arsenic under in soils with higher water content (Leung et al., 2010; Yang et al., 2019), grow well in water in phytofiltration applications (Huang et al., 2004), and take up arsenic(III) under reducing conditions (Yang et al., 2012), our findings suggest that the long-term stress of poorly drained and aerated soils is fatal to *P. vittata*.

***P. vittata* not hardy in Mediterranean climate**

We further attribute high *P. vittata* mortality to frost/freeze damage and flooding. Even in coarse-textured soils, survival ranged widely from 40-100%. Although *P. vittata* has been described as hardy (Ma et al., 2001), we found *P. vittata* is not hardy under field conditions in the relatively mild Mediterranean climate, even with frost/freeze protection. The high number of field experiments and surveys of natural populations in humid subtropical climates (Liao et al., 2004; Wei et al., 2006; Chen et al., 2007b; Wan et al., 2013; Lei et al., 2018; Li et al., 2018; Yang et al., 2019) implies the fern is well-suited to such conditions, yet even in a humid subtropical climate one field study reported extensive (97%) *P. vittata* mortality after winter frost/freeze damage (Kertulis-Tartar et al., 2006). Especially in Mediterranean and temperate climates, perennial *P. vittata* cultivation would require intensive overwinter management including temporary field greenhouses, maintenance of ferns during freezing conditions, and fern replacement after mortality, ultimately increasing phytoextraction costs, especially in large-scale applications. Otherwise, *P. vittata* cultivation outside of humid subtropical or tropical climates would be limited to annual cultivation over a short growing season, again increasing costs and remediation time.

Phytoextraction does not decrease arsenic bioaccessibility

We calculated highly variable remediation times extending from decades to centuries, assuming linear depletion of total arsenic from soil. In an attempt to shorten remediation times, the bioavailable contaminant stripping theory applies an alternative threshold for remediation based on depletion of bioaccessible, not total arsenic, since the fraction that is not bioaccessible does not contribute to risk (Fitz et al., 2003). However, we found phytoextraction did not decrease relative arsenic bioaccessibility compared to controls, suggesting the bioaccessible contaminant stripping theory would not help make phytoextraction more feasible at this site. Even though phytoextraction decreases phytoavailable arsenic from rhizosphere soil (Fitz et al., 2003), we

found phytoextraction does not deplete bioaccessible arsenic in bulk soil. If phytoavailable and bioaccessible arsenic are related forms of arsenic in soil, our results suggest phytoextraction is limited to rhizosphere soils, which is likely, considering the importance of rhizosphere processes for arsenic uptake in *P. vittata* (Fitz et al., 2003; Gonzaga et al., 2006). It is also likely that phytoavailable and bioaccessible arsenic are overlapping but not identical fractions, due to differences in soil vs. gastric pH. Arsenic(V) is more soluble at gastric pH (2.5) than soil pH (5-7), due to dissolution of iron oxides (Hem, 1972) and protonation of arsenate (Lu and Zhu, 2011) which decreases arsenic sorption.

Limited feasibility for phytoextraction with *P. vittata*

Over four years of arsenic phytoextraction with *P. vittata*, we found low arsenic accumulation in ferns, slow remediation rates, and high mortality, especially in fine-textured soils, and those were not improved upon fertilization. This suggests that phytoextraction with *P. vittata* is at best limited to very specific conditions: coarse-textured soils with low arsenic enrichment in humid subtropical climate conditions. A rough estimate based on the best remediation rate we found (5.06 kg/ha/yr) suggests arsenic could be removed linearly in about a decade from coarse-textured soil with 30 mg As/kg. Ironically, conditions promoting growth and arsenic accumulation would promote phytoenrichment of arsenic from deeper to surface depth intervals *via* uptake, translocation to aboveground biomass, and litter fall, a liability for phytoextraction. Further work is needed to quantify long-term phytoenrichment and investigate ways to curtail phytoenrichment through short harvest intervals without impacting fern survival through harvest-related stress. Finally, our findings suggest the best methods to ameliorate field stress and increase fern survival and arsenic accumulation are ecologically-based methods, including compost application.

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Table 1. Soil characteristics of unphytoextracted control soil

Depth, cm	Analyte[1]	Fine[2]	Coarse
0-15	As[3]	73.66±9.68	79.26±1.93
15-30	As	81.70±10.59	56.40±2.27
0-15	Al[4]	2.97±0.13	2.71±0.22
15-30	Al	4.14±0.15	4.91±0.26
0-15	Ca	3157.73±162.82	1281.39± 33.92
15-30	Ca	3192.63±103.41	923.96± 36.18
0-15	Cu	0.47±0.07	0.58±0.02
15-30	Cu	0.45±0.07	0.67±0.03
0-15	Fe	3.55±0.37	2.22±0.10
15-30	Fe	2.99±0.56	1.68±0.10
0-15	K	252.90±19.10	112.92± 2.60
15-30	K	172.49± 8.28	57.44± 2.49
0-15	Mg	719.10±36.33	209.58± 3.85
15-30	Mg	848.00±52.47	218.18± 5.88
0-15	Mn	17.85±1.79	10.09±1.30
15-30	Mn	16.25±1.84	6.96±1.07
0-15	P	24.85±4.71	14.48±0.82
15-30	P	10.71±1.71	6.14±0.49
0-15	Pb	4.43±0.59	3.59±0.32
15-30	Pb	5.78±2.13	1.60±0.11
0-15	S	21.34±3.05	7.88±0.20
15-30	S	16.94±1.32	5.10±0.17
0-15	Zn	20.99±2.71	15.05±0.82
15-30	Zn	25.21±6.62	7.39±3.08
0-15	CEC, meq/100g	26.42±1.23	10.13±0.21
15-30	CEC, meq/100g	27.48±1.08	6.75±0.27
0-15	pH	6.52±0.04	6.56±0.02
15-30	pH	6.70±0.05	7.08±0.06
0-15	Bulk density, g/cc	1.06±0.04	1.20±0.04
0-15	Sand[5], %	19.92	64.96
0-15	Clay, %	34.73	13.27
0-15	Texture	Silty clay loam	Sandy loam
15-30	Sand, %	17.01	66.23
15-30	Clay, %	35.94	10.61
15-30	Texture	Silty clay loam	Sandy loam

[1]All units mg/kg unless otherwise noted

[2]Mean and standard error of three replicates unless otherwise noted

[3]Total arsenic

[4]All elements other than As are Modified Morgan extractable

[5]Texture data is mean of 2 replicates

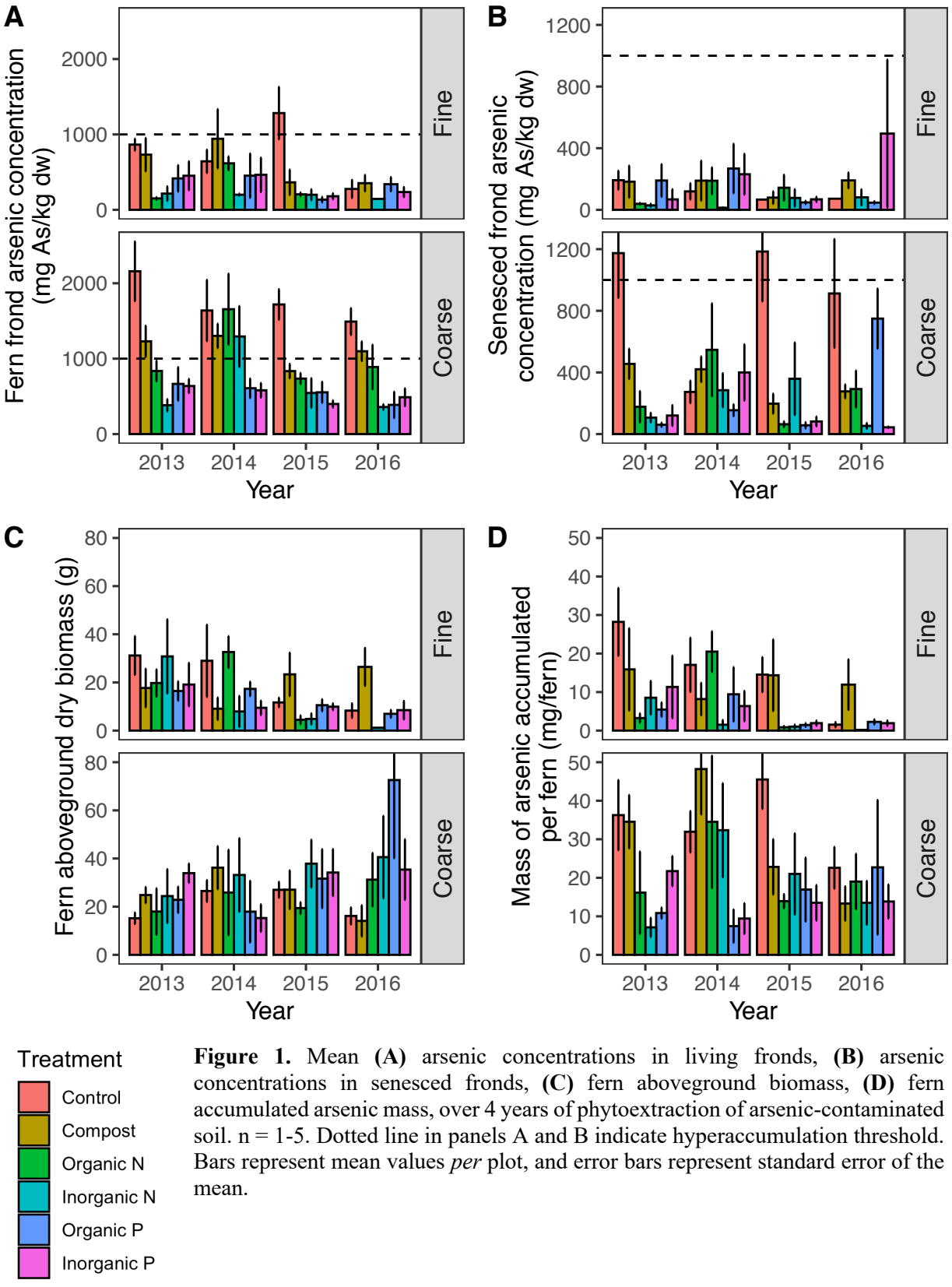

Figure 1. Mean **(A)** arsenic concentrations in living fronds, **(B)** arsenic concentrations in senesced fronds, **(C)** fern aboveground biomass, **(D)** fern accumulated arsenic mass, over 4 years of phytoextraction of arsenic-contaminated soil. n = 1-5. Dotted line in panels A and B indicate hyperaccumulation threshold. Bars represent mean values *per* plot, and error bars represent standard error of the mean.

Table 2. Remediation rates and estimated time to remove arsenic to background levels.

Year	Texture	Treatment	Soil As (mg/kg)	Remediation rate (kg/ha/yr)	Remediation time (yr)
2015	Fine	Control	59.87	1.61±0.51	58.6±17.7
2015	Fine	Compost	42.74	1.60±1.03	486.7±326.2
2015	Fine	Organic N	59.28	0.10±0.04	1515.7±676.0
2015	Fine	Inorganic N	45.97	0.11±0.06	2928.5±1522.9
2015	Fine	Organic P	40.62	0.16±0.05	558.1±235.4
2015	Fine	Inorganic P	40.66	0.22±0.07	327.4±90.1
2015	Coarse	Control	89.69	5.06±0.85	30.6±3.7
2015	Coarse	Compost	99.96	2.54±0.80	94.1±26.7
2015	Coarse	Organic N	94.61	1.55±0.20	105.9±16.4
2015	Coarse	Inorganic N	61.52	2.33±1.17	78.5±24.1
2015	Coarse	Organic P	62.58	1.88±0.92	287.2±149.4
2015	Coarse	Inorganic P	64.86	1.50±0.51	281.4±218.8
2016	Fine	Control	66.42	0.17±0.07	930.7±310.0
2016	Fine	Compost	61.3	1.33±0.73	280.4±158.4
2016	Fine	Organic N	NA	NA	NA
2016	Fine	Inorganic N	78.05	0.02±NA	5563.2±NA
2016	Fine	Organic P	62.34	0.25±0.07	528.1±201.5
2016	Fine	Inorganic P	52.54	0.21±0.07	3751.6±3371.9
2016	Coarse	Control	85.07	2.51±0.60	88.3±40.9
2016	Coarse	Compost	105.16	1.48±0.49	152.3±29.9
2016	Coarse	Organic N	105.08	2.11±0.80	267.9±174.6
2016	Coarse	Inorganic N	76.28	1.50±0.63	121.2±28.6
2016	Coarse	Organic P	117.56	2.52±1.94	234.0±105.2
2016	Coarse	Inorganic P	97.89	1.54±0.49	154.1±48.4

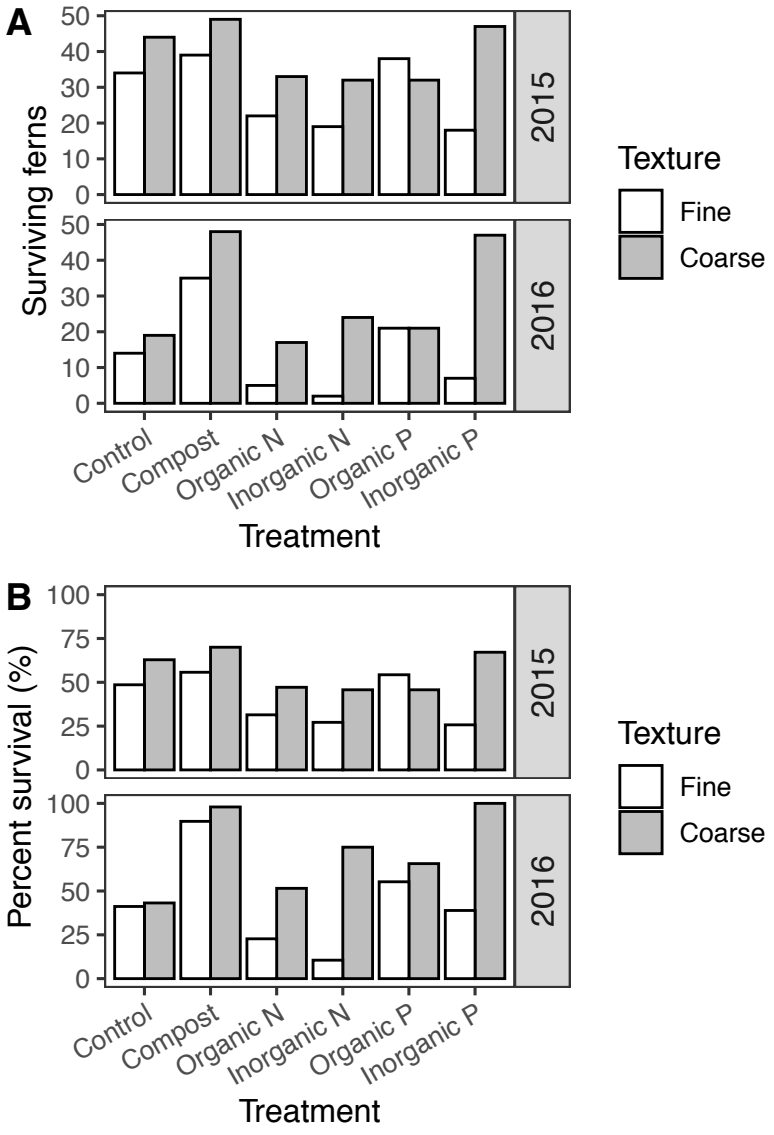

Figure 2. Number of surviving ferns **(A)** and percent fern survival **(B)** in June 2015 and September 2016. In summer 2014, dead ferns were replaced to equal 70 ferns/texture increment.

Table 3. Nutrient concentrations (mg/kg, mean±standard error) in fern fronds living at harvest.

Year	Texture	Treatment	P	K	Ca	Mg	Zn	Cu	Mn	Fe
2013	Fine	Control	3403.3±203.3	20093.3±1707.6	3266.7±166.7	2156.7±80.9	15.3±7.7	10.7±2.0	21.1±2.0	146.3±21.2
2013	Fine	Compost	3663.3±575.0	20616.7±592.5	2993.3± 97.4	2193.3±198.8	8.1±8.1	7.9±2.4	20.4±2.5	132.9±21.5
2013	Fine	Organic N	2598.0±99.6	18674.0±1289.5	3536.0±220.1	2074.0±60.6	18.9±8.0	9.6±1.7	14.5±1.4	298.7±194.7
2013	Fine	Inorganic N	2450.0±50.0	19700.0±1000.0	2750.0±450.0	1850.0±50.0	29.6±5.2	8.9±3.2	15.4±6.5	106.3± 6.3
2013	Fine	Organic P	3266.7±384.4	22200.0±2402.8	3100.0±458.3	2066.7±66.7	40.3±11.7	11.5±2.1	22.4±4.2	138.9± 2.9
2013	Fine	Inorganic P	2900.0±321.5	17666.7±5938.7	3533.3±272.8	2466.7±318.0	38.2±13.2	8.4±3.0	19.5±6.3	138.1±17.0
2013	Coarse	Control	2913.3±197.3	19411.1±415.1	3254.4±198.9	2256.7±119.4	20.8±6.8	10.7±0.9	44.6±4.9	263.6±68.1
2013	Coarse	Compost	3027.1±170.9	23161.4±796.8	3211.4±126.7	2125.7±61.1	20.0±5.7	10.6±0.9	38.3±3.7	307.1±180.3
2013	Coarse	Organic N	2790.0±362.5	22566.7±982.1	3536.7±345.0	2313.3±161.5	7.9±7.9	7.8±1.4	37.6±7.0	149.4±19.5
2013	Coarse	Inorganic N	3630.0±315.8	26540.0±984.0	2810.0±189.9	2148.0±110.8	19.0±7.8	11.1±1.0	38.8±7.1	125.6±10.2
2013	Coarse	Organic P	3600.0±229.5	27033.3±2376.5	3133.3±267.9	2066.7±95.5	47.0±11.2	12.7±1.2	23.0±7.1	491.2±365.5
2013	Coarse	Inorganic P	3060.0±103.0	23840.0±1715.1	3200.0±288.1	1980.0±86.0	30.0±5.6	10.3±1.3	21.5±2.2	375.2±271.4
2016	Fine	Control	3375.0±256.2	19875.0±1397.2	2000.0± 70.7	1975.0±62.9	22.3±2.5	7.8±0.6	12.5±1.5	217.2±61.2
2016	Fine	Compost	3080.0±224.5	17740.0±997.8	2020.0±146.3	1840.0±67.8	19.7±0.9	7.2±0.4	15.0±1.8	125.2± 7.4
2016	Fine	Organic N	NA	NA	NA	NA	NA	NA	NA	NA
2016	Fine	Inorganic N	NA	NA	NA	NA	NA	NA	NA	NA
2016	Fine	Organic P	2740.0±128.8	17800.0±1213.7	2100.0±247.0	1840.0±112.2	24.1±1.8	8.5±0.7	14.4±1.9	139.6±16.8
2016	Fine	Inorganic P	3100.0±152.8	17333.3±1507.0	2333.3±176.4	1966.7±33.3	20.3±1.5	8.1±0.2	13.8±2.3	148.3±18.7
2016	Coarse	Control	3060.0±299.3	18080.0±363.9	2100.0±109.5	1860.0±81.2	21.3±1.3	6.7±0.3	24.4±2.1	119.0± 8.6
2016	Coarse	Compost	3280.0±182.8	19380.0±924.9	2000.0±178.9	1780.0±86.0	20.6±1.0	7.0±0.5	19.0±2.6	95.6±11.9
2016	Coarse	Organic N	1750.0±184.8	14350.0±792.1	2475.0±232.3	1800.0±70.7	26.6±1.3	6.9±0.3	32.7±4.8	173.5±29.0
2016	Coarse	Inorganic N	2320.0±190.8	17800.0±407.4	2000.0± 83.7	1680.0±49.0	26.6±0.9	7.4±0.2	25.4±3.6	159.8±27.8
2016	Coarse	Organic P	2066.7±133.3	15200.0±814.5	3966.7±1225.2	2500.0±650.6	29.0±1.9	6.4±0.7	21.8±6.5	212.0±40.3
2016	Coarse	Inorganic P	3040.0±51.0	19160.0±908.1	1900.0±170.3	1660.0±24.5	23.2±1.0	7.1±0.3	14.7±1.3	140.6±11.2

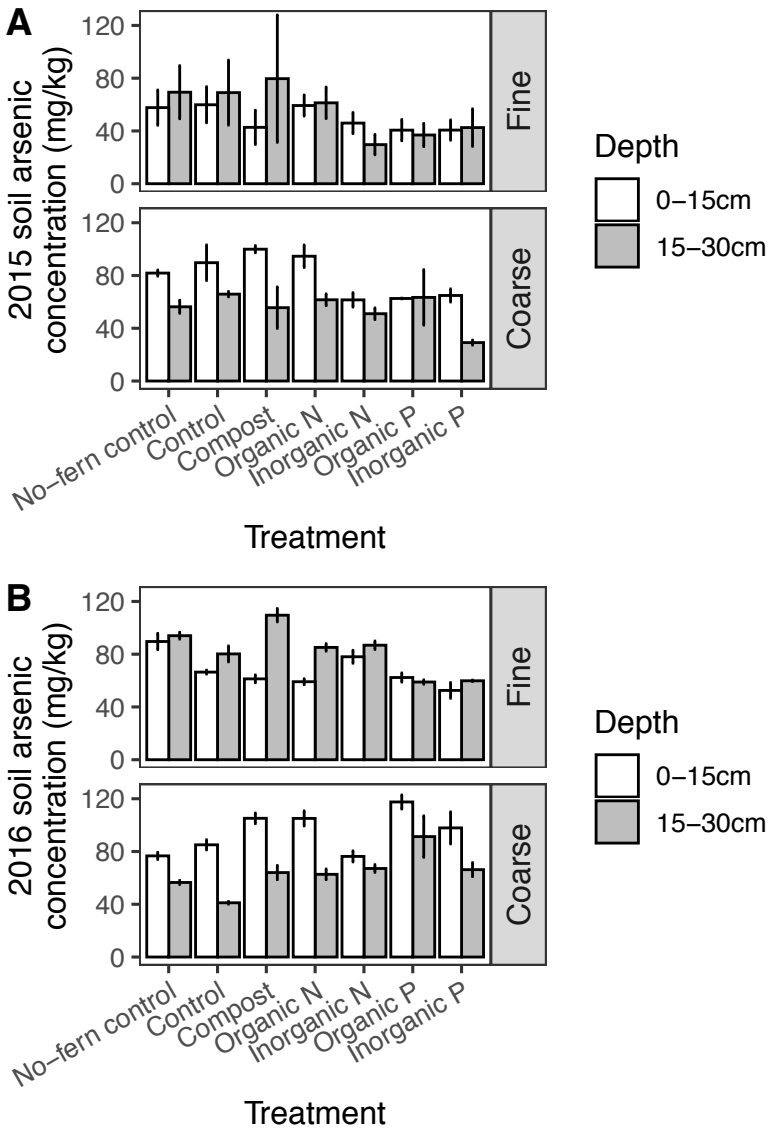

Figure 3. Soil arsenic concentrations after harvest in 2015 **(A)** and 2016 **(B)**. Bars indicate mean and standard error of 3 replicates.

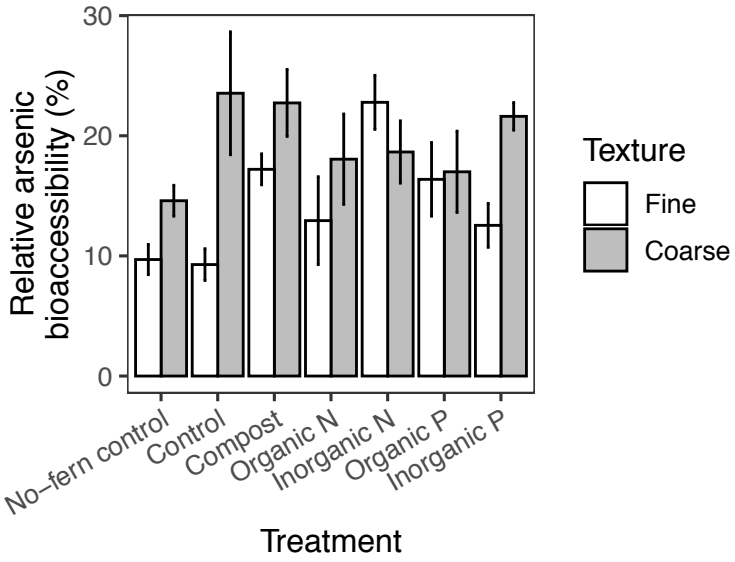

Figure 4. Relative arsenic bioaccessibility. Bars indicate mean and standard error of 3 replicates.

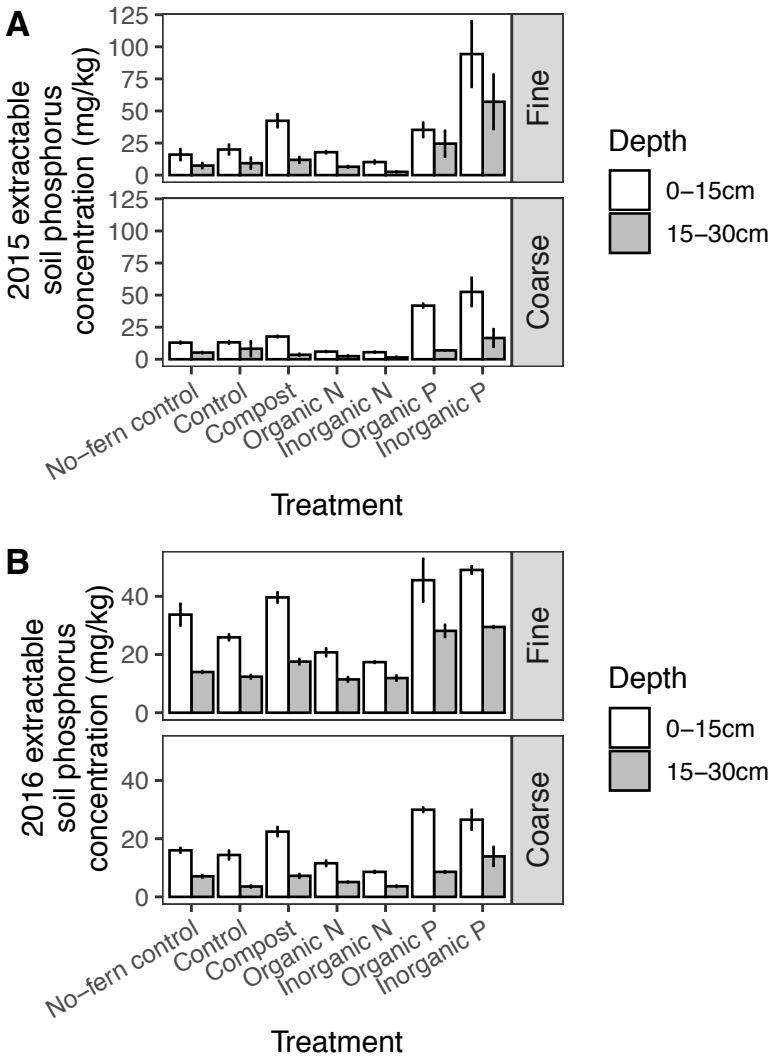

Figure 5. Soil phosphorus concentrations after harvest in 2015 **(A)** and 2016 **(B)**. Bars indicate mean and standard error of 3 replicates.

Chapter 3. *Pteris vittata* arsenic accumulation only partially explains soil arsenic depletion during field-scale phytoextraction

Abstract

Slow arsenic removal rates with arsenic hyperaccumulator *Pteris vittata* under heterogeneous field conditions limit practical use of phytoextraction. In a 58-week field study, we determined effects of soil arsenic concentrations, fertilizer application, and mycorrhizal fungi inoculation on arsenic uptake rates, soil arsenic depletion, and arsenic mass balances. Initial soil arsenic concentrations were positively correlated with arsenic uptake rates, but did not affect remediation time estimates (12 to 170 yrs). Soil inoculation with the mycorrhizal fungus *Funneliformis mosseae* led to higher fern aboveground biomass. Ferns accumulated a mean of 3.6% of initial soil arsenic and mean soil arsenic concentrations decreased up to 44%. Regardless of treatment, arsenic accumulation in *P. vittata* matched soil arsenic depletion in the 0-10 cm depth interval. However, over the entire 0-20 cm depth fern arsenic accumulation could not account for 61.5% of the soil arsenic depletion. The discrepancy could be attributed to leaching of arsenic released through rhizosphere processes. Arsenic speciation indicated the rhizosphere is a reactive zone distinct from bulk soil, due to the presence of arsenic(III) (12.8 to 71.5%). To our knowledge, this is the first mass balance relating arsenic accumulation in *P. vittata* to significant decreases in soil arsenic concentrations under field conditions.

Introduction

Soil arsenic contamination leads to elevated concentrations of arsenic in plant tissue and soil porewater globally, increasing potential human exposure to this carcinogen (Crecelius et al., 1974; Colbourn et al., 1975; Wang and Mulligan, 2006; Moreno-Jimenez et al., 2012; Singh et al., 2015). Plant-based removal of arsenic from soil, or phytoextraction, with the fern *Pteris vittata* is an emerging *in situ* technology potentially suitable to remediate moderately contaminated areas where soil excavation is cost-prohibitive and where risk is less acute (McGrath et al., 2002). While theoretically promising, phytoextraction with *P. vittata* faces practical limitations, especially under field conditions where heterogeneous soil arsenic concentrations lead to slow and variable arsenic uptake rates (Kertulis-Tartar et al., 2006; Niazi et al., 2012; Robinson et al., 2015). Remediation time estimates derived from the few existing field studies (Kertulis-Tartar et al., 2006; Niazi et al., 2012; Lessl and Ma, 2013) range from 10 to 78 years to remove 390 kg As/ha (100 mg As/kg) from soils.

Soil fertilization or soil inoculation with mycorrhizal fungi has been investigated in container and hydroponic systems to increase arsenic uptake rates, either through direct effects on biomass or indirect effects on arsenic availability in soil and/or effects on volume of soil accessed by roots. Nitrogen fertilization led to higher biomass and therefore higher arsenic accumulation *per* fern (Liao et al., 2007). Fertilization with phosphate rock increased fern arsenic concentrations and biomass more effectively than fertilization with soluble phosphorus, which could have competed with arsenic for uptake into the fern (Lessl and Ma, 2013; da Silva et al., 2018). However, in other cases phosphate rock (Cao et al., 2003; Fayiga and Ma, 2006) or soluble phosphorus (Chen et al., 2002b; Tu and Ma, 2003b) did not affect arsenic uptake in *P. vittata*. Soluble phosphorus led to higher arsenic concentrations in porewater in soils contaminated with arsenic and metals, because phosphorus competitively desorbed arsenic from soil (Caille et al., 2004). Inoculation with the

mycorrhizal fungus *Funneliformis mosseae* led to a larger *P. vittata* biomass (Liu et al., 2005; Trotta et al., 2006) but did not affect arsenic accumulation *per* fern (Trotta et al., 2006), especially in moderately contaminated soils (Liu et al., 2005). While promising, all the work published so far has been performed under hydroponic and greenhouse conditions or in outdoor container studies. It is unclear whether those findings obtained in simplified conditions might apply to *in situ* conditions (Gerhardt et al., 2009) where plant roots are not confined to a limited volume of growth medium (Neugschwandtner et al., 2008).

Mass balances comparing arsenic accumulated in plants to changes in arsenic stocks in soil are needed to realistically quantify phytoextraction effectiveness, especially under field conditions. Critically, mass balances can indicate the existence of other processes depleting arsenic from soils during phytoextraction. For example, leaching of contaminants occurs during chelant-assisted phytoextraction of cationic metals (Madrid et al., 2003) and could occur in arsenic phytoextraction due to higher availability of arsenic in the presence of applied phosphorus (Cao et al., 2003; Caille et al., 2004; Gonzaga et al., 2009). However, field-scale mass balances are rarely calculated (Robinson et al., 2015) and are challenging to obtain because the high spatial variability in soil arsenic distribution masks changes in arsenic levels (Gerhardt et al., 2009; Niazi et al., 2012; Robinson et al., 2015). Relying on plant uptake alone to quantify remediation rates overcomes issues with soil heterogeneity (Gerhardt et al., 2009; Niazi et al., 2012) but is not a substitute for a mass balance.

We present a 58-week field study that 1) determines temporal changes in arsenic concentrations in *P. vittata*, 2) investigates the effects of soil arsenic concentrations and soil treatment (fertilization and mycorrhizal fungi inoculation) on arsenic uptake rates, 3) compares *P. vittata* arsenic accumulation to soil arsenic depletion during phytoextraction, and 4) characterizes arsenic speciation in the rhizosphere to hypothesize mechanisms for arsenic mobilization from soil. To our knowledge, this is the first mass balance in the *P. vittata* literature linking fern arsenic accumulation to significant decreases in soil arsenic concentrations during phytoextraction under field conditions.

Materials and Methods

Study site and experimental design

A field site (81 m^2) was established at a former industrial property on the San Francisco Bay shore (Richmond, CA, USA) under remediation order from the State of California for removal of pyrite roasting residues (Department of Toxic Substances Control, 2006; Tetra Tech EM, 2008). Soil arsenic, lead, copper, zinc, and iron concentrations were elevated and heterogeneous, with mean arsenic levels *per* plot ranging from 23.5 to 118.6 mg/kg (Table 1). Major soil minerals identified with X-ray diffraction (PANalytical) following USGS protocols (Poppe et al., 2002) include quartz, albite, hematite, and clays including nontronite, trioctahedral montmorillonite, and/or vermiculite (data not shown).

Before starting experiments, the soil was moistened and well-mixed to a depth of 20 cm where a clay pan occurred. Soil was limed (16,000 kg/ha to reach target pH of 7) because *P. vittata* grows well in soils with higher pH (Chen et al., 2002c). After liming, the soil pH was 6.08. We compared six soil treatments: (i) organic fertilization with compost (1% N, 0.21% P; 1374 kg N/ha, 283 kg P/ha), inorganic fertilization with (ii) nitrogen ($(NH_4)_2SO_4$; 50 kg N/ha) or (iii-iv) phosphorus (CalPhos™, 7.9% P, 18% Ca; 85 kg P/ha or 737 kg P/ha), (v) inoculation with the fungus *F. mosseae* (INVAM; 44 mL inoculum/fern), and (vi) controls without amendments. Each treatment

was applied to individual square plots (1.5 m^2), with 6 replicates *per* treatment. Powdered fertilizers and compost were tilled into plots and were resupplied *via* top dressing (same rate) at 45 weeks. Mycorrhizal fungal inoculant was applied directly to each hole at planting. Fungus-treated plots were lined in aluminum sheeting to prevent hyphae migration to adjacent plots.

Twenty-five young ferns (30 cm spacing) with bare roots were planted in each plot in November 2016 and watered *via* drip irrigation (mean 7 L/day). The study field was protected by hoophouses (mean high temperature 27±0.4ºC, mean low temperature 9±0.2ºC, mean relative humidity 94±0.4%). Experiments lasted 58 weeks.

Soil, fern and porewater sample collection

At the beginning and end of the experiments, triplicate soil samples were collected using an incremental sampling method (Brewer et al., 2017) where each plot was divided into 49 equally sized increments. Because the soil had been mixed well, initial soil samples were collected from the surface (0-3 cm) only and assumed to represent the 0-20 cm depth interval. Final soil samples were collected from two depths, 0-10 cm and 10-20 cm, after rhizome removal and without regard to fern location. Soil samples were air-dried and ground (2 mm) before analyses.

Pinnae (1 middle pair from each frond) from 4-9 ferns were sampled every 6 weeks and were washed three times with deionized water. At the end of the experiments, the aboveground biomass of each fern was cut 20 cm above the rhizome. Fronds living at harvest (hereafter, fronds) were separated from senesced fronds, which were not analyzed. Samples were dried at 55ºC (pinnae) or 37ºC (fronds) and ground (20 gauge mesh).

Soil porewater was sampled every 6 weeks from a depth of 5-10 cm using Rhizon porewater samplers inserted between fern crowns. Porewater was vacuum-extracted using acid-washed syringes and acidified to approximately 120 mM with HCl. Samples were stored at -18 ºC in the dark until analyses.

Root and soil samples for microprobe and bulk X-ray absorption spectroscopy (XAS) were collected at the end of the experiments from soil adjacent to fern crowns 0-10 cm deep in triplicate control plots and immediately stored at 4 ºC. Samples were dried, processed, and maintained under anoxic conditions.

Fern and soil sample analyses

Dry biomass was measured on both pinnae and final frond samples. Plant tissue and soil samples were digested following a modified EPA 3050B protocol. Total arsenic, phosphorus, iron, copper, zinc, and lead concentrations of soil and fern digests were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES), whereas total arsenic concentrations in filtered porewater samples (0.45 μm) was analyzed using hydride-generation-ICP-OES after addition of 0.8 mL of 40% KI/8% ascorbic acid to 4 mL sample and 2.7 mL 1.1 M HCl.

Samples of roots with rhizospheric soil and of soil aggregates were embedded in EPO-TEK 301 epoxy under anoxic conditions and prepared as thin sections (30 μm; Spectrum Petrographics) with roots sliced longitudinally for microprobe analyses. For bulk XAS, rhizosphere soil was sampled with a small brush. Aggregates with no visible roots were considered bulk soil. Under anoxic conditions, bulk soil, rhizosphere soil, and roots were finely ground and mounted on a 0.22 μm nitrocellulose filter. X-ray microprobe analyses were performed at Advanced Light Source (ALS, USA) XFM beamline 10.3.2 (Marcus et al., 2004). Briefly, arsenic and iron spatial distribution in the samples was determined using micro-focused X-ray fluorescence (μXRF) elemental mapping using a 12 keV incident beam. In sample regions of interest, arsenic speciation

was determined using arsenic K-edge X-ray absorption near edge structure (μXANES) spectroscopy. Bulk XANES spectra were collected at Stanford Synchrotron Radiation Laboratory (SSRL, USA) beamline 7.3. Filter membranes were sealed under anoxic conditions and mounted on sample holders with Kapton tape. Micro and bulk XANES spectra were plotted using the Athena package (Ravel and Newville, 2005) and linear combination fitting was performed with customized beamline software. Details of the spectroscopy measurements are available in the Supplemental Information.

Calculations

Arsenic accumulated *per* fern (mg/fern) was calculated by multiplying fern arsenic concentration by the dry fern aboveground biomass. Arsenic uptake rate (kg/ha/y) was calculated by dividing the arsenic accumulated *per* fern by the soil surface area *per* fern and study duration. Remediation time (y) to remove all soil arsenic in the 0-20 cm depth interval was calculated by dividing the mass of soil arsenic *per* unit surface area by the arsenic uptake rate. To assess the mass balance, the mean arsenic accumulation *per* fern, normalized to mass soil *per* depth, was compared to the difference in initial and final soil arsenic concentrations *per* depth (i.e., soil arsenic depletion) (Eq. 1). The mass of soil in a 30 cm × 30 cm × 10 cm volume for the 0-10 cm depth or 10-20 cm depth, or 30 cm × 30 cm × 20 cm volume for the 0-20 cm depth, was used to normalize the mass arsenic accumulated *per* fern or depleted from soil.

$$\frac{\text{mass As}_{\text{fern}}}{\text{mass soil}} = \frac{\text{mass As}_{\text{soil,init}}}{\text{mass soil}} - \frac{\text{mass As}_{\text{soil,final}}}{\text{mass soil}} \quad (1)$$

Statistical analysis

Statistical analysis was performed with R (R Core Development Team, 2013). Analyses of covariance with soil arsenic concentrations as a covariate were performed on linear models to analyze effects of treatment on fern arsenic concentrations, biomass, arsenic accumulation, arsenic uptake rate, and changes in soil arsenic concentrations during phytoextraction, though initial soil arsenic concentration was not included as a covariate in the analysis of variance for remediation time. Regression summaries were used to compare effects of treatments to the control, and differences in means were determined with Tukey's Honestly Significant Difference test on means normalized to soil arsenic concentration where appropriate. A paired t test was used to determine the mass balance across all plots, comparing mean soil arsenic depletion *per* plot to mean arsenic accumulation *per* fern *per* plot (Eq. 1).

Results and Discussion

Temporal evolution of fern biomass and arsenic uptake

Time after transplanting affected fern pinnae arsenic concentrations ($P<0.001$; Figure 1A) and biomass ($P<0.001$; Figure 1B). Pinnae arsenic concentrations and biomass were correlated ($R^2=0.78$; data not shown), suggesting arsenic uptake is related to pinnae growth. Pinnae arsenic concentrations and biomass did not change between 12 and 18 weeks after transplanting (Figure 1A-B). However, significant increases in arsenic concentrations and biomass occurred later, between 18 and 30 weeks after transplanting (Figure 1A-B), with pinnae arsenic concentrations increasing an order of magnitude (Figure 1A). For all soils with amendments, pinnae arsenic concentrations reached the hyperaccumulation threshold and stabilized after 30 weeks. This

occurred later, after 48 weeks, in the control soils (Figure 1A). Pinnae biomass did not stabilize but continued increasing until 48 weeks after transplanting (Figure 1B), though this increase did not carry through to higher arsenic accumulation in pinnae at 48 weeks (data not shown). In soils with similar arsenic concentrations to ours, but in a pot study, arsenic concentrations in *P. vittata* fronds passed the hyperaccumulation threshold before 8-12 weeks of growth (Cao et al., 2003; Fayiga et al., 2004). Our results suggest the onset of phytoextraction could be delayed in sub-ideal field conditions and triggered by specific climate conditions. Our findings suggest frond harvest should occur at 12 weeks after frond growth commences (i.e., at 30 weeks here) for efficient field-scale phytoextraction.

Fern arsenic uptake varies with soil arsenic content

Final fern frond arsenic concentrations ($P<0.001$) and biomass ($P<0.01$), and hence arsenic accumulation *per* fern ($P<0.001$) and arsenic uptake rates ($P<0.001$), increased with initial soil arsenic concentrations and ranged over 1-2 orders of magnitude (Figure 2A-D). Others reported similar arsenic uptake rates (Niazi et al., 2012), similar (Niazi et al., 2012; Ciurli et al., 2014) or higher (Kertulis-Tartar et al., 2006) frond arsenic concentrations, and higher biomass (Niazi et al., 2012) for *P. vittata* in arsenic phytoextraction field studies. Lower mean soil arsenic concentrations here than in the other studies (Kertulis-Tartar et al., 2006; Niazi et al., 2012; Ciurli et al., 2014) could explain the lower frond arsenic concentrations and biomass, since we found that arsenic uptake and biomass varies with soil arsenic concentration. Our results are consistent with previous reports of higher *P. vittata* biomass in the presence of low to moderate soil arsenic concentrations compared to soils with no arsenic (Tu and Ma, 2002), suggesting arsenic hyperaccumulation stimulates growth instead of costing metabolic energy when soil arsenic concentrations are low to moderate.

We found *P. vittata* can hyperaccumulate arsenic from soils with arsenic concentrations as low as 60 mg As/kg soil (Figure 2A). In soils with arsenic concentrations lower than 70 mg/kg, hyperaccumulation has only rarely been reported (Mandal et al., 2012), with *P. vittata* frond arsenic concentrations typically lower than 600 mg/kg (Caille et al., 2004; Gonzaga et al., 2008; Lessl and Ma, 2013). However, our results suggest hyperaccumulation might be unimportant for efficient removal of arsenic from soils with lower arsenic concentrations, since we found that soil arsenic concentrations did not affect remediation times (Figure 2E). Our results suggest remediation occurs equally efficiently with slower plant uptake of arsenic from soils with lower arsenic levels, or with faster plant uptake from soils with higher arsenic levels.

Effects of soil fertilization and inoculation with *F. mosseae* on fern arsenic uptake

When effects of initial soil arsenic concentrations were also considered, treatment did not affect pinnae arsenic concentrations or biomass (Figure 1A and B), but it significantly affected frond arsenic concentrations ($P<0.05$, Figure 2A) and biomass ($P<0.05$, Figure 2B). More specifically, when considering initial soil arsenic concentrations, frond arsenic concentrations were significantly lower in ferns grown in compost- ($P<0.01$) and fungi- ($P<0.05$) treated soils than in the control ferns, while biomass of ferns grown in fungi-treated soils ($P<0.01$) was significantly higher than in the control soils. The negative effect of fungi inoculation on fern arsenic concentrations observed in fronds but not in pinnae could be due to dilution of arsenic concentrations by stipes (midribs), which do not store arsenic (Lombi et al., 2002). Fungi inoculation is likely leading to longer fronds, and consequently to more pairs of pinnae *per* frond and higher arsenic storage capacity, but not to higher biomass of individual pinnae. We found that

arsenic concentrations across treatments in *P. vittata* were correlated with pinnae biomass (R^2=0.78), but not whole aboveground biomass (R^2=0.0059).

The increase in fern biomass in the fungi-treated soils appeared to carry through to higher uptake rates and shorter remediation times. The effect of treatment on arsenic accumulation *per* fern (Figure 2C) and arsenic uptake rate (Figure 2D) was only significant at P<0.1, with ferns in fungi-treated soils removing significantly more arsenic (P<0.05) than in the control plots, when initial soil arsenic concentrations were considered. Treatment significantly affected remediation time (Figure 2E) (P<0.05), with remediation times for fungi-treated ferns significantly lower (P<0.05) than the control.

When frond arsenic concentrations were normalized to initial soil arsenic concentrations, treatment did not significantly affect frond arsenic concentrations (Figure SI-1A), but mean biomass of fungi-inoculated ferns, normalized to soil arsenic concentrations, was twice greater than that of control and nitrogen-treated ferns, a significant difference (Figure SI-1B).

Our field-observations confirm previous findings in controlled greenhouse conditions of higher *P. vittata* biomass upon inoculation with *F. mosseae* (Liu et al., 2005; Trotta et al., 2006; Leung et al., 2010, 2013), leading to higher arsenic accumulation *per* fern even when frond arsenic concentrations did not increase (Liu et al., 2005; Leung et al., 2010). *F. mosseae* could increase fern growth by increasing transport of phosphorus to the fern (Liu et al., 2005; Trotta et al., 2006; Leung et al., 2013). However, we found significantly lower phosphorus concentrations in ferns grown in *F. mosseae*-inoculated soils (Figure SI-2), which could be due to repression of the arbuscular mycorrhizal fungi phosphorus uptake pathway, though such downregulation was reported in high phosphorus status plants (Nagy et al., 2009) and *P. vittata* phosphorus status has not been considered high (Meharg and Hartley-Whitaker, 2002; Zhao et al., 2002). Our results suggest that fungi inoculation can increase *P. vittata* growth through processes other than nutrient transport, perhaps through effects on arsenic reductase activity (Leung et al., 2013) to increase arsenic tolerance and free metabolic energy for growth. *F. mosseae* inoculation could be especially important to support growth of *P. vittata* under field stress.

Effects of phosphorus treatments on phosphorus and arsenic uptake by *P. vittata*

Our findings suggest that phosphorus can interfere with arsenic uptake on a mole-for-mole basis under field conditions. In ferns grown in compost-treated soils, mean phosphorus concentrations were significantly higher than in control plots (Figure SI-2), while arsenic concentrations were lower (Figure 2A). Likewise, soil arsenic concentrations (P<0.001; data not shown) and treatment (P<0.001; Figure SI-2) also significantly affected fern phosphorus concentrations. Soil arsenic concentrations were negatively correlated with fern phosphorus concentrations, suggesting competition of arsenic and phosphorus at the phosphate intake pathway (Wang et al., 2002). Interference of phosphorus with arsenic uptake in *P. vittata* has been observed on time scales of hours to months, over wide phosphorus concentration ranges (Wang et al., 2002; Tu and Ma, 2003b; Natarajan et al., 2009). However, compost application did not affect arsenic accumulated *per* fern (Figure 2C), due to a slight increase in biomass in the presence of compost.

In contrast, calcium phosphate, regardless of application rate, did not affect *P. vittata* biomass (Figure 2B), nor arsenic (Figure 2A) or phosphorus concentrations (Figure SI-2) in *P. vittata*. Although calcium phosphate at our high application rate supplied approximately twice the available phosphorus of compost, our results suggest compost-supplied phosphorus could be more available to *P. vittata* than calcium phosphate. Calcium phosphate could have reacted with lead in soil (mean 143.9 mg Pb/kg; Table 1) to form insoluble lead-phosphates (Ma et al., 1993) and

therefore not been available for fern uptake, whereas compost application has been shown to decrease phosphorus sorption and increase phosphorus solubility in soil (Giusquiani et al., 1988; Mkhabela and Warman, 2005).

Our results contrast with recent observations that phosphate rock application at rates similar to ours led to higher *P. vittata* frond arsenic concentrations, biomass, and phosphorus accumulation than when phosphorus was applied as soluble form, or not applied at all (Lessl and Ma, 2013; da Silva et al., 2018). In that loamy sand soil, available phosphorus (0.38 mg/kg) was low enough that a form of supplemental phosphorus with low phosphorus availability was needed to maintain fern survival (Lessl and Ma, 2013). In our sandy loam soil, available phosphorus (2.7 mg/kg) appears to have been sufficient for fern survival and concomitant arsenic uptake such that we found no effect of either compost-derived or calcium phosphate on arsenic accumulated *per* fern. Phosphorus application might be necessary to support *P. vittata* and increase arsenic uptake only in soils with extremely low (<< 2.7 mg/kg) available phosphorus.

Decreases in soil arsenic concentrations during phytoextraction

After phytoextraction, soil arsenic concentrations were significantly lower than initial values, both in 0-10 cm ($P<0.001$) and 10-20 cm ($P>0.001$) depth intervals, with significantly lower final values in the deeper depth interval ($P<0.001$; Table 2). Over the 58 week experiments, mean arsenic concentrations in the 0-10 cm depth interval decreased by up to 20.5% (field mean 9.8%) in 34 of 36 plots (Table 2), whereas in the 10-20 cm depth interval, they decreased by up to 43.6% (field mean 18.5%) in 35 of 36 plots (Table 2). Treatment had little to no effect on final soil arsenic concentrations.

The greater soil arsenic depletion we observed in soils at deeper depth intervals is similar to field observations with *P. vittata* (Kertulis-Tartar et al., 2006) and *Pityrogramma calomelanos,* another arsenic-hyperaccumulating fern (Niazi et al., 2012). We found *P. vittata* roots primarily in the 0-10 cm depth (data not shown), as did others (Liao et al., 2004; Kertulis-Tartar et al., 2006), with 84% of roots in the 0-10 cm depth (Liao et al., 2004).

Comparing fern arsenic accumulation to soil arsenic depletion

To our knowledge, this is the first mass balance relating arsenic phytoextraction with *P. vittata* to significant decreases in soil arsenic concentrations under field conditions. Over 58 weeks, ferns accumulated a mean of 3.6% of initial soil arsenic (Table 2). The amount of arsenic depleted from the 0-10 cm depth interval was not significantly different from the amount of arsenic accumulated in ferns (Table 2 and Figure SI-4A; $P=0.1007$). Considering that the greater *P. vittata* root density is observed in this depth, our finding could suggest *P. vittata* took up all arsenic depleted from the 0-10 cm depth, an outcome promising for practical use of phytoextraction.

However, in the larger 0-20 cm depth interval, the amount of arsenic depleted from soil was significantly larger than fern arsenic accumulation (Table 2 and Figure SI-4C; $P<0.001$). Fern arsenic accumulation accounted for a mean of 38.5% of the arsenic depleted from soil across 0-20 cm depth, in the 35 plots where depletion was observed (Table 2). Therefore, sequestration of arsenic in fern fronds does not explain the entirety of soil arsenic depletion.

Similarly, although soil arsenic concentrations decreased significantly during phytoextraction under field conditions with *P. calomelanos*, fern arsenic accumulation could not account for the depletion (Niazi et al., 2012). The discrepancy was attributed to soil sampling error induced by high heterogeneity in soil arsenic concentrations (Niazi et al., 2012), a problem masking changes in soil metal concentrations in other phytoextraction studies (Wilde et al., 2005). To circumvent

issues of contaminant heterogeneity, the authors suggested fern arsenic accumulation is a better indicator of the arsenic removed from soil during phytoextraction (Niazi et al., 2012). However, similar discrepancies, with *P. vittata* accumulation accounting for only 13-22% of soil arsenic depletion, have occurred in pot studies where soil is presumably well-mixed (Gonzaga et al., 2008). We used a well-replicated study design, extensively mixed soil before planting the ferns to decrease within-plot heterogeneity, collected incremental, representative soil samples (Hadley and Petrisor, 2013; Brewer et al., 2017), and calculated a mass balance to show that the discrepancy in frond arsenic accumulation and soil arsenic depletion is not an artifact of sampling error, but indicates the possibility of other processes leading to loss of soil arsenic, including storage in rhizomes and leaching of arsenic.

Explaining arsenic loss from soil

Rhizomes can be a secondary storage organ for arsenic in *P. vittata* (Liao et al., 2004). Rhizome arsenic concentrations, biomass, and arsenic accumulation were 50-60% that of pinnae in a naturally occurring *P. vittata* population (Liao et al., 2004), though arsenic concentrations in rhizomes were only 19-29% of frond concentrations in cultivated ferns (Zhang et al., 2002). We found arsenic accumulation in rhizomes was 0.097±0.04 mg As/kg soil, 1-2 orders of magnitude less than in fronds (data not shown). Furthermore, we found that the mass of arsenic contained in ferns, even including an additional 60% arsenic to account for rhizome accumulation in well-established populations (Liao et al., 2004), was still significantly lower ($P<0.001$) than soil arsenic depletion across both depths (0-20 cm), suggesting the importance of still other processes leading to arsenic loss from soils.

Arsenic loss by leaching could explain the arsenic not accounted for by frond or rhizome accumulation in our mass balance. However, we found low arsenic concentrations in porewater at a depth of 5-10 cm (<6 μg/L; Figure SI-3), suggesting either arsenic was not highly leachable in the 0-10 cm depth from bulk soil, arsenic leaching was spatiotemporally heterogeneous and not well represented in our samples, or leachable arsenic had been depleted by the time porewater measurements commenced. We expected the potential for arsenic leaching from soil during phytoextraction to be limited due to strong sorption of arsenic(V) onto iron(III) oxides (Lin and Puls, 2000; Lombi et al., 2000; Niazi et al., 2012), especially at pH 6.0. Bulk XANES spectra showed that most arsenic in bulk soil in the 0-10 cm depth is present as arsenic(V) (Figure 3D and Table SI-1), and bulk and micro spectra show abundant iron oxides and silicates (Table SI-2). However, arsenic reduction and therefore mobility could have increased in the 10-20 cm depth interval due to poor drainage above the clay pan (Roberts et al 2011). The lack of treatment effect here, in contrast to observations that compost and phosphorus soil treatments can lead to arsenic desorption and increase arsenic concentrations in the soil solution (Caille et al., 2004; Dobran and Zagury, 2006), suggests arsenic leaching is due to fern cultivation.

Rhizosphere processes could increase arsenic leaching

We found evidence to suggest the rhizosphere is a reactive zone with mobilization processes distinct from bulk soil and which could lead to arsenic leaching. Indeed, bulk and micro XANES spectra showed arsenic in rhizosphere soil is present as mixed arsenic(III) (12.8-71.5%) and arsenic(V) (29.1-89.8%). Furthermore, bulk XANES spectra collected from whole root samples showed high abundance of arsenic(III) (69.0-78.7%) with limited arsenic(V) (21.3-31.0%).

Arsenic reduction could be coupled to dissolved organic carbon (DOC) mobilization and/or oxidation in the *P. vittata* rhizosphere. Root exudates have been suggested to be play an important

role in arsenic release from soil solid surfaces for uptake into *P. vittata* (Gonzaga et al., 2006; Liu et al., 2016). As the mixed redox status of rhizosphere arsenic suggests (Figure 3, Figure SI-5, Figure SI-6), arsenic and DOC cycles in *P. vittata* rhizosphere soil could intersect through a combination of processes including: 1) release of arsenic(V) from iron oxide surfaces due to ion exchange or ligand-enhanced dissolution with DOC (Fitz et al., 2003; Dobran and Zagury, 2006), leading to fern uptake of arsenic(V) and/or leaching of arsenic(V) (Masscheleyn et al., 1991; Hutton et al., 2005; Dobran and Zagury, 2006; Roberts et al., 2011) and/or DOC (Vinther et al., 2006; Uselman et al., 2007); 2) enhanced weathering of sulfide residues in pyrite cinders (Tiberg et al., 2017), releasing arsenic(III) and/or arsenic(V) and decreasing pH locally (Ciurli et al., 2014); 3) reduction of arsenic(V) within roots (Duan et al., 2005); 4) microbially-mediated arsenic reduction in the rhizosphere coupled to DOC oxidation (Dobran and Zagury, 2006; Das et al., 2017); 5) fern uptake of arsenic(III) (Wang et al., 2011); and/or 6) replenishment of soil arsenic through leaching from foliage (Yan et al., 2009) or excretion of arsenic(III) from roots to soil (Chen et al., 2016b).

Arsenic reduction coupled to DOC oxidation could increase leaching from rhizosphere and bulk soils, particularly if low pH microsites exist near pyrite cinders (Ciurli et al., 2014). With decreasing pH, arsenic(III) sorption decreases, in contrast to arsenic(V) sorption (Dixit and Hering, 2003). We found evidence to suggest soluble species could be transported from the rhizosphere to bulk soil when gravitational flow exceeds transpiration flux. Contrary to expected arsenic redox gradients within aggregates (Masue-Slowey et al., 2013), we found more arsenic(III) on the exterior (49.1 to 64.8%) than interior (27.9 to 40.0%) of soil aggregates (Figure 3C-D and Table SI-1). The aggregate represents bulk root zone soil, such that the greater abundance of arsenic(III) on the surface suggests transport of arsenic(III) from rhizosphere to bulk soil. However, others have found low concentrations of arsenic(III) in oxic soil porewater (Masscheleyn et al., 1991; Hutton et al., 2005; Roberts et al., 2011). Leaching of arsenic(III) could be hyperlocalized, or arsenic(III) could reoxidize after mobilization, perhaps *via* reduction of manganese oxides (Amirbahman et al., 2006).

But arsenic reduction is not necessary for arsenic leaching. At pH 6, similar amounts of arsenic(III) and (V) sorb to iron oxides (Dixit and Hering, 2003), indicating both species are equally leachable at the soil pH we observed. Others observed arsenic(V) leaching from oxic soils (Masscheleyn et al., 1991; Hutton et al., 2005; Roberts et al., 2011), especially from soils upon addition of organic matter (Dobran and Zagury, 2006). Here, arsenic(V) could be mobilized from rhizosphere and bulk soil due to ion exchange with leaching of root-derived organic carbon from the rhizosphere to lower soil horizons (Vinther et al., 2006; Uselman et al., 2007). Leaching of arsenic(III) and (V) from soils below the root zone could help explain the greater depletion in arsenic concentrations in the 10-20 cm than in the 0-10 cm depth (Table 2). Our findings suggest the 10-20 cm depth is not a sink for arsenic, but instead is a zone of active arsenic mobilization processes coupled to *P. vittata* growth even if below the root zone. Moreover, our results confirm other reports that arsenic leached from soil after *P. vittata* planting and irrigation (Yang et al., 2012).

Higher resolution data on arsenic mobilization and transport from root zone to bulk soil, particularly at deeper depths, is needed to determine to what extent arsenic leaching occurs and the fate of this leached arsenic. Depending on concentration and fate of mobilized arsenic, arsenic leaching during phytoextraction could be considered a remediation process analogous to monitored natural attenuation (Reisinger et al., 2005), or a process contaminating soil and groundwater.

Acknowledgements

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Table 1. Properties of field site soil (0-10 cm depth) before phytoextraction

Soil characteristic		Value	Range
pH[1]		5.5	
		Total[2]	
As	(mg/kg)	78.3	23.5 - 118.6
P	(mg/kg)	290.3	98.0 - 735.5
Fe	%	3.4	1.9 - 4.6
Pb	(mg/kg)	143.9	95.2 - 223.0
Cu	(mg/kg)	550.6	404.5 - 772.0
Zn	(mg/kg)	401.5	293.6 - 562.3
		Modified Morgan extractable [3]	
P	(mg/kg)	2.7	n/a
K	(mg/kg)	129.0	n/a
Ca	(mg/kg)	2479.1	n/a
Mg	(mg/kg)	341.2	n/a
Zn	(mg/kg)	38.1	n/a
B	(mg/kg)	0.3	n/a
Mn	(mg/kg)	44.7	n/a
Cu	(mg/kg)	18.6	n/a
Fe	(mg/kg)	24.4	n/a
Pb	(mg/kg)	4.4	n/a
Al	(mg/kg)	15.2	n/a
Na	(mg/kg)	32.0	n/a
S	(mg/kg)	46.3	n/a
CEC[3]	meq/100g	23.7	n/a
Organic matter [3]	%	8.6	n/a
Bulk density [4]	g/cc	1.1	n/a
Sand[4]	%	53.6	n/a
Silt[4]	%	29.8	n/a
Clay[4]	%	16.6	n/a
Texture [4]		Sandy loam	n/a

[1]Mean of 3 replicates, in water

[2]Field mean and range of plot means in 36 plots, after soil treatments applied

[3]Mean of 2 replicates, before soil treatments applied

[4]Mean of 3 replicates

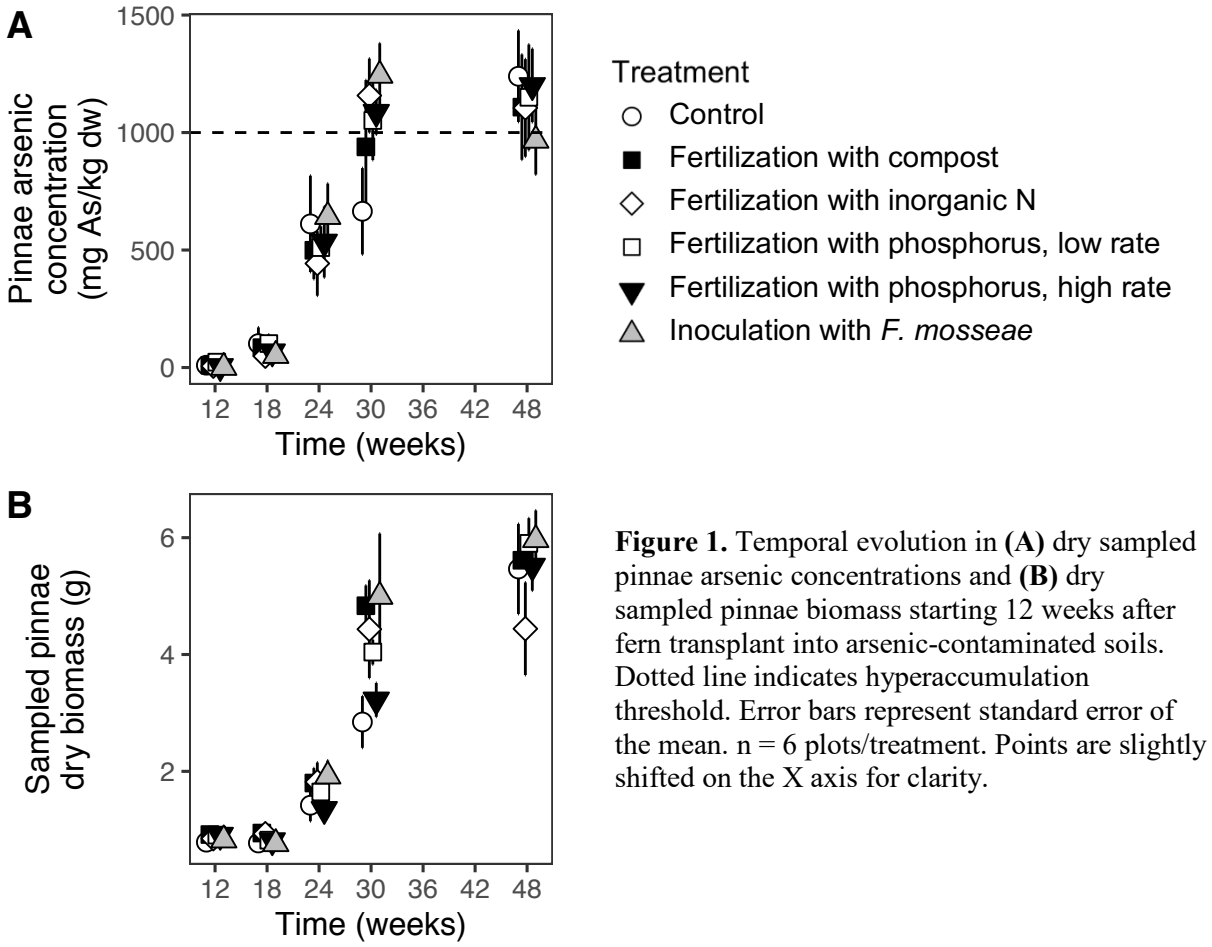

Figure 1. Temporal evolution in **(A)** dry sampled pinnae arsenic concentrations and **(B)** dry sampled pinnae biomass starting 12 weeks after fern transplant into arsenic-contaminated soils. Dotted line indicates hyperaccumulation threshold. Error bars represent standard error of the mean. n = 6 plots/treatment. Points are slightly shifted on the X axis for clarity.

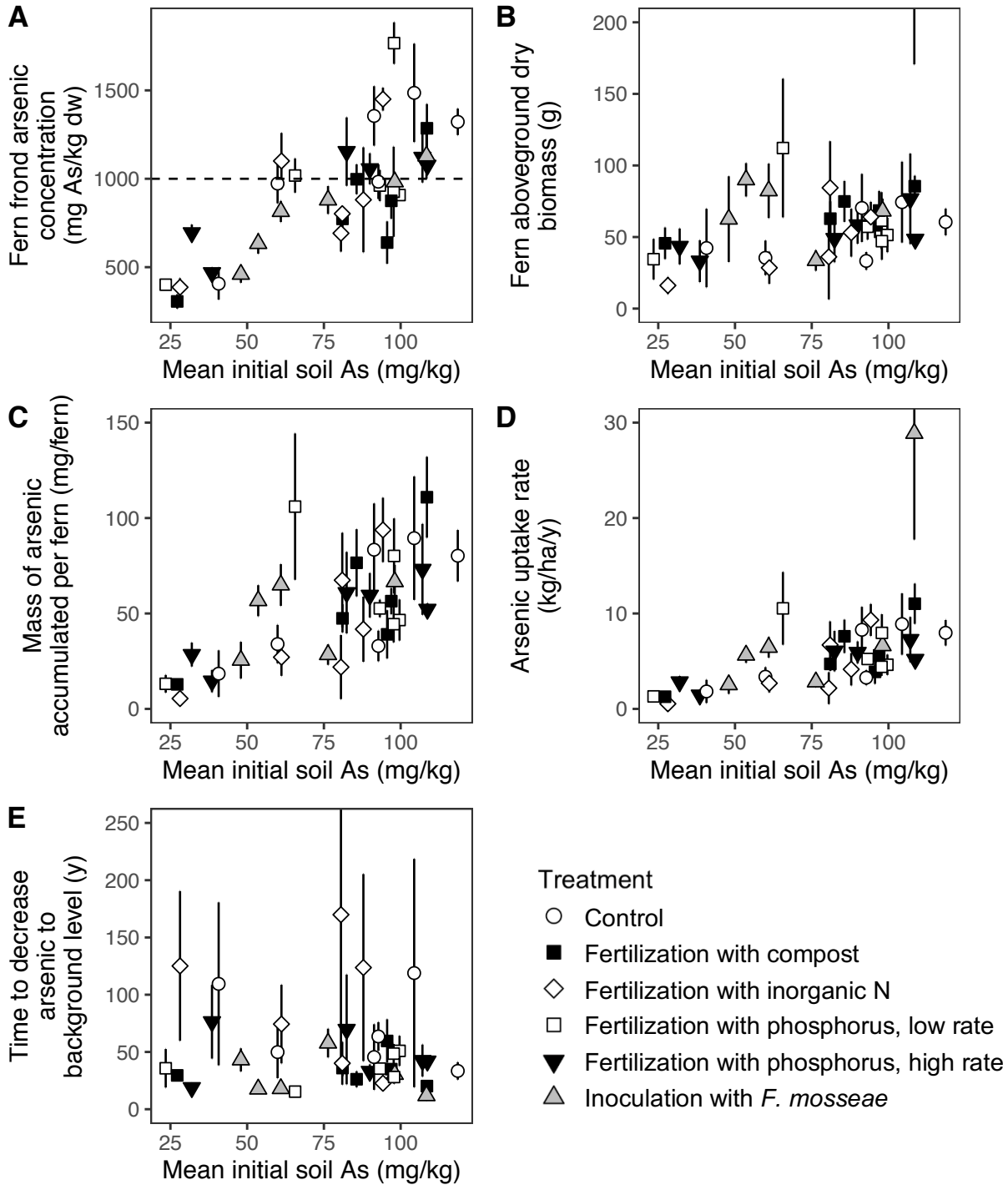

Figure 2. Mean **(A)** fern arsenic concentrations, **(B)** fern aboveground biomass, **(C)** fern accumulated arsenic mass, **(D)** arsenic uptake rates, and **(E)** remediation times at final harvest, 58 weeks after fern transplanting in arsenic-contaminated soils. n = 4. Dotted line in panel A indicates hyperaccumulation threshold. Symbols represent mean values *per* plot, and error bars represent standard error of the mean.

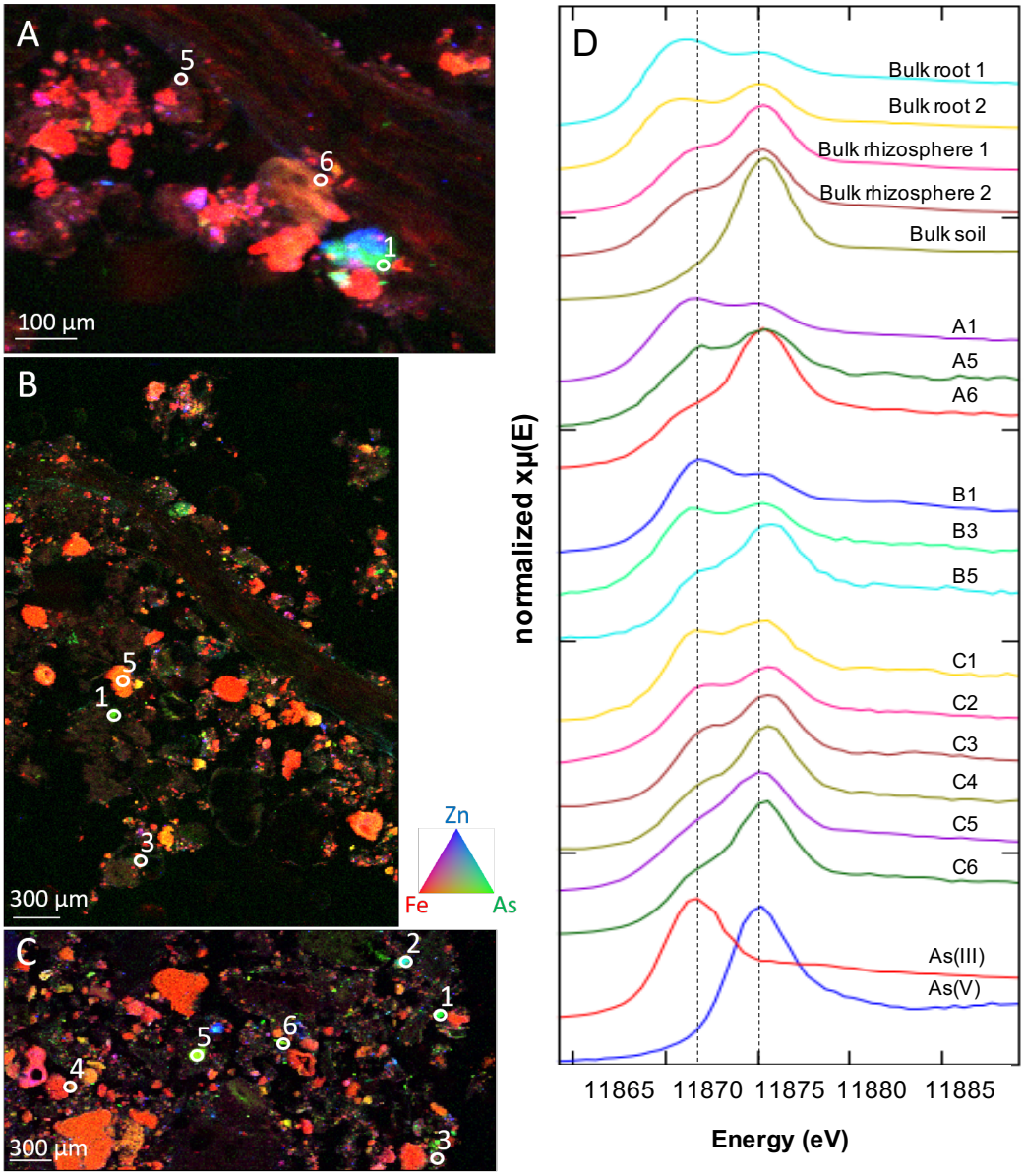

Figure 3. Tricolor-coded microscale X-ray fluorescence tri-color images of As, Fe, and Zn in **(A, B)** untreated (control) roots with rhizosphere soil and **(C)** untreated (control) soil aggregate, collected at 12keV. The tri-color shield indicates color in panels A, B, and C. Within an image, brighter colors indicate higher fluorescence signals from the imaged elements. Selected arsenic K-edge bulk and microscale X-ray absorption near-edge structure (XANES) spectra reported for bulk spectra from powdered untreated (control) roots, rhizosphere soil, and bulk soil; 3 spots across root cross-section A; 3 spots across root cross-section B; and 6 spots across aggregate cross-section C (spots 1-3 are on aggregate edge; spot 4 is approximately aggregate center). Spectra for additional spots in panels A, B, and C are available in the Supplemental Information. As(III) and As(V) spectra that were included in the best-fit results from linear combination fitting analysis are included in panel D for reference. The complete library of standards available in the Supplemental Information. Vertical lines denote energies of 11872 and 11875 eV that were used to differentiate As(V) and As(III) species, respectively.

Table 2. Mass balance comparing soil arsenic depletion to fern arsenic accumulation after 58 weeks of phytoextraction. Plots are listed in order of decreasing initial soil arsenic. Mass balance compares fern arsenic accumulation per kg soil (per 1 depth)*2 to soil arsenic depletion across 0-20 cm, or fern arsenic uptake per kg soil (per 1 depth) to soil arsenic decrease in either 0-10 cm or 10-20 cm depth.

			0-20 cm				0-10 cm		10-20 cm	
Plot	**Treat-ment**	**Fern As accumulation per kg soil (per 1 depth)**	**Initial soil As**	**% of initial soil As accumulated in fern after 58 weeks**	**Soil As depletion after 58 weeks**	**% of depleted soil As accumulated in fern after 58 weeks**	**Soil As depletion after 58 weeks**		**Soil As depletion after 58 weeks**	
		mg As/ kg soil	**mg/kg**	**%**	**mg/kg**	**%**	**mg/kg**	**% decr**	**mg/kg**	**% decr**
16	Control	8.1	118.6±4.0	3.4	1.4	281.4	-2.4	-2.0	5.3	4.4
11	P - high	5.3	108.7±3.6	2.4	31.0	8.5	15.3	14.1	46.6	42.9
17	Compost	11.2	108.6±5.0	5.2	-2.5	NA	-2.3	-2.2	-2.7	-2.5
21	Fungi	29.3	108.4±1.7	13.5	14.0	104.6	20.6	19.0	7.4	6.9
18	P - high	7.4	107.1±3.5	3.4	11.9	31.1	10.0	9.3	13.8	12.8
9	Control	9.0	104.4±4.9	4.3	10.0	45.3	8.1	7.7	11.9	11.4
3	P - low	4.7	99.6±2.3	2.4	24.0	9.8	7.0	7.0	41.1	41.2
8	Fungi	6.7	98.1±1.3	3.4	6.2	54.6	3.8	3.8	8.6	8.7
25	P - low	8.1	97.8±4.7	4.1	15.0	26.9	16.9	17.2	13.2	13.5
5	P - low	4.5	97.7±0.9	2.3	30.3	7.4	17.9	18.4	42.6	43.6
6	Compost	5.7	97.0±1.9	2.9	21.2	13.5	9.4	9.7	33.0	34.1
2	Compost	3.9	95.6±0.6	2.1	12.4	15.9	8.5	8.9	16.2	17.0
24	Nitrogen	9.5	94.2±6.1	5.0	13.0	36.6	14.2	15.1	11.7	12.4
14	P - low	5.3	93.2±3.3	2.9	11.0	24.2	9.0	9.7	13.0	13.9
36	Control	3.3	92.7±6.1	1.8	12.0	13.9	14.7	15.8	9.4	10.1
23	Control	8.4	91.3±7.6	4.6	13.4	31.5	12.1	13.2	14.7	16.1
7	P - high	6.0	90.0±2.7	3.3	11.7	25.6	4.7	5.3	18.7	20.8

Table 2. (continued) Mass balance comparing soil arsenic depletion to fern arsenic accumulation after 58 weeks of phytoextraction.

			0-20 cm				0-10 cm		10-20 cm	
Plot	**Treat-ment**	**Fern As accumulation per kg soil (per 1 depth)**	**Initial soil As**	**% of initial soil As accumulated in fern after 58 weeks**	**Soil As depletion after 58 weeks**	**% of depleted soil As accumulated in fern after 58 weeks**	**Soil As depletion after 58 weeks**		**Soil As depletion after 58 weeks**	
		mg As/ kg soil	**mg/kg**	**%**	**mg/kg**	**%**	**mg/kg**	**% decr**	**mg/kg**	**% decr**
10	Nitrogen	4.2	87.9±3.1	2.4	16.3	12.9	3.8	4.4	28.9	32.8
35	Compost	7.7	85.6±2.4	4.5	5.8	66.4	4.2	4.9	7.4	8.7
22	P - high	6.2	82.4±4.4	3.7	4.4	70.2	3.2	3.8	5.6	6.8
15	Compost	4.8	81.1±1.0	3.0	10.5	22.8	5.8	7.1	15.2	18.8
19	Nitrogen	6.8	81.0±0.9	4.2	6.8	50.0	4.5	5.5	9.2	11.3
1	Nitrogen	2.2	80.6±2.6	1.4	14.7	7.5	11.5	14.3	18.0	22.3
4	Fungi	2.9	76.3±2.1	1.9	13.9	10.3	3.6	4.8	24.2	31.7
33	P - low	10.7	65.6±2.0	8.2	9.6	55.5	10.8	16.4	8.5	13.0
34	Nitrogen	2.7	61.2±2.8	2.2	6.6	20.6	10.9	17.8	2.4	4.0
20	Fungi	6.6	61.0±0.5	5.4	2.9	114.6	2.7	4.5	3.0	4.9
12	Control	3.4	59.9±0.2	2.9	10.2	16.7	12.3	20.5	8.2	13.7
32	Fungi	5.7	53.6±1.8	5.3	8.4	34.2	8.0	14.9	8.8	16.4
27	Fungi	2.6	48.0±1.7	2.7	7.1	18.3	5.4	11.3	8.7	18.1
28	Control	1.9	40.7±1.8	2.3	9.4	10.0	4.7	11.6	14.0	34.4
26	P - high	1.5	38.5±0.9	1.9	6.9	10.7	2.8	7.1	11.0	28.6
13	P - high	2.9	32.0± NA	4.5	5.8	24.8	5.6	17.4	6.1	18.9
30	Nitrogen	0.5	28.1±0.4	1.0	4.6	6.0	0.3	1.1	8.9	31.6
31	Compost	1.3	27.2±0.3	2.4	6.4	10.1	3.6	13.3	9.3	34.1
29	P - low	1.3	23.5±1.1	2.8	1.2	55.7	0.2	1.0	2.2	9.2

Chapter 3. Supplemental Information

Micro-focused and bulk X-ray spectromicroscopy

X-ray microprobe analyses were performed at the Advanced Light Source XFM beamline 10.3.2 (Marcus et al., 2004). Thirty micron-thick thin sections were mounted with silicon vacuum grease onto a Peltier-cooling stage. All data were collected in fluorescence mode at -20 ºC and ambient pressure using a Canberra 7-element Ge solid state detector and processed using a suite of LabVIEW custom software available at the beamline.

μXRF Mapping: Arsenic and iron spatial distribution in the samples was determined using micro-focused X-ray fluorescence (μXRF) elemental mapping with a 12 keV incident beam, using a 50 ms/pixel dwell time, a 2 × 2.5 μm beam spot size, and a pixel size of 3 × 3 μm (Figure 3A), 7 × 7 μm (Figure 3B), or 10 × 10 μm (Figure 3C). Maps were deadtime-corrected. Decontamination was not required.

μXANES: In sample regions of interest, arsenic and iron speciation were determined using arsenic K-edge and iron K-edge μX-ray absorption near edge structure (μXANES) spectroscopy. Spectra were calibrated using sodium arsenate (E0=11875 eV) and elemental iron (E0=7110.75 eV), respectively. All spectra recorded in the range 11770-12177 eV (As) and 7011-7416 eV (Fe) were deadtime-corrected, deglitched, calibrated, pre-edge background subtracted, and post-edge normalized using a suite of custom LabVIEW software available at the beamline. If counts were low, spectra were collected at 2 adjacent spots and merged.

Bulk XAS: Bulk XANES spectra were collected at SSRL beamline 7.3. Filter membranes were sealed and mounted on sample holders with Kapton tape. Data were collected in fluorescence mode using a cryostat sample holder at 10 K and a Canberra 30 element Ge solid state detector. Spectra collected in the range 11635-12877 eV (As) and 6880-8126 eV (Fe) were calibrated to a reference gold (E0=11919 eV) foil (As) or iron (E0=7112 eV) foil (Fe) in SixPak (Webb 2005), and normalized and plotted in Athena.

Least-square linear combination fitting: LSQ LC fitting of experimental XANES spectra (micro and bulk) was performed using custom LabVIEW software for arsenic in the range of 11770-12177 eV using a library of 68 standard arsenic compounds, and for iron in the range of 7011-7416 eV using a library of 79 standard iron compounds. The best LCF was obtained by minimizing the normalized sum of squares residuals (NSS=0=perfect fit), according to the formula $NSS = 100 \times \{\sum(\mu exp - \mu fit)^2 / \sum (\mu exp)^2\}$ where μ represents the normalized absorbance. Some spectra were corrected for over-absorption induced distortion using μ corrected = μ exp / (1 + a (1 - μ exp)) where the parameter a was adjusted. The error on the percentages of species found via LC fitting is estimated to ± 10%. 2D scatter valence plots were generated from Fe and As XANES data according to methods previously described in Marcus et al. (2008, Fe XAS database updated since) and a Matlab code described elsewhere (Fakra et al., 2018) and available at beamline 10.3.2. For iron valence plots, kappa and mu represent normalized absorption values at 7113 and 7117.5eV respectively. For arsenic valence plots, kappa and mu represent normalized absorption values at 11870.8 and 11889.9 eV respectively. Standards are plotted as black empty squares and sample data points are in color.

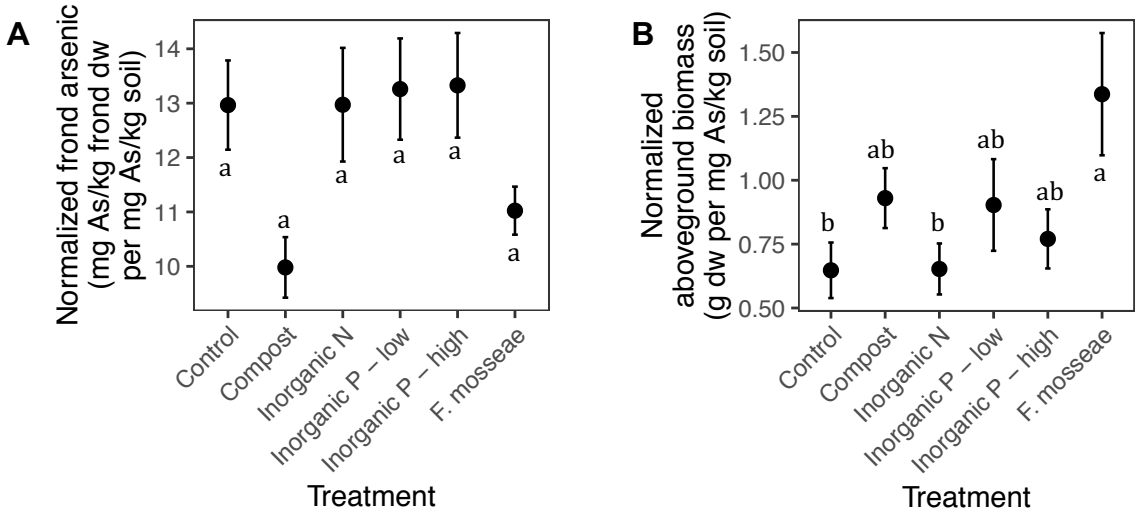

Figure SI-1. Mean fern arsenic concentrations **(A)** and fern aboveground biomass **(B)** by treatment, normalized to soil arsenic concentrations, at final harvest in December, 58 weeks after fern transplanting into arsenic-contaminated soils. n = 24.

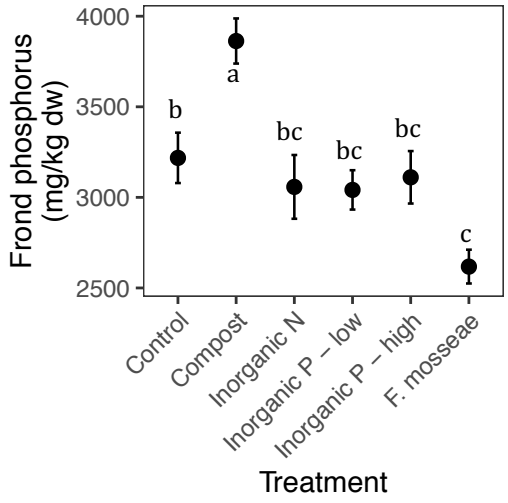

Figure SI-2. Phosphorus concentrations in dry fern aboveground biomass by treatment at final harvest in December, 58 weeks after fern transplanting into arsenic-contaminated soils. n = 24.

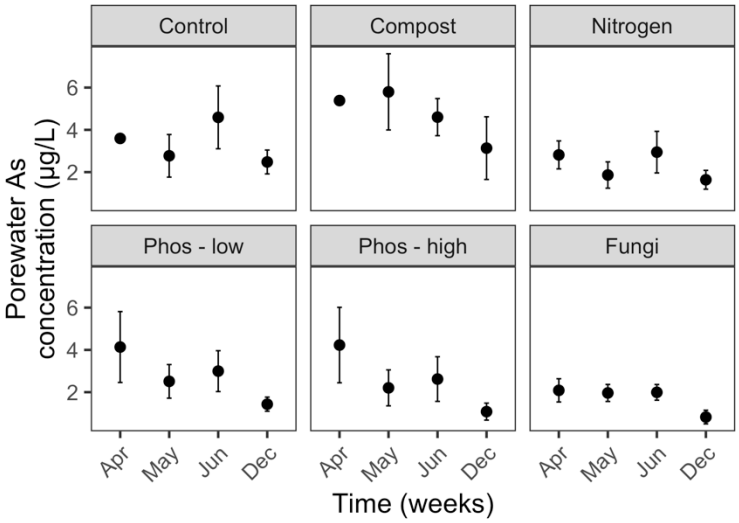

Figure SI-3. Temporal changes in arsenic concentrations in porewater collected from soil 0-10 cm deep 15 cm from fern crowns starting 5 months after fern transplant into arsenic-contaminated soils. Panels represent soil treatments. Error bars represent standard error of the mean. n = 6 plots/treatment.

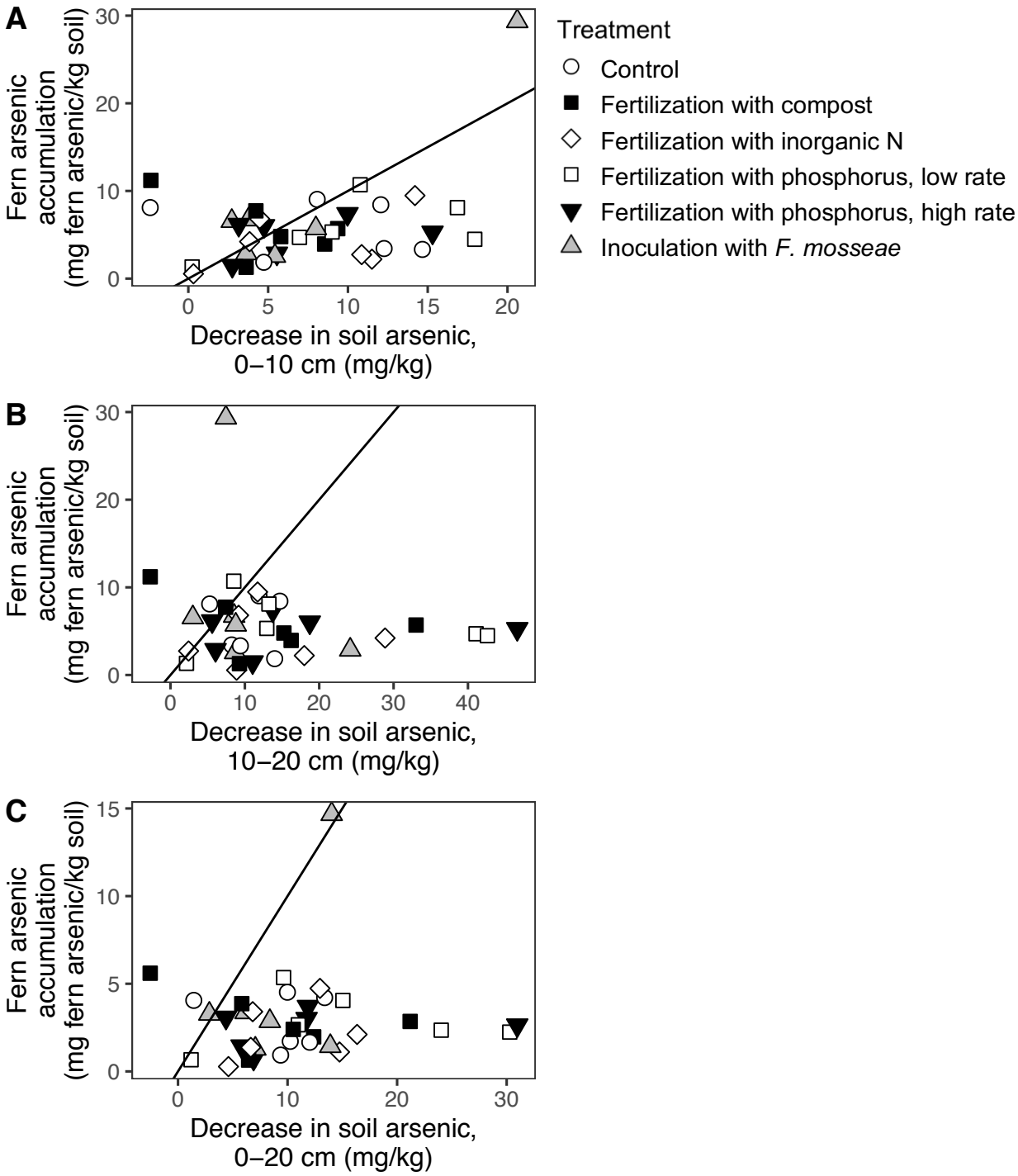

Figure SI-4. Mean arsenic contained *per* fern, normalized *per* kilogram soil, plotted by decrease in soil arsenic concentrations in **(A)** 0-10 and **(B)** 10-20 cm depth, assuming all arsenic came from 1 depth, and **(C)** across 0-20 cm depth. The line indicates a 1-1 relationship between fern arsenic accumulation (Y axis) and soil arsenic depletion (X axis). Fern arsenic uptake is mean of 4 replicates, soil arsenic mean final arsenic (3 replicates) subtracted from mean initial arsenic (3 replicates).

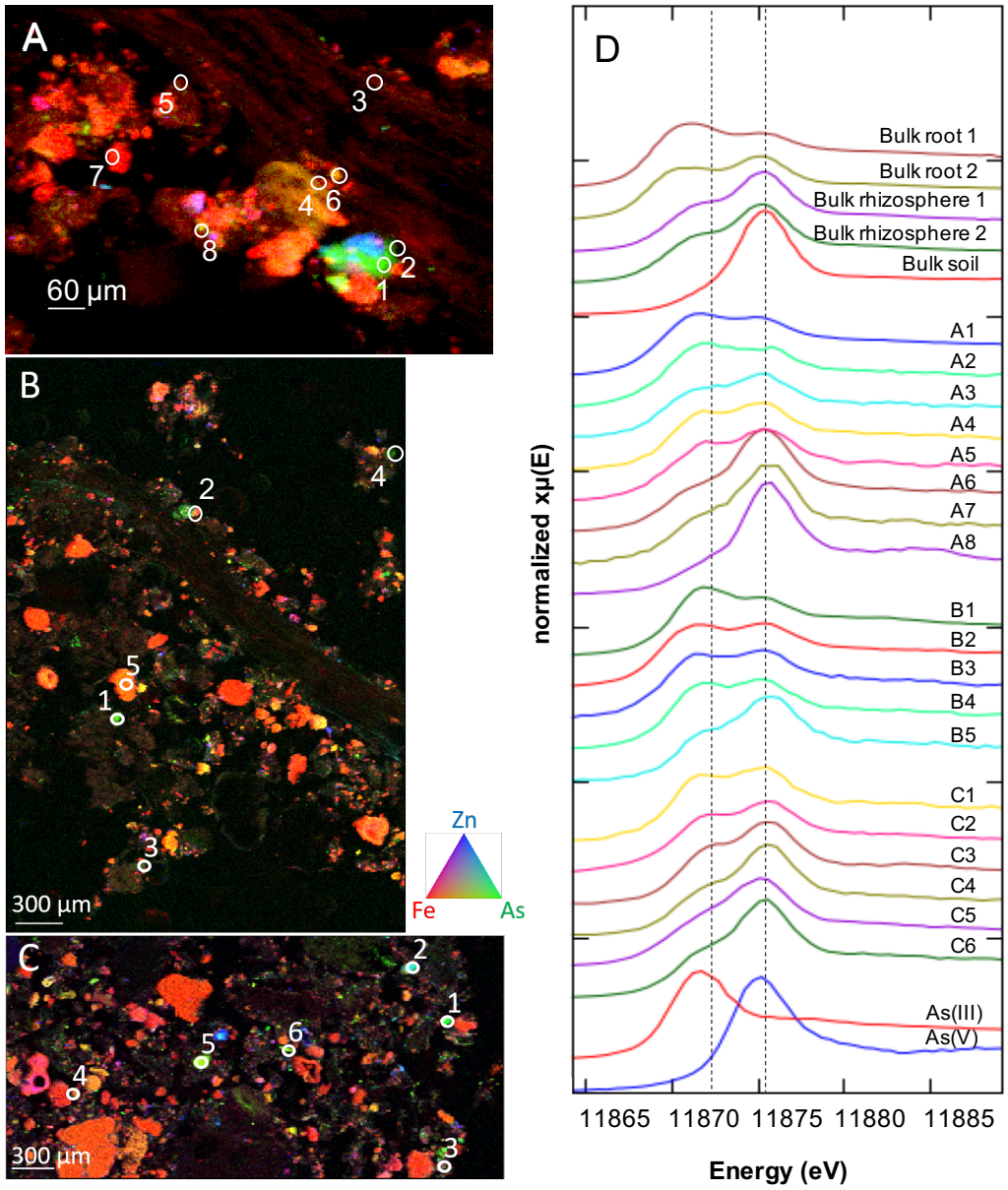

Figure SI-5. Tricolor-coded microscale X-ray fluorescence tri-color images of As, Fe, and Zn in **(A, B)** untreated (control) roots with rhizosphere soil and **(C)** untreated (control) soil aggregate, collected at 12keV. The tri-color shield indicates color in panels A, B, and C. Within an image, brighter colors indicate higher fluorescence signals from the imaged elements. Arsenic K-edge bulk and microscale X-ray absorption near-edge structure (XANES) spectra reported for bulk spectra from powdered untreated (control) roots, rhizosphere soil, and bulk soil; 8 spots across root cross-section A; 5 spots across root cross-section B; and 6 spots across aggregate cross-section C (spots 1-3 are on aggregate edge; spot 4 is approximately aggregate center). As(III) and As(V) spectra that were included in the best-fit results from linear combination fitting analysis are included in panel D for reference. The complete library of standards available in the Supplemental Information. Vertical lines denote energies of 11872 and 11875 eV that were used to differentiate As(V) and As(III) species, respectively.

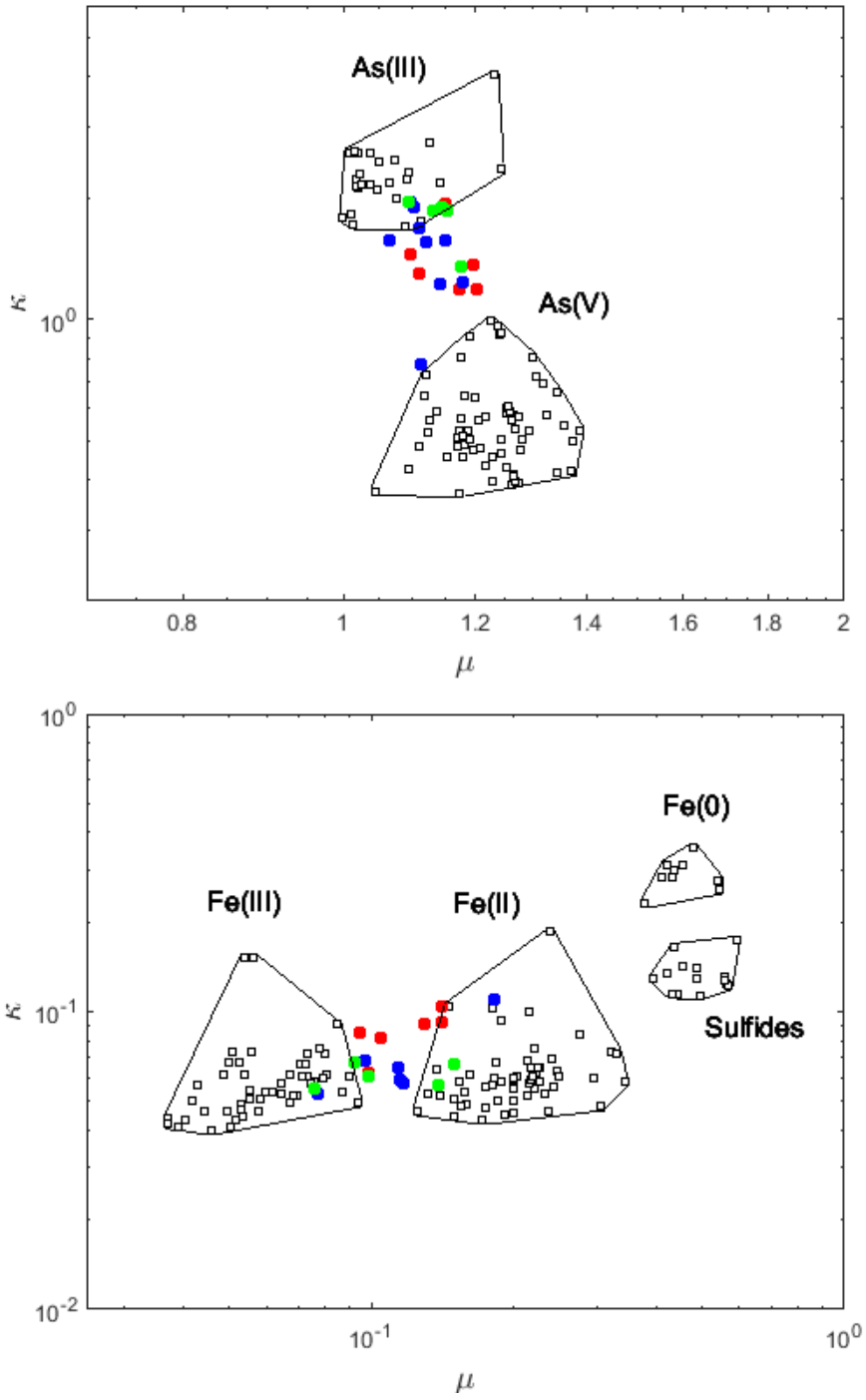

Figure SI-6. Valence plots of arsenic (top) and iron (bottom) speciation in root 3A and soil samples Figure 3A (blue), Figure 3B (green), and Figure 3C (red). Standards are plotted as black empty squares. Kappa and mu represent normalized absorption values at 11870.8 and 11889.9 eV respectively.

Table SI-1. Linear combination fits for As K-edge bulk and micro (A1-C6) XAS spectra in Figure 3D.

Spectra	As(III) %	As(V) %	Normalized Sum of Squares
Bulk root 1	101.8	6.1	2.59E-04
Bulk root 2	75.5	26.3	2.90E-04
Bulk rhizosphere 1	48.6	52.5	4.04E-04
Bulk rhizosphere 2	48.3	50.4	4.25E-04
Bulk soil	9.9	102.9	4.20E-04
A1	71.5	29.1	1.81E-04
A2	71.2	30.8	6.41E-04
A3	57.7	42.6	8.26E-04
A4	55.0	46.1	5.76E-04
A5	54.1	46.9	1.05E-03
A6	30.4	70.5	5.06E-04
A7	31.7	71.0	1.56E-03
A8	12.8	89.8	1.51E-03
B1	70.3	31.8	4.93E-04
B2	67.6	34.3	3.40E-04
B3	64.0	38.3	7.75E-04
B4	61.5	40.3	5.42E-04
B5	42.9	60.1	1.47E-03
C1	64.8	37.7	8.15E-04
C2	53.4	46.9	4.60E-04
C3	49.1	52.0	7.11E-04
C4	40.0	61.0	6.08E-04
C5	38.4	63.8	4.75E-04
C6	27.9	73.9	3.44E-04

Table SI-2. Linear combination fits for Fe K-edge bulk and micro (A1-C6) XAS spectra collected from bulk samples or microXANES spots in Figure 3(A-C) and/or Figure SI-5(A-C).

Spectra	%	Fe Component 1	%	Fe Component 2	%	Fe Component 3	Normalized Sum of Squares
Bulk root 2	28.4	Iron (III) oxyhydroxide	40.4	Iron (III) oxide	29.9	Iron (II-III) silicate	7.06E-05
Bulk rhizosphere 1	5.9	Iron (II-III) silicate	82.6	Iron (III) oxide	9.8	Iron (III) silicate	5.45E-05
Bulk rhizosphere 2	6.2	Iron (II-III) silicate	72.9	Iron (III) oxide	19.4	Iron (II-III) silicate	5.73E-05
Bulk soil	3.2	Iron (II-III) silicate	83.1	Iron (III) oxide	11.9	Iron (III) silicate	5.65E-05
A1	10.7	Iron (II-III) silicate	10.6	Iron (II) sulfate	79.7	Iron (II-III) silicate	4.22E-04
A2	12.7	Iron (II-III) silicate	49.8	Iron (II-III) silicate	37.7	Iron (II) sulfate	1.89E-04
A3	12.8	Iron (II-III) silicate	62.2	Iron (II-III) silicate	24.5	Iron (II) sulfate	7.21E-05
A4	39.6	Iron (III) oxyhydroxide	45.2	Iron (III) oxyhydroxide	15.0	Iron (II) silicate	1.12E-04
B1	13.6	Iron (II-III) silicate	61.5	Iron (II-III) silicate	24.6	Iron (II) sulfate	4.73E-05
B2	18.6	Iron (III) oxyhydroxide	12.8	Iron (II) silicate	68.3	Iron (III) oxide	7.12E-05
B3	38.5	Iron (II-III) silicate	10.7	Iron (III) silicate	52.7	Iron (III) oxyhydroxide	1.35E-04
B4	6.4	Iron (III) silicate	54.8	Iron (II) sulfate	38.2	Iron (III) oxyhydroxide	6.84E-05
C1	13.9	Iron (III) silicate	55.5	Iron (II-III) silicate	30.0	Iron (II) sulfate	1.80E-04
C2	8.1	Iron (II) silicate	25.7	Iron (III) silicate	67.1	Iron (III) oxide	1.11E-04
C3	7.7	Iron (III) silicate	18.9	Iron (II) silicate	73.5	Iron (III) oxide	8.80E-05
C4	9.0	Elemental iron	11.2	Iron (II) silicate	79.2	Iron (III) oxide	2.51E-04
C5	11.1	Iron (II-III) silicate	66.8	Iron (II-III) silicate	21.3	Iron (II) sulfate	5.00E-05
C6	13.0	Elemental iron	49.6	Iron (III) oxyhydroxide	37.7	Iron (II) sulfate	1.66E-04

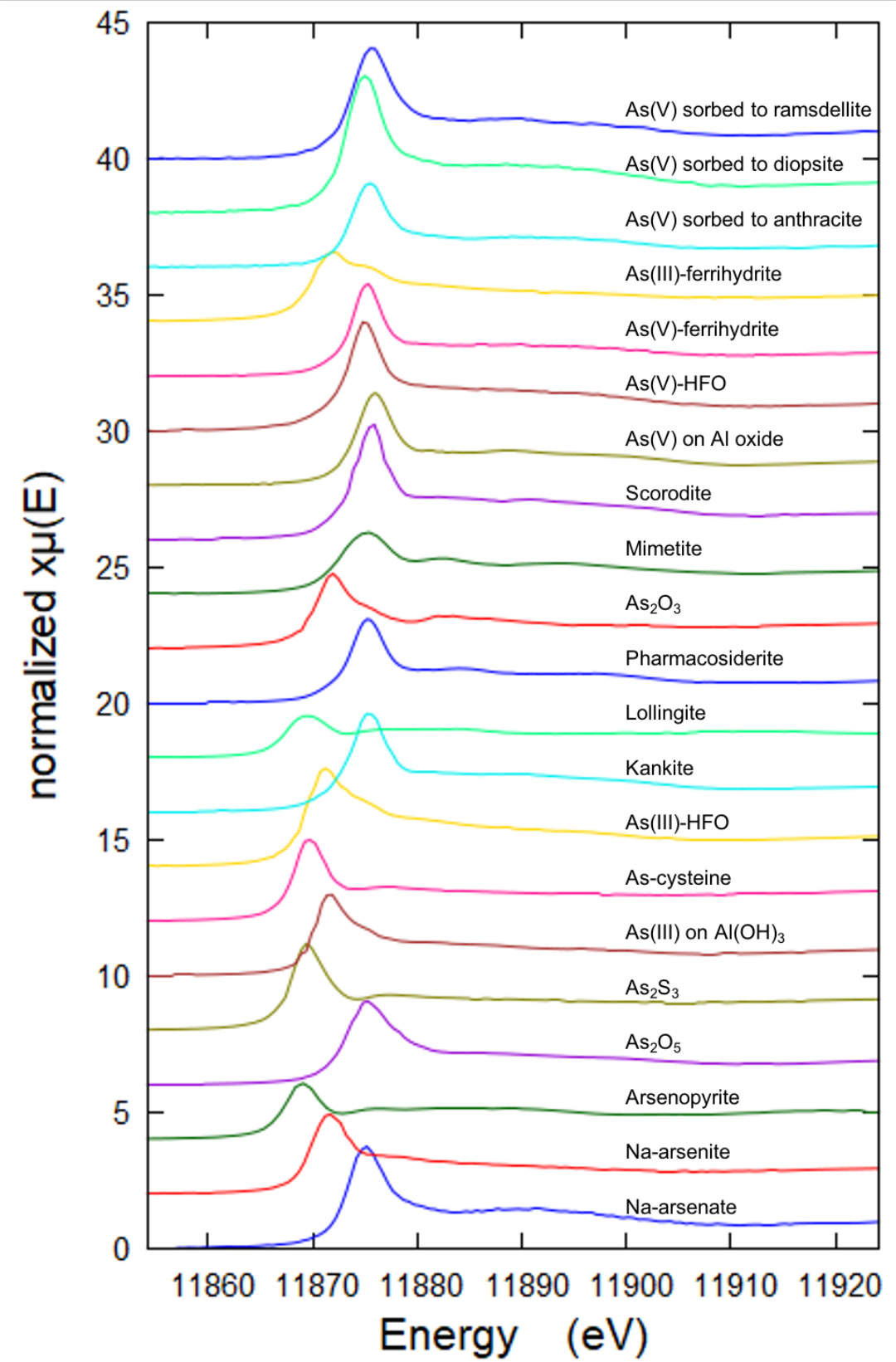

Figure SI-7. Standard spectra used in linear combination fitting of arsenic K-edge spectra in Figure 3D and Figure SI-5.

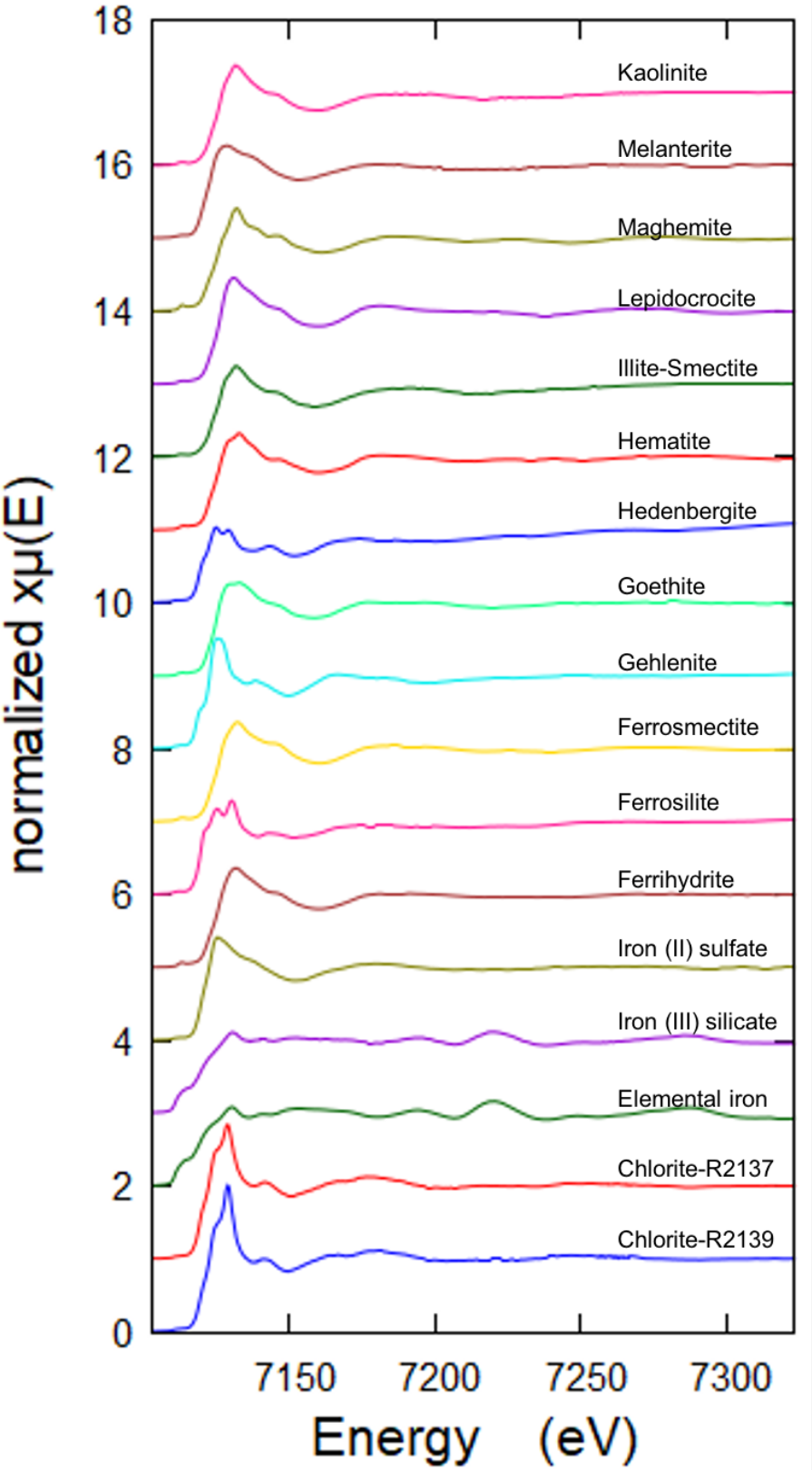

Figure SI-8. Standard spectra used in linear combination fitting of iron K-edge spectra in Table SI-2.

Chapter 4. Higher arsenic accumulation and leaching are coupled to lower biomass in hyperaccumulator *Pteris vittata*

Abstract

Arsenic and nutrient availability to the arsenic-hyperaccumulating fern *Pteris vittata* depends on soil characteristics including clay content. In soils with low nutrient availability, rhizosphere processes scavenging phosphorus and iron might increase arsenic availability in the fern rhizosphere. If available arsenic is not taken up by the fern, it could leach. In 22-week long unsaturated flow-through soil column experiments, we determined effects of phosphorus fertilization and mycorrhizal fungi inoculation on arsenic uptake by *P. vittata* and arsenic leaching from 2 soils of different clay contents. To determine mechanisms of arsenic release from soil, we investigated arsenic speciation in rhizosphere and bulk soil using synchrotron-based spectromicroscopy. We found that *P. vittata* hyperaccumulated arsenic from the coarse-textured soil to a mean of 3,062 mg/kg in aboveground biomass, but did not hyperaccumulate arsenic in the medium-textured soils. Conversely, fern biomass was 74% smaller in the coarse- than in the medium-textured soil, but nonetheless mass of accumulated arsenic was 1.2 to 2.4 times greater in ferns growing in coarse- compared to medium-textured soil. In both soils, fern arsenic uptake was greater than loss by leaching. Mean arsenic loss by leaching was 195% greater from coarse- compared to medium-textured soil, and in both soils was higher in the presence of ferns. Only 4 to 5% of arsenic in ferns growing in coarse-textured soil, and 17 to 34% of arsenic growing in medium-textured soil, was contributed to ferns from bulk soil *via* transpiration flux-driven uptake of porewater, suggesting most uptake occurs from rhizosphere soil. Supplemental phosphorus had no effect in the coarse-textured soil but in the medium-textured soil delayed growth and led to lower transpiration. Mycorrhizal fungi did not affect fern arsenic uptake or arsenic leaching. We found that arsenic availability for plant uptake and leaching are related and greater in coarse-textured soil. Our results suggest that arsenic hyperaccumulation comes at a biomass cost in soils with high phytoavailable arsenic and low nutrient content, but that the fern is sensitive to supplemental phosphorus. Furthermore, our results indicate that rhizosphere processes are critical to the release of arsenic, and that this arsenic can leach.

Introduction

Hyperaccumulator plants take up, translocate, and store metal(loids) in their aboveground biomass (Reeves, 2005; Rascio and Navari-Izzo, 2011). Worldwide, over 450 hyperaccumulator species are found in metal(loid)-enriched soils (Rascio and Navari-Izzo, 2011). Great interest has emerged in using hyperaccumulators for remediation of soil and water (e.g. phytoextraction) contaminated with arsenic, cadmium, nickel, and zinc (Chaney et al., 2007; Tack and Meers, 2010; Rascio and Navari-Izzo, 2011; Li et al., 2018). Metal(loid) accumulation in aboveground biomass, including mechanisms of transport and sequestration, is relatively well-understood (Baker and Whiting, 2002; Xie et al., 2009; Rascio and Navari-Izzo, 2011; Ent and Baker, 2013; Danh et al., 2014). An understanding of how hyperaccumulator rhizosphere chemistry affects metal availability in soil for uptake is developing (Kidd et al., 2009; Alford et al., 2010), including the theory that metal release from soil is tied to nutrient scavenging (Meharg and Hartley-Whitaker, 2002; Audet, 2012). Indeed, the idea that metal solubility in soils can be manipulated to increase hyperaccumulator uptake and therefore phytoextraction rates is highly attractive (Wenzel et al.,

2003; Kidd et al., 2009; Saifullah et al., 2009; Lessl and Ma, 2013). Critically, we must increase our understanding of how hyperaccumulators impact metal(loid) cycling in and below the rhizosphere, because increases in metal(loid) availability in soil could lead to metal leaching if the mobilized metal(loid) fraction is not completely taken up by the target plant. Here, we used the arsenic hyperaccumulating fern *Pteris vittata* to investigate arsenic cycling in a plant-soil-water system. We varied soils and nutrient treatments to determine the effects of arsenic and nutrient availability on arsenic uptake and leaching.

Pteris vittata hyperaccumulates arsenic to concentrations reaching 23,000 mg/kg dry weight in aboveground biomass when grown in soils with high arsenic levels (Ma et al., 2001). Extensive work has explored arsenic phytoextraction with *P. vittata* (Xiao et al., 2008; Xie et al., 2009; Lessl and Ma, 2013; Danh et al., 2014; Fayiga and Saha, 2016) as an *in situ* alternative to soil excavation-based remediation methods (Tack and Meers, 2010; Wan et al., 2016). The fern hyperaccumulates arsenic under a range of conditions (Caille et al., 2004; Kertulis-Tartar et al., 2006; Ciurli et al., 2014; da Silva et al., 2018). However, phytoextraction rates are slow compared to the mass of arsenic in soil even at moderate concentrations (~100 mg/kg), leading to long estimated remediation times on the order of decades or more to deplete soil arsenic to background levels (Shelmerdine et al., 2009; Niazi et al., 2012; Lessl and Ma, 2013). Manipulations to soil arsenic availability, fern biomass, and fern arsenic uptake through nutrient application have been investigated to increase phytoextraction rates.

Indeed, arsenic and nutrient availability could be intimately linked in the fern. *P. vittata*, like many hyperaccumulators, is well-adapted to low-nutrient soils (Koide et al., 1988; Wang et al., 2019). According to the phosphorus starvation theory (Meharg and Hartley-Whitaker, 2002; Audet, 2012), arsenic uptake in *P. vittata* could be a byproduct of nutrient acquisition, especially iron and phosphorus. Phosphorus and arsenic are chemical analogues of one another, are both found in soils associated with iron oxides (Lombi et al., 2000; Smith et al., 2006), and can be released in the rhizosphere through the same biogeochemical processes involving dissolved organic carbon (Marschner and Romheld, 1994). Phosphorus application increases arsenic solubility in soil (Peryea, 1991; Caille et al., 2004). Furthermore, arsenic is taken up in the fern through the phosphate intake pathway (Wang et al., 2002; Sun et al., 2020), and phosphorus has been shown to compete with arsenic for plant uptake (Wang et al., 2002). Frond arsenic concentrations and biomass were higher in *P. vittata* supplied with sparingly soluble compared to soluble phosphorus (Lessl and Ma, 2013; da Silva et al., 2018), though in other cases phosphorus application did not affect arsenic uptake in *P. vittata* regardless of solubility (Chen et al., 2002b; Cao et al., 2003; Tu and Ma, 2003b; Fayiga and Ma, 2006). Furthermore, soluble phosphorus increased arsenic concentrations in porewater but not in *P. vittata* fronds (Caille et al., 2004). These finding indicate that the relationships between arsenic and phosphorus availability in soil and uptake in the fern vis-à-vis fern nutrient demand are complex and deserve further elucidation, including the effects of soil phosphorus relative to supplemental phosphorus. These relationships are particularly important because phosphorus could play a double role, releasing arsenic from soil yet outcompeting arsenic for *P. vittata* uptake and therefore increasing arsenic leaching.

Alternately, effects of mycorrhizal fungi inoculation on nutrient and metal(loid) availability to hyperaccumulators deserve further attention (Audet, 2012) as inoculation with mycorrhizal fungi could increase *P. vittata* nutrient, water, and possibly arsenic access (Karandashov and Bucher, 2005). *P. vittata* associates with mycorrhizal fungi (Leung et al., 2006; Wu et al., 2007). Inoculated *P. vittata* had higher biomass (Liu et al., 2005; Trotta et al., 2006; Chapter 3) but did not accumulate more arsenic (Trotta et al., 2006), especially in moderately contaminated soils (Liu et

al., 2005; Chapter 3). Mycorrhizal fungi also evolved under phosphorus starvation conditions and support plants under nutrient, drought, and metal stress, but have stress tolerance mechanisms that differ from ferns (Audet, 2012).

Moreover, soil characteristics, particularly clay content (i.e., soil texture), affect nutrient and arsenic availability (Smith et al., 2006). Clay-sized particles have the greatest surface area of soil particles and include mineral phases such as iron oxides, aluminum oxides, and clays that provide surfaces on which arsenic and phosphorus as well as other nutrients can adsorb (Manning and Goldberg, 1996a; Lombi et al., 2000; Redman et al., 2002). Arsenic has been shown to be more plant available (Jacobs and Keeney, 1970; Woolson et al., 1973) and more leachable (Masscheleyn et al., 1991; Hale et al., 1997) from soil with lower clay content.

Few arsenic phytoextraction studies investigate arsenic leaching from soil during *P. vittata* growth or compute arsenic budgets. Arsenic was found in soil leachate after *P. vittata* growth (Yang et al., 2012). In a pot study, more arsenic was depleted from soil than accumulated in ferns (Gonzaga et al., 2008), and in long-term field studies, greater depletion of soil at depths below the fern root zone has been observed, compared to surface depths (Kertulis-Tartar et al., 2006; Niazi et al., 2012; Chapter 3), which could indicate leaching of arsenic below the root zone. However, no studies have quantified arsenic cycling in the *P. vittata*-soil-water system or, more specifically, compared arsenic accumulation in *P. vittata* to arsenic leaching from soil during phytoextraction.

Unsaturated flow-through soil column studies with plants are a challenging yet powerful setup to quantify arsenic cycling in controlled whole plant-soil-water systems that approximate natural systems. With such systems, water balances including transpiration can be calculated, and arsenic transport in soil porewater can be modeled (Zhang and Selim, 2006). Combined with X-ray absorption near edge spectroscopy and X-ray fluorescence microprobe imaging, soil columns with plants allow us to investigate rhizosphere processes (Kopittke et al., 2017) and relate rhizosphere processes to arsenic cycling at the system scale. We conducted a 22-week soil column study with *P. vittata* to determine the effects of soil texture (a proxy for nutrient and arsenic availability), phosphorus application, and fungi inoculation on arsenic cycling in the plant-soil-water system. We used synchrotron-based spectromicroscopy to determine arsenic speciation in bulk and rhizosphere soils and to suggest mechanisms of arsenic mobilization for plant uptake and/or leaching. We hypothesized that both arsenic accumulation and leaching would be higher in *P. vittata* grown in soil with lower clay content. We observed greater arsenic uptake and leaching with smaller fern biomass in soil with lower clay content across all treatments, suggesting significant plant growth trade-offs associated with arsenic hyperaccumulation and phytoextraction.

Methods

Soils

Two soils moderately contaminated with arsenic and with contrasting textures (medium-textured soil, 16.7% clay, 138.0±1.5 mg/kg; coarse-textured soil, 4.0% clay, 95.3±1.1 mg/kg; Table 1) were excavated from the 0-30 cm depth in a former railroad right-of-way (Berkeley, CA). Soils were sieved to 4 mm and stored field-moist in sealed containers at 5 °C. Containers were opened regularly to maintain oxic conditions. Bulk density was measured in triplicate for a volume of soil excluding the volume of particles >2 mm, and porosity was calculated from bulk density (USDA, 2001). Soil texture was measured in triplicate with the hydrometer method (Gee and Or, 2002). Clay minerals identified with X-ray diffraction (PANalytical) following USGS protocols (Poppe et al., 2002) included nontronite, trioctahedral montmorillonite, and/or vermiculite.

Soil column study

Experiments with novel unsaturated flow-through soil columns with plants were performed in triplicate in order to estimate arsenic uptake by *P. vittata*, arsenic transport in soil, and arsenic leaching. We compared two soil treatments, (i) phosphorus fertilization (CalPhos^{TM}, 7.9% P, 18% Ca; 1.2 g/kg), and (ii) inoculation with the fungus *F. mosseae* (INVAM; 44 mL inoculum/fern), with (iii) control columns without amendments, and (iv) control columns without plants. For columns without plants, only phosphorus-treated and control columns were established, because fungal inoculant was not expected to survive in the absence of ferns, with 1 replicate *per* treatment. Phosphorus was mixed into soil before packing columns. Fungal inoculant was applied to each hole at transplanting.

Soil columns were made of acrylic tubes (15 cm diameter, 45 cm length) with holes for porewater samplers and tensiometers at 5, 10, and 27 cm depths. At the bottom of each column a 0.2 μm filter (contacting soil) and glass filter separated soil from effluent port, which was filled with quartz sand with glass wool on either end. Columns were packed at field site bulk density for the medium-textured soil and a higher bulk density for the coarse-textured soils, because the field bulk density was not dense enough to support the coarse-textured soil in the column before root growth. Soil was well-mixed before weighing into 20 increments of equal mass appropriate for packing into a 2 cm depth. Soil was packed to a total depth of 40 cm using a custom-made 3-pronged device to apply downward force over a small surface area, adapting the protocol from Pallud et al. (2004), to avoid formation of layers. *P. vittata* ferns with bare roots and 3-7 fronds 10-20 cm in length were planted with roots 0-10 cm deep in column. Porewater samplers (Soilmoisture Equipment Corp.) and tensiometers (Soilmoisture Equipment Corp.) were filled with degassed synthetic rain and inserted horizontally at depth (5, 10, and 27 cm) with the tip 7.5 cm inside column. Columns were covered in thick black fabric to keep soil in the dark, and the soil surface was covered lightly with aluminum foil to decrease evaporation.

A synthetic rain solution that served as column influent was made following rain composition local to the soil (Whitehead and Feth, 1964) adjusted to current pH (5.33) and was kept in the dark during experiments. The influent solution was supplied by a single port at the rhizome, and was eluted through columns for 22 weeks at a flow rate (3.52±0.06 ml/hr) equivalent to maximum daily rainfall (0.508 cm/day) (Western Regional Climate Center, 2016) using a peristaltic pump.

The column experiments were conducted in a greenhouse (mean day temperature 25.8±0.1°C, mean night temperature 17.3±0.05°C, mean relative humidity 56.5±0.07%) with a 16 hour photoperiod.

Soil, porewater, effluent, and fern sample collection

Porewater samples (< 60 mL) were extracted approximately every 2 weeks using acid-washed syringes maintained at 4 °C during collection. Water potential was recorded before and after sample collection. Porewater samples were immediately filtered (0.45 μm) under anoxic conditions, subsampled for analyses, and stored at -18 °C in the dark until analysis. Effluent was collected weekly into polyethylene bottles (500 mL) pre-acidified with concentrated HNO_3 (70% TM grade). After collection, effluent was adjusted to 2% HNO_3, filtered (0.45 μm), and stored (4 °C) for analysis. Pinnae samples (1 middle pair from each frond) were collected at 11 and 21 weeks of the column study.

At the end of the experiments, all aboveground biomass was removed with clippers 2 cm above the rhizome, and separated based on development of sori and tissue senescence (young = sori

absent to forming, mature = sori brown but not fully distended, senescent = sori fully distended, tissue browning to fully brown). Rhizomes were removed from soil. Root and soil samples (collected from 0-7.5 cm, 7.5-17 cm, and 17-30 cm depth intervals) were immediately stored at 4 °C. Whole root samples for microprobe analyses were stored immediately at 4 °C, flash-frozen in liquid nitrogen within 6 hours of harvesting and stored at -80 °C until analyses. Root samples for X-ray absorption spectroscopy (XAS) were dried, processed, and maintained under anoxic conditions for X-ray absorption spectroscopy (XAS).

Aboveground biomass (hereafter, frond) was washed 3 times in deionized water. Rhizomes and root samples for elemental analysis were washed in deionized water until clean of soil. Fronds, rhizomes, and root elemental analysis samples were dried in a drying oven (55 °C) for 24 hours or until constant mass was achieved.

Soil samples were either air-dried for elemental analysis or dried, processed, and maintained under anoxic conditions for XAS.

Sample analysis

Dry biomass was measured on pinnae, frond, and rhizome samples. Pinnae biomass served as a proxy for whole plant biomass during growth. Plant tissue and soil samples were digested following a modified EPA 3050B protocol. Total arsenic, phosphorus, and iron concentrations of soil and fern digests were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES). The detection limit was 20 μg/L for each analyte.

Total arsenic concentrations in effluent and porewater samples were analyzed using either ICP-OES or, if concentrations were less than 50 μg/L, hydride-generation-ICP-OES (HG-ICP-OES; detection limit 2 μg/L) after addition of 0.8 mL of 40% KI/8% ascorbic acid to 4 mL sample and 2.7 mL 1.1 M HCl. In porewater, arsenic(III) concentrations were determined using HG-ICP-OES in samples buffered at pH 5 through addition of 2.5 mL 0.5 M disodium citrate to 2.5 mL sample, dissolved organic carbon (DOC) concentrations were analyzed using an O-I-Analytical analyzer, and pH was determined using a Denver Instruments meter with pH/ATC Sartorius ATC combination electrode.

Samples of roots with rhizospheric soil and of soil aggregates were embedded in EPO-TEK 301 epoxy under anoxic conditions and prepared as thin sections (30 μm; Spectrum Petrographics) with roots sliced longitudinally for microprobe analyses. For bulk XAS, rhizosphere soil was sampled with a small brush. Aggregates with no visible roots were considered bulk soil. Under anoxic conditions, bulk soil, rhizosphere soil, and roots were finely ground and mounted on a 0.22 μm nitrocellulose filter. X-ray microprobe analyses were performed at Advanced Light Source (ALS, USA) XFM beamline 10.3.2 (Marcus et al., 2004). Briefly, arsenic and iron spatial distribution in the samples was determined using micro-focused X-ray fluorescence (μXRF) elemental mapping using a 12 keV incident beam. In sample regions of interest, arsenic speciation was determined using arsenic K-edge X-ray absorption near edge structure (μXANES) spectroscopy. Bulk XANES spectra were collected at Stanford Synchrotron Radiation Laboratory (SSRL, USA) beamline 7.3. Filter membranes were sealed under anoxic conditions and mounted on sample holders with Kapton tape. Micro and bulk XANES spectra were plotted using the Athena package (Ravel and Newville, 2005) and linear combination fitting was performed with customized beamline software. Details of the spectroscopy measurements are available in the Supplemental Information.

Fern arsenic accumulation, cumulative leaching, and mass balance calculations

Arsenic accumulated *per* fern aboveground biomass (mg/frond type) or *per* rhizome (mg/rhizome) was calculated by multiplying the arsenic concentration by the dry biomass of aboveground biomass or rhizome, respectively. Volume of transpired water *per* fern was calculated by subtracting effluent volume from influent volume *per* column, assuming all influent not collected as effluent was transpired. This assumption overestimates transpiration in columns where soil water content increased during the flow-through study. The contribution of arsenic, phosphorus, or iron in porewater to fern accumulation was calculated by multiplying the mean arsenic, phosphorus, or iron concentration (respectively) in porewater at 10 cm depth by the volume of transpired water *per* column. To calculate cumulative arsenic and phosphorus leaching *per* column, porewater and effluent volume were added together and multiplied by effluent arsenic or phosphorus concentration *per* week, assuming the porewater removed during sampling would have contributed to effluent. Final soil arsenic concentrations were normalized to initial soil arsenic concentrations *per* column. To calculate the mass balance of arsenic after the study, the masses of arsenic accumulated in the fern, retained in soil in each depth interval (0-7.5, 7.5-17, and 17-40 cm), extracted in porewater, and leached in effluent, were each normalized to initial mass of arsenic in soil and then summed. Normalized mass of arsenic accumulated in fern was compared to normalized mass of leached arsenic (in extracted porewater and effluent) using a t test.

Statistical analysis

Statistical analysis was performed in R (R Core Development Team, 2013). Analyses of covariance were performed on linear models to analyze main effects of explanatory variables including time (continuous), depth (continuous), soil, and treatment on response variables including fern arsenic concentrations, biomass, arsenic accumulation, arsenic uptake rate, and changes in soil arsenic concentrations during phytoextraction. Models were selected based on AIC criteria using the MuMIn package (Barton, 2019). Two-way interactions were included when those models were most highly ranked. Regression summaries were used to compare effects of explanatory variables, and differences in means were determined with Tukey's Honestly Significant Difference test on means normalized to initial soil arsenic concentration where appropriate. A paired t test was used to determine the mass balance across all plots, comparing arsenic leaching *per* column to arsenic accumulation *per* fern.

Results

P. vittata arsenic and nutrient uptake

Pinnae arsenic concentrations differed dramatically with soil type ($P<0.001$), with higher values in ferns growing in coarse-textured soil. For the coarse-textured soil, arsenic concentrations in sampled pinnae ranged from 1122 to 1890 mg/kg after 11 weeks, but increased ($P<0.001$) to 3,157 to 4,358 mg/kg after 21 weeks (Figure 1A). Pinnae arsenic concentrations were considerably lower ($P<0.001$) for the medium-textured soil, where concentrations ranged between 555 and 821 mg/kg for all time points, never reaching the hyperaccumulation threshold (Figure 1A). The interaction of soil by time ($P<0.001$) indicated that pinnae arsenic concentrations were lower at 21 weeks in ferns growing in the medium-textured soil compared to coarse-textured soil. The mass of arsenic accumulated in sampled pinnae also increased ($P<0.001$) over time, from 0.21 to 0.26 mg at 11 weeks to 0.95 to 1.1 mg at 21 weeks in the coarse-textured soil, though accumulated mass

was always lower ($P<0.05$) in the medium-textured soil, only 0.21 to 0.30 mg at 11 weeks increasing to 0.49 to 0.67 mg at 21 weeks (Figure 1B).

At final harvest, soil type similarly affected fern frond arsenic concentrations ($P<0.001$) and mass of accumulated arsenic *per* fern ($P<0.01$), yet had the reverse effect on whole plant biomass ($P<0.001$) (Figure 2A-C). Fern arsenic concentrations in coarse-textured soil ferns ranged between 2,666 and 3,570 mg/kg for the whole plant, up to 10 times higher ($P<0.001$) than the values in medium-textured soil ferns, which were only 363 to 574 mg/kg for the whole plant. The total mass of accumulated arsenic in coarse-textured soil ferns ranged from 15.2 to 20.2 mg/fern, higher ($P<0.01$) than in the medium-textured soil, where values ranged from 8.5 to 11.9 mg/fern (Figure 2B). However, the fern dry biomass was much higher ($P<0.001$) in the medium-textured soil, with values between 20.1 and 23.5 g for the whole plant, while they were only 5.1 to 6.1 mg for the whole plants in the coarse-textured soil (Figure 2C). Soil treatment did not affect whole plant arsenic concentrations ($P=0.34$), mass of accumulated arsenic ($P=0.82$), or biomass ($P=0.32$).

Whole plant phosphorus concentrations were lower ($P<0.05$), in ferns grown in medium-textured soil, with a mean of 1,814 mg/kg, compared to in coarse-textured soil, with a mean of 2,277 mg/kg (Figure SI-1A). In contrast, iron concentrations were higher ($P<0.05$) in ferns grown in the medium-textured soil, with a mean of 239 mg/kg, but were lower with a mean of 60 mg/kg in the coarse-textured soil (Figure SI-1B). Phosphorus concentrations were higher in phosphorus-treated ferns ($P<0.05$).

As expected, arsenic concentrations were greater ($P<0.001$) in fern aboveground biomass compared to the rhizome and roots (Figure 2A). In both soils, root concentrations were less than 92 mg/kg and rhizome concentrations less than 311 mg/kg, while aboveground concentrations ranged from 1,553 to 4,661 mg/kg in aboveground biomass in coarse-textured soil ferns, and 196 to 1610 mg/kg in medium-textured soil ferns. Mass of accumulated arsenic was also greater ($P<0.001$) in aboveground biomass compared to rhizomes (Figure 2B). Interactions of plant part biomass by soil ($P<0.001$) and by treatment ($P<0.05$) showed that senescent fronds were larger in the medium-textured soil ferns compared to coarse-textured soil, yet were smaller in phosphorus-treated ferns across both soils compared to in other treatments.

Within aboveground biomass alone, arsenic concentrations were significantly lower in mature ($P<0.05$) and senescent ($P<0.001$) fronds, compared to young fronds. In coarse-textured soil ferns, arsenic concentrations ranged from 1,552 to 4,032 mg/kg in mature and senescent fronds, compared to 3,980 to 4,662 mg/kg in young fronds, while in medium-textured soil, mature and senesced fronds contained 197 to 896 mg/kg, compared to 920 to 1,610 mg/kg in young fronds. However, total mass of accumulated arsenic was greater ($P<0.05$) in senesced fronds, where values ranged from 2.6 to 9.4 mg/senescent fronds, compared to young fronds, where values ranged from 2.0 to 6.0 mg/young fronds across both soils. Here, the interaction of frond age and treatment ($P<0.05$) indicated phosphorus-treated senesced fronds accumulated less total arsenic than young fronds.

Bulk soil arsenic concentrations

Across soils, soil arsenic concentrations decreased ($P<0.001$) to 75-104% of initial values over the study period (Figure 3). Final concentrations were lower ($P<0.001$) in the surface 0-7.5 cm depth interval, where they were 75 to 91% of initial concentrations, compared to the 7.5-17 cm and 17-40 cm depth intervals, where concentrations were 85 to 104% of initial values. In the medium-textured soil only, final soil arsenic concentrations were lower ($P<0.001$) in *F. mosseae*-inoculated and phosphorus-treated soil compared to the control.

Porewater elemental concentrations

Across soils, soil ($P<0.001$), time ($P<0.001$), and depth ($P<0.001$) affected porewater total arsenic concentrations (Figure 4A-B). Across both soils, arsenic concentrations decreased with time. In the coarse-textured soil, an early peak at 152 to 171 μg/L in arsenic concentrations shifted downwards over weeks 3 to 7, with concentrations higher in the 27 cm depth than surface depths for the remainder of the study. By contrast, in the medium-textured soil, concentrations decreased in the 5 cm depth from 220 to 62 μg/L, with the exception of variable concentrations in the phosphorus-treated medium-textured soil columns, where concentrations peaked at a mean of 420 μg/L in weeks 7 and 11. In the medium-textured soil, porewater could be extracted from the phosphorus-treated columns till 17 weeks, longer than the control and *F. mosseae*-inoculated columns, where porewater extraction was not possible as early as 5 to 11 weeks into the study. Interactions of soil and time ($P<0.001$), soil and depth ($P<0.001$), soil and treatment ($P<0.01$) showed that in the medium-textured soil, arsenic concentrations slightly increased with time, decreased with depth, and increased with phosphorus treatment. Arsenic(III) concentrations were very low in the coarse-textured soil porewater, less than 4.4 μg/L, but were higher ($P<0.001$) in the medium-textured soil, with a mean of 13.6 μg/L increasing up to a peak of 109 μg/L in weeks 5 to 9 in the phosphorus treated columns (Figure SI-2).

Phosphorus concentrations were less than 0.60 mg/L in coarse-textured soil porewater but were higher ($P<0.001$), up to 3 times those values, in the medium-textured soil porewater, and decreased ($P<0.001$) with time in both soils (Figure 4C-D). Phosphorus and total arsenic concentrations were moderately correlated ($R^2 = 0.41$).

Iron concentrations were less than 66 μg/L in the coarse-textured soil porewater but higher ($P<0.001$) in the medium-textured soil porewater, ranging from 10 to 164 μg/L, and decreased with time ($P<0.001$) (Figure SI-2). A significant soil by depth ($P<0.001$) interaction showed that iron concentrations decreased with depth in the medium-textured soil.

DOC concentrations were less than 32 mg/L in coarse-textured soil porewater but higher ($P<0.001$) in the medium-textured soil porewater, where they reached 165 mg/L (Figure 4E-F). Depth affected ($P<0.001$) porewater DOC concentrations. In the medium-textured soil porewater, concentrations peaked in the first 7 weeks porewater in the 5 and 10 cm depths, but increased over time at 27 cm depth in the medium-textured soil from 62 to 162 mg/L. Interactions of soil by week ($P<0.001$) and soil by depth ($P<0.001$) show that DOC concentrations increased with time and depth in the medium-textured soil but not coarse-textured soil.

Soil ($P<0.001$), time ($P<0.001$), depth ($P<0.001$), and treatment ($P<0.001$) affected porewater pH, which ranged from 6.0 to 8.9 in both soils and increased significantly with depth across both soils (Figure SI-1). Interactions of soil by time ($P<0.001$), by depth ($P<0.001$), and by treatment ($P<0.01$) showed in the medium-textured soil that pH increased over time, decreased with depth, and increased with phosphorus treatment.

Effluent elemental concentrations, volume, and cumulative leaching

Across both soils, effluent arsenic concentrations were higher in the presence of ferns ($P<0.001$). In the presence of ferns, effluent arsenic concentrations in the coarse-textured soil increased from 95 to a peak of 241 μg/L at 7 weeks and then decreased to 123 μg/L, whereas in the absence of ferns values ranged from 20 to a peak of 207 ug/L. In the medium-textured soil, effluent concentrations were lower ($P<0.001$), 126 to 153 μg/L in the presence of ferns and less than 92 μg/L in the absence of ferns. Although across both soils arsenic concentrations increased

with time (P<0.001), a significant time by soil interaction (P<0.001) indicated that concentrations plateaued with no peak in the medium-textured soil. Phosphorus treatment lowered arsenic concentrations in effluent of both soils (P<0.05), regardless of whether ferns were present.

Effluent volumes were greater in the coarse-textured soil (P<0.001), where effluent flow lasted to 22 weeks (Figure 6A-B) and led to cumulative arsenic loss of 5.6 to 5.9 μg/day by leaching, more (P<0.001) than from the medium-textured soil. In medium-textured soil, effluent flow ceased at 7 weeks (Figure 5B) with only 1.4 to 2.7 μg/day lost by leaching. Effluent volumes were greater in phosphorus-treated columns (P<0.05), but this did not translate into greater cumulative loss of arsenic from phosphorus-treated soil (P=0.409).

Mass balance

Across all soils and treatments, a mean of 90.7% (79.9 to 101.2%) of initial arsenic was recovered in final soil, plant tissue, porewater samples, and effluent (data not shown). Approximately 1.5 to 1.8% of the initial arsenic present in soil was taken up into ferns growing in the coarse-textured soil, more (P<0.001) than in the medium-textured soil, where only 0.8 to 1.2% of the initial arsenic was accumulated. In both soils, the percent of initial soil arsenic taken up into plants exceeded (P<0.001) the percent of initial arsenic leached (Figure 7). About 0.07% of initial arsenic was lost to leaching from the coarse-textured soil, but only 0.007 to 0.025% was lost from the medium-textured soil.

A smaller mass of arsenic (P<0.001), phosphorus (P<0.001), and iron (P<0.01) was transported to fern aboveground biomass from bulk soil *via* transpiration flux-driven uptake of porewater in ferns growing in coarse- compared to medium-textured soil (Figure 8). Only 4 to 5% of arsenic in coarse-textured soil ferns was contributed from transpiration flux of bulk porewater, compared to 17 to 34% of arsenic in medium-textured soil ferns (Figure 8).

X-ray absorption spectroscopy (XAS)

Bulk arsenic K-edge XAS spectra indicated greater abundance of arsenic(III) in rhizosphere soil (33.2% to 41.3% As(III)), compared to in whole roots (18.8% to 26.6% As(III)) or in bulk soil (10.1% to 15.1% As(III)) (Table SI-5), in the coarse textured soils. In contrast, in medium-textured soils, bulk spectroscopy showed arsenic(III) fractions lesser than (7.9% in control soil) or equal to (18.7% in phosphorus-treated soil) to those in roots (19.2% As(III) in control soil, 18.1% in phosphorus-treated soil), with even lower abundance in bulk soil (0% to 10.5% As(III)) (Table SI-5). In both coarse and medium-textured soil, a higher fraction of arsenic(III) was found in phosphorus compared to control samples, except for in whole root samples from fine textured soil, where arsenic(III) fractions were similar (19.2% in control roots, 18.1% in phosphorus-treated roots) (Table SI-5).

Microprobe arsenic K-edge X-ray absorption near-edge structure (XANES) spectra (Figures 9-10, Tables SI-1 and SI-2, and Figure SI-3) showed that across all sample types, a higher fraction of arsenic(III) was present in medium- than coarse-textured soil (Figure SI-3). In both textures we found very little evidence of arsenic reduction in aggregates from control soil (0 to 7.4% As(III) in coarse-textured soil, Figure 9A; 0 to 8.2% As(III) in medium-textured soil, Figure 10A). A higher fraction of arsenic(III) was found in aggregates of phosphorus-treated soil in coarse- (13.4 to 15.9% As(III), Figure 9C) and medium- (0 to 68.7% As(III), Figure 10C) textured soil, compared to control aggregates. In rhizosphere soil, microprobe data showed a lower fraction of arsenic(III) (<10%) in control coarse- and medium-textured soils, but a higher fraction in phosphorus-treated coarse- (5.0 to 21.5%) and medium- (7.1 to 44.5%) textured soil (Figures 9B-

10B). On whole root surfaces, the fraction of arsenic(III) fraction was very high, up to 100% arsenic(III). In coarse-textured soil, the fraction of arsenic(III) on the root surface ranged from 6.3 to 28.5% on control roots, 6.3 to 80.9% on *F. mosseae*-inoculated roots, and 0.0 to 20.0% on phosphorus-treated roots (Figure 9A). In medium-textured soil, the fraction of arsenic(III) on the root surface ranged from 74.1 to 99.8% on control roots, 97.7 to 106.0% on *F. mosseae*-inoculated roots, and 8.2 to 44.8% on phosphorus-treated roots, whereas on adjacent particles the fraction of arsenic(III) was only 18.2% in control soil and 3.5% in *F. mosseae*-inoculated soil (Figure 10A).

Bulk iron K-edge XANES spectra indicated iron(III) oxyhydroxides were the most abundant species (52.2 to 76.6%) in coarse textured bulk soil, rhizosphere soil, and roots (Table SI-6). Other mineral groups detected in coarse-textured soil included iron(II) silicates and iron(III) silicates (Figure SI-4). In medium-textured bulk soil, rhizosphere soil, and roots, iron(III) oxyhydroxides were less abundant (40.6 to 44.9%) and were not present at all in phosphorus-treated rhizosphere soil (Table SI-6). Iron(III) silicates and iron(II-III) silicates made up the difference.

Discussion

Hyperaccumulation at a biomass cost

Surprisingly, we observed hyperaccumulation in ferns growing in coarse-textured soil (4% clay, a loamy sand), but not in medium-textured soil (13% clay, a sandy loam). The large increase in frond arsenic concentrations we observed with decreasing clay content suggests changes in clay content could have a strong effect on arsenic phytoavailability. Arsenic strongly associates with the clay particle size fraction and the iron oxides therein (Lombi et al., 2000; Smith et al., 2006), such that arsenic phytoavailability is lower in soils with higher clay contents (Jacobs and Keeney, 1970; Woolson et al., 1973). Our findings build on previous work showing *P. vittata* frond arsenic concentrations decrease as clay content increases in medium to fine-textured soils (Liao et al., 2004) and across wide clay content intervals (e.g., 10 to 59%) (Xu et al., 2010), suggesting that clay content and arsenic availability limit arsenic phytoextraction rates.

Although hyperaccumulation (i.e. arsenic translocation to fronds) in *P. vittata* is well established (Ma et al., 2001; da Silva et al., 2018; Li et al., 2018), the rhizome has been proposed to function as a secondary storage organ enabling tolerance of highly available arsenic (Liao et al., 2004), consistent with the genetically independent nature of tolerance and hyperaccumulation (Maestri et al., 2010). High rhizome arsenic concentrations (37-157% of pinnae concentrations) were observed in naturally occurring *P. vittata* populations growing in soils with high arsenic availability (Liao et al., 2004). However, even with the apparent higher phytoavailability of arsenic in the coarse-textured soil, we found that arsenic concentrations were the highest in the fronds, with low concentrations in the rhizome. Our findings suggest that, at least over timescales of 5-6 months, *P. vittata* does not use the rhizome as a secondary storage organ, confirming that under high phytoavailability conditions, both arsenic tolerance and hyperaccumulation are functional traits in *P. vittata*.

Even as hyperaccumulation works effectively, hyperaccumulation—and/or tolerance—appears to exact a metabolic cost. In arsenic hyperaccumulation, energy is used for active transport *via* phosphate transporters, glutathione production, arsenic reduction, transport, and sequestration (Meharg and Hartley-Whitaker, 2002; Maestri et al., 2010; Danh et al., 2014). We found lower biomass coupled with the higher arsenic concentrations in ferns growing in the coarse-textured soil, suggesting that arsenic tolerance and hyperaccumulation take metabolic energy away from biomass production. Although *P. vittata* biomass increases in the presence of arsenic in soil (Tu

and Ma, 2002), our results could suggest that at higher levels of available arsenic (between that in medium-textured soil and coarse-textured soil), biomass decreases as resources are allocated to tolerance and hyperaccumulation mechanisms.

Greater arsenic availability leads to leaching despite hyperaccumulation

We observed greater fern arsenic accumulation, yet also greater loss of arsenic by leaching in the coarse-textured soil, compared to the medium-textured soil, suggesting that plant available arsenic is also available to leach, or that arsenic uptake in *P. vittata* requires a mobilization step with the next step either uptake or leaching. In the coarse-textured soil, post-study soil arsenic concentrations did not differ significantly with depth, suggesting arsenic leached from soil at all depths, such that porewater arsenic concentrations increased with depth and effluent arsenic concentrations were higher than in the medium-textured soil. The peak in effluent arsenic concentrations in the coarse-textured soil suggests rapid leaching of the most available arsenic fraction, similarly to leaching of arsenic from soils with 8% clay (Beesley et al., 2010), followed by a decrease in concentrations as the most available arsenic fractions are depleted. Moreover, we found greater effluent flowrates and duration in the coarse-textured soil, which we attribute to lower transpiration from the smaller aboveground fern biomass. Our results suggest a biogeochemical domino effect where fern-scale impacts community-scale arsenic cycling. High arsenic availability leads to resource reallocation away from biomass production towards arsenic tolerance and hyperaccumulation, in turn leading to lower transpiration, greater infiltration and leaching of available arsenic to potentially impact other ecological receptors. Yet even though the most leachable fraction was depleted early on from the coarse-textured soil, the highest arsenic concentrations were found in young fronds produced later in the study, which could suggest that arsenic continued to be highly plant available and that the pools of arsenic available for leaching and plant uptake are overlapping but not identical.

Lower arsenic availability and larger ferns limit leaching

In contrast, in the medium-textured soil with a higher clay content, arsenic leaching was lower. Arsenic sorption and desorption processes appeared to occur at all depths, such that porewater arsenic concentrations did not change with depth, effluent arsenic concentrations were stable and lower than in the coarse-textured soil, and soil arsenic concentrations increased with depth, indicating retention of arsenic released from the surface (0-7.5 cm) depth. Nonetheless, porewater and effluent concentrations in our study were still 4 to 40 times higher than in soils with similar or higher clay content (17 to 29%) (Yang et al., 2012; Chapter 3). While effluent flow lasted, arsenic leached from the system at lower but still environmentally problematic concentrations (Hartley et al., 2004). But crucially, strong initial fern growth in the medium-textured soil, likely due to higher nutrient content, led to higher senescent frond biomass and a decrease in effluent flow as transpiration (applied water minus effluent) exceeded water application. Our results confirm the critical role transpiration can play in limiting water percolation and therefore leaching of soluble, plant available constituents (Medalie et al., 1994; Benjamin et al., 1996). However, lower biomass of mature and young fronds indicates that once the fern was under drought stress, growth slowed. Nonetheless, pinnae arsenic accumulation shows that arsenic accumulation continued even under drought stress.

Fern growth enhances arsenic availability for uptake and leaching

Critically, we found that arsenic concentrations in effluent were higher in the presence of *P. vittata* ferns, a troubling finding for phytoextraction. Under field conditions arsenic leached from soil after *P. vittata* growth and irrigation (Yan et al., 2009; Yang et al., 2012) yet bulk leachate concentrations did not change in presence *vs*. absence of ferns (Yang et al., 2012), suggesting that arsenic leaching under field conditions could be heterogeneous and higher from rhizosphere-impacted soil, as represented by the soil columns, than bulk field soil.

Greater arsenic leaching in the presence of fern growth suggests the importance of rhizosphere processes to arsenic release for uptake and leaching. We found arsenic depletion from soil was greatest from surface 0-7.5 cm depths, where *P. vittata* roots are primarily located (Liao et al., 2004). Moreover, we deduced that arsenic concentrations in rhizosphere porewater must be greater than in bulk soil porewater. When arsenic concentrations were assumed to be the same in rhizosphere and bulk porewater, a mass balance calculation showed that uptake of arsenic through transpiration flux of arsenic at the porewater concentrations we measured accounted for less than 30% of arsenic accumulated in the fern. Our findings therefore suggest that processes other than mass flow of soluble arsenic from bulk soil to roots are important for arsenic uptake (Barber, 1995). If diffusive flux of nutrients is lower than *P. vittata* demand, *P. vittata* could employ nutrient scavenging processes that release iron, phosphorus, and therefore arsenic in the rhizosphere (Meharg and Hartley-Whitaker, 2002; Gerke, 2015). Such processes would increase arsenic concentrations in the rhizosphere compared to bulk porewater. We hypothesize the majority of arsenic taken up into *P. vittata* was mobilized directly in the rhizosphere as a byproduct of nutrient scavenging processes, and that some of this released arsenic was not taken up by the fern and instead leached, especially when gravitational flow exceeded transpiration flux.

Specifically, arsenic release could be coupled to phosphorus and iron release from soil iron oxide minerals (Manning and Goldberg, 1996a; Lombi et al., 2000; Redman et al., 2002). Release processes could include ion exchange, ligand-enhanced dissolution, and reductive dissolution (Marschner, 1995; Fitz et al., 2003; Li et al., 2007; Gerke, 2015), likely tied to release of root exudates from *P. vittata* roots (Gonzaga et al., 2006; Liu et al., 2016). We found primarily oxidized arsenic in well-drained rhizosphere soil, suggesting processes including ion exchange and ligand-enhanced dissolution, likely coupled to rhizosphere DOC, are more important than reductive dissolution. Nonetheless, we found evidence of reductive processes in the rhizosphere, with up to 41% arsenic(III) in rhizosphere soil. In particular, we found up to 100% arsenic(III) on root surfaces. This high abundance of surficial arsenic(III) could indicate transport of arsenic toward the root and accumulation in the rhizoplane, with slower uptake of arsenic(III) (Wang et al., 2002) enriching arsenic(III) relative to arsenic(V) on the root surface. Alternately, arsenic(III) could indicate efflux of arsenic(III) from roots, which has been proposed to be a secondary tolerance mechanism in *P. vittata* and other plants under arsenic stress (Chen et al., 2016b).

In bulk porewater, bulk soils, and soil aggregates, the predominance of arsenic(V) indicates arsenic can leach under oxic conditions, and that arsenic mobilized as arsenic(III) is oxidized (perhaps coupled to manganese oxide reduction (Amirbahman et al., 2006). Leaching of root-derived dissolved organic carbon (Vinther et al., 2006; Uselman et al., 2007) could also increase arsenic release from bulk soil for leaching. Indeed, arsenic(V) mobility in soil increases at the circumneutral to alkaline soil pH we observed (Dixit and Hering, 2003).

Phosphorus starvation theory explains fern response to supplemental phosphorus

Rhizosphere nutrient acquisition processes have specific significance in the case of hyperaccumulators. The phosphorus starvation theory, where hyperaccumulation is the result of plants taking up and sequestering excess metal(loid)s in response to nutrient imbalances, is a possible explanation for evolution of arsenic hyperaccumulators (Meharg and Hartley-Whitaker, 2002; Audet, 2012). The ecological niche of *P. vittata*, like other hyperaccumulators, could be characterized by infertile soils (Koide et al., 1988), such that *P. vittata* employs scavenging techniques to acquire necessary phosphorus and other nutrients. In the very low nutrient coarse-textured soil, we hypothesize that extensive use of these scavenging processes cost metabolic energy, increased already high arsenic availability, led to high uptake of arsenic and consequently even more energy expenditure to sequester this arsenic, and ultimately resulted in low biomass containing arsenic at high concentrations. The lack of effect of supplemental phosphorus in the coarse-textured soil suggests it is not only phosphorus but other nutrients as well which are required to meet *P. vittata* nutritional needs, and/or a nutrient imbalance if other nutrients (e.g. copper and zinc) sorbed to the calcium phosphate (Cao et al., 2004), as it appears arsenic did (Neupane and Donahoe, 2013), leading to lower arsenic in effluent of phosphorus-treated soils.

In contrast, the moderate arsenic concentrations in ferns grown in the medium-textured soil ferns suggests the soil nutrient content was sufficient. We hypothesize these ferns used less energy to search for nutrients and therefore did not release from soil and subsequently take up as much arsenic. Hence, *P. vittata* growing in the medium-textured soil experienced lower metabolic costs and consequently higher biomass until drought stress limited biomass production.

Finally, our results suggest *P. vittata* is less tolerant to phosphorus availability greater than that of the medium-textured soil. *P. vittata* has a low nutrient demand. Fronds of *P. vittata* growing in its native habitat in China were only 0.08% phosphorus and 1.83% N, and ferns including *P. vittata* had the lowest % phosphorus of any flora group in China (Han et al., 2005). We found phosphorus application delayed fern growth in both soils, as has been shown for tropical forest ferns (Walker et al., 1996), leading to smaller senescent fronds containing lower amounts of arsenic. In the phosphorus-treated medium-textured soil, delayed growth led to lower transpiration, higher effluent volume, and more cumulative arsenic loss due to the greater effluent volume. We hypothesize the greater infiltration in this less-well drained soil led to reducing conditions. On the root surface of phosphorus-treated ferns in medium-textured soil we found lower a lower fraction of arsenic(III), suggesting less transpiration drawing arsenic(III) to accumulate on the root surface, yet arsenic(III) was more abundant in phosphorus-treated than control rhizosphere soil and soil aggregates, and arsenic(III) and iron were released to porewater. In the coarse-textured soil, we also found greater abundance of arsenic(III) in rhizosphere soil and soil aggregates for phosphorus-treated compared to control columns, suggesting a similar though less pronounced effect of supplemental phosphorus in this more well-drained soil.

Mycorrhizal fungi inoculation did not affect phosphorus or iron concentrations in *P. vittata*, suggesting the fern is not reliant on *F. mosseae* for nutrient uptake. Alternately, *F. mosseae* could support *P. vittata* arsenic tolerance through effects on arsenic reductase activity (Leung et al., 2013). We found greater abundance of arsenic(III) on the root surface of fungi-inoculated roots in both soils, which could indicate an increase in arsenic tolerance mechanisms leading to greater arsenic(III) efflux from roots (Chen et al., 2016b). If the released arsenic leaches, this could explain the greater arsenic depletion we found in fungi-inoculated soils. Arsenic concentrations in effluent of fungi-inoculated soils were higher initially, though not significantly so. Effluxed arsenic(III) could oxidize to arsenic(V), coupled to reduction of manganese oxides in soil (Hartley et al., 2004),

and then leach due to mobility of arsenic(V) at the circumneutral to alkaline soil pH we observed (Dixit and Hering, 2003).

Significance of leaching

We found that in both soils, arsenic uptake in ferns exceeded cumulative loss by leaching by an order of magnitude. Even though frond arsenic concentrations were an order of magnitude greater in coarse-textured soil ferns, mass of accumulated arsenic in coarse-textured soil ferns was only 1.2 to 2.4 times that of medium-textured soil ferns, while leached arsenic was also greater in coarse-textured soil. High arsenic accumulation comes with an ecological tradeoff, and the system price of the tradeoff depends on ability of lower soil horizons to sorb arsenic leached during *P. vittata* growth. Counterintuitively, phytoextraction could be best suited for soils with lower arsenic availability, to limit arsenic leaching.

Summary

High phytoavailable arsenic coupled to low nutrient content led to lower *P. vittata* biomass, likely a response to the higher metabolic cost of arsenic hyperaccumulation and nutrient scavenging. The biomass cost resulted in greater arsenic leaching. Ironically, in nutrient-poor soils rhizosphere nutrient scavenging activities could increase arsenic availability where it matters most —the rhizosphere—since most arsenic is taken up from rhizosphere, not bulk soils. These rhizosphere processes increase arsenic availability for leaching, leading to greater leaching with fern growth. Even though here arsenic uptake exceeded leaching, the fate of this leached arsenic must be determined to avoid soil and groundwater contamination during phytoextraction.

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Table 1. Properties of soil before phytoextraction

Soil characteristic		Soil texture	
		Medium	**Coarse**
pH[1]		6	6.93
Total[2]			
As	(mg/kg)	138.0±1.5	95.3±1.1
Extractable[3]			
P	(mg/kg)	17.7	8.1
K	(mg/kg)	288.8	56.5
Ca	(mg/kg)	2591.8	729.4
Mg	(mg/kg)	384.2	103.6
Zn	(mg/kg)	57.4	4.6
B	(mg/kg)	0.9	0.2
Mn	(mg/kg)	4.0	4.4
Cu	(mg/kg)	2.0	1.7
Fe	(mg/kg)	7.8	2.5
Pb	(mg/kg)	12.3	2.2
Al	(mg/kg)	3.7	5.4
Na	(mg/kg)	30.6	13.5
S	(mg/kg)	23.6	6.1
CEC	meq/100g	20.0	4.6
Organic matter	%	5.9	0.9
Bulk density[4]	g/cc	1.06	1.6
Clay[4]	%	16.7	4.0
Texture		Sandy loam	Loamy sand

[1]Mean of 3 replicates, in water

[2]Mean and standard error of soil in each column, before soil treatments applied

[3]Mean of 2 replicates, Modified Morgan extractable, before soil treatments applied

[4]Texture and bulk density are means of 3 replicates

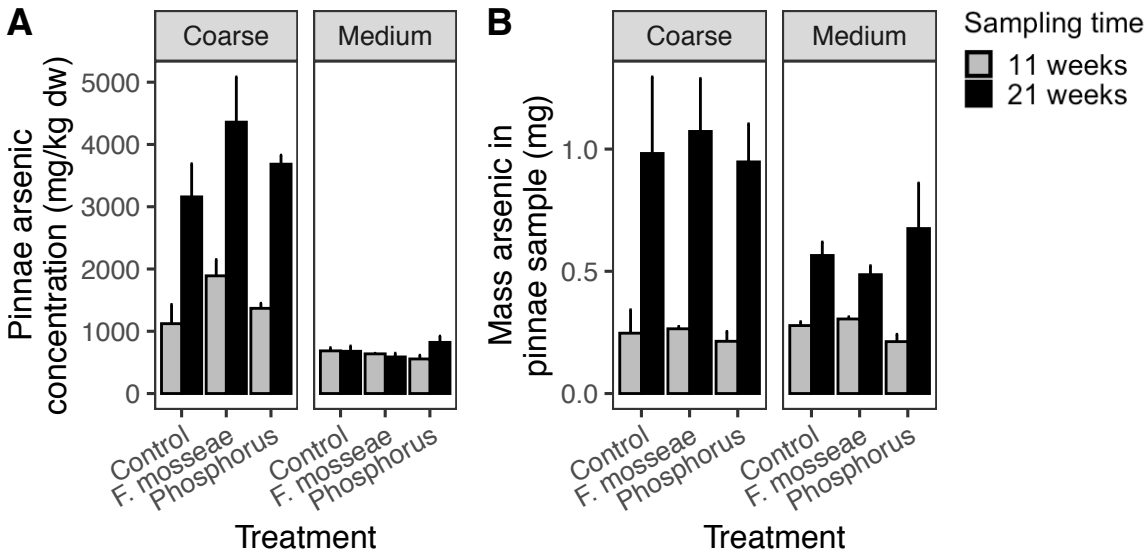

Figure 1. Arsenic concentrations **(A)** and mass arsenic accumulated **(B)** in pinnae at 11 and 21 weeks of growth, in coarse- (left panels) or medium-textured soil (right panels). Bars represent the mean and standard error of 2-3 replicates.

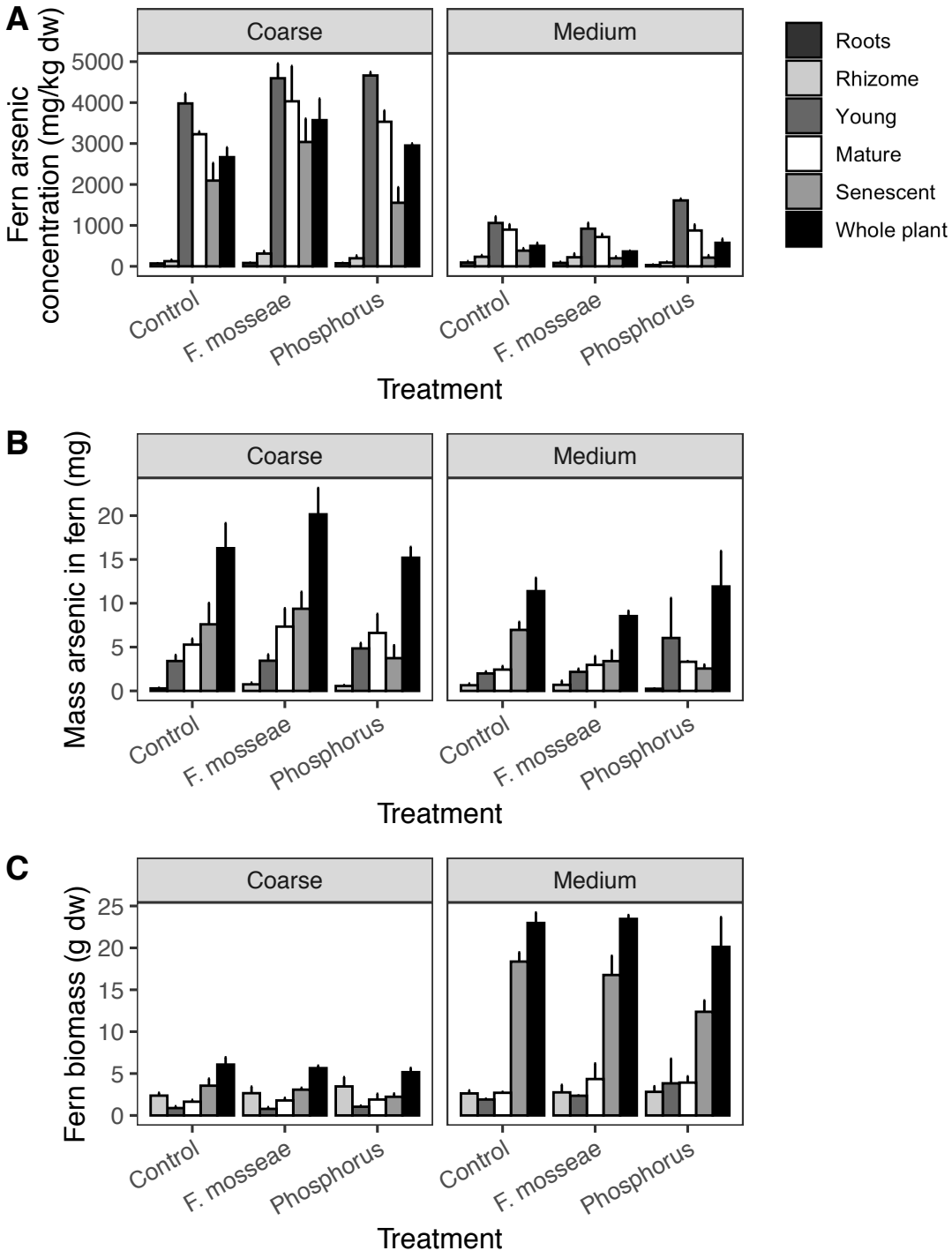

Figure 2. Arsenic concentrations **(A)**, mass arsenic accumulated **(B)** and dry fern biomass **(C)** in ferns at final harvest at 22 weeks of growth, in coarse- (left panels) or medium-textured soil (right panels). Bars represent the mean and standard error of 2-3 replicates. Note: roots are present in panel A only, though legend applies to all plots.

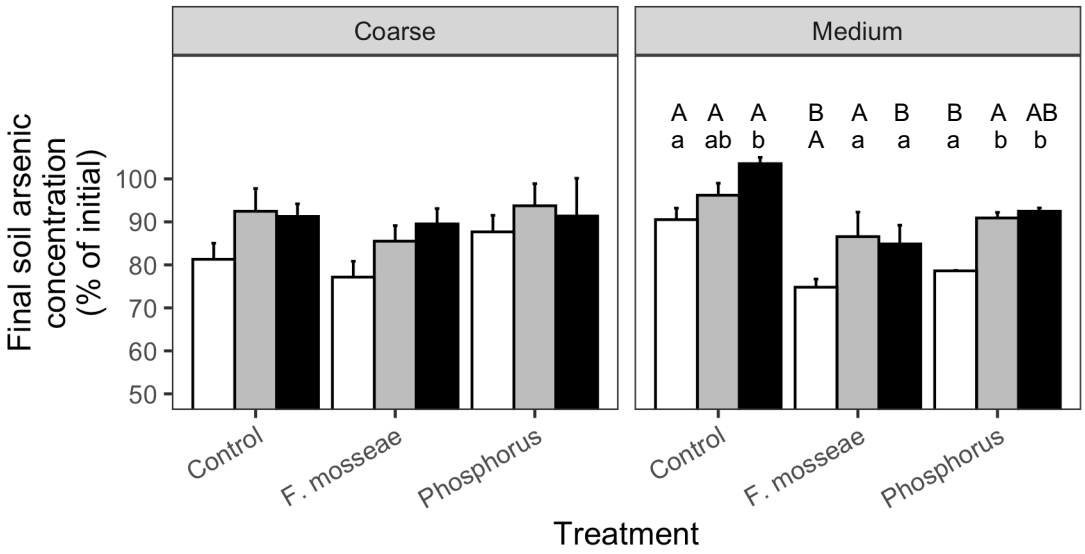

Figure 3. Final soil arsenic concentrations at 3 depths in soil columns, normalized to initial soil arsenic concentrations, after 22 weeks of *P. vittata* growth in coarse- (left panel) or medium-textured soil (right panel). Bars represent mean and standard error of 3 replicates. Means with the same lower case letter are not significantly different at $P<0.05$ within a soil and treatment, and means with the same upper case letter are not significantly different at $P<0.05$ within a soil and depth. In the loamy sand soil, no significant differences were found within a treatment or depth.

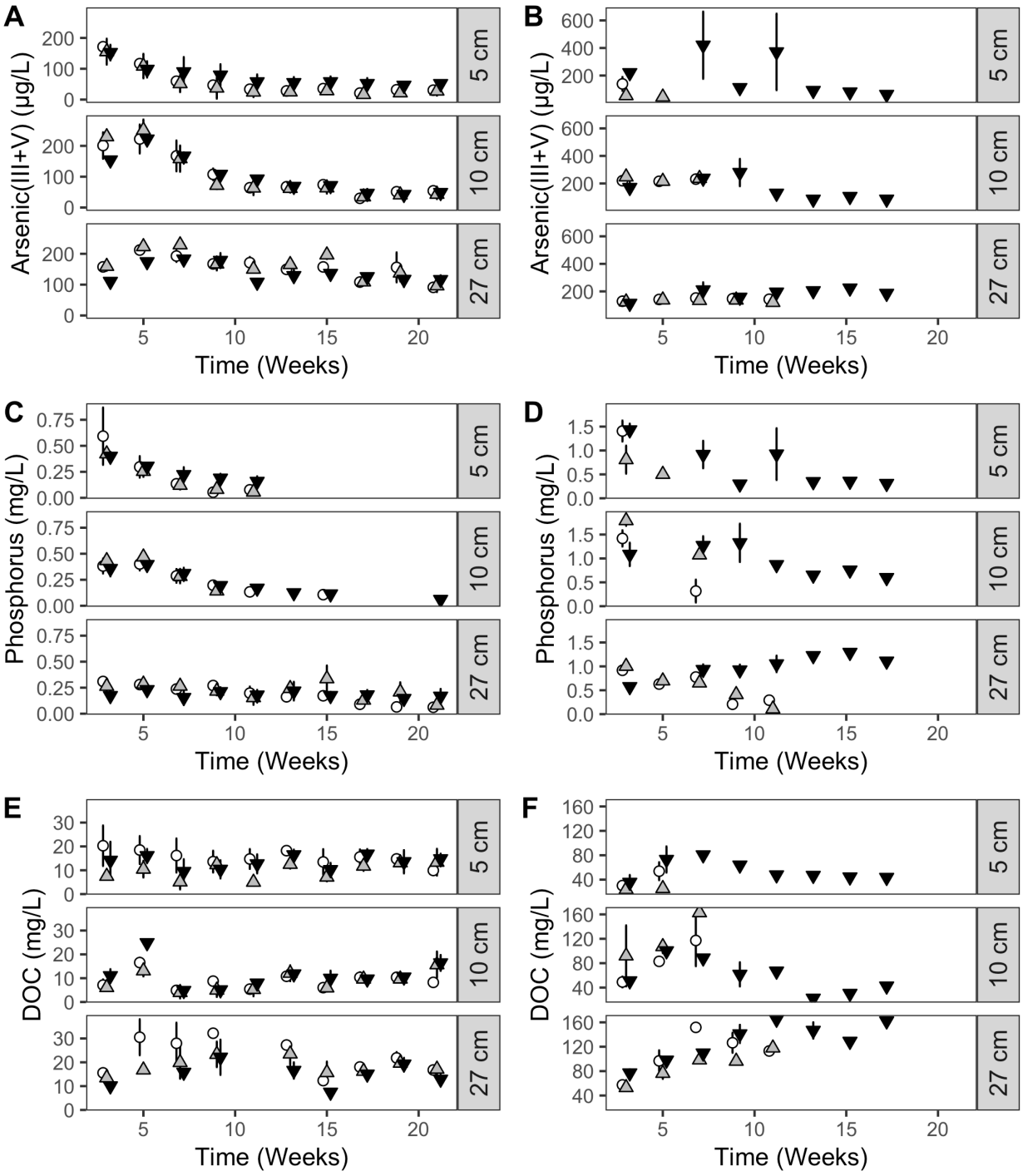

Figure 4. Concentrations of arsenic(III+V), phosphorus, and dissolved organic carbon (DOC) in porewater extracted from coarse- (**A, C,** and **E,** respectively), or medium-textured soil (**B, D,** and **F,** respectively). Open circles represent control columns, grey triangles represent *F. mosseae* inoculated columns, and black triangles phosphorus-treated columns. Note the different y-axis scales. Points represent mean and standard error of porewater extracted from 2-3 replicates columns. In some cases, porewater could not be extracted from columns.

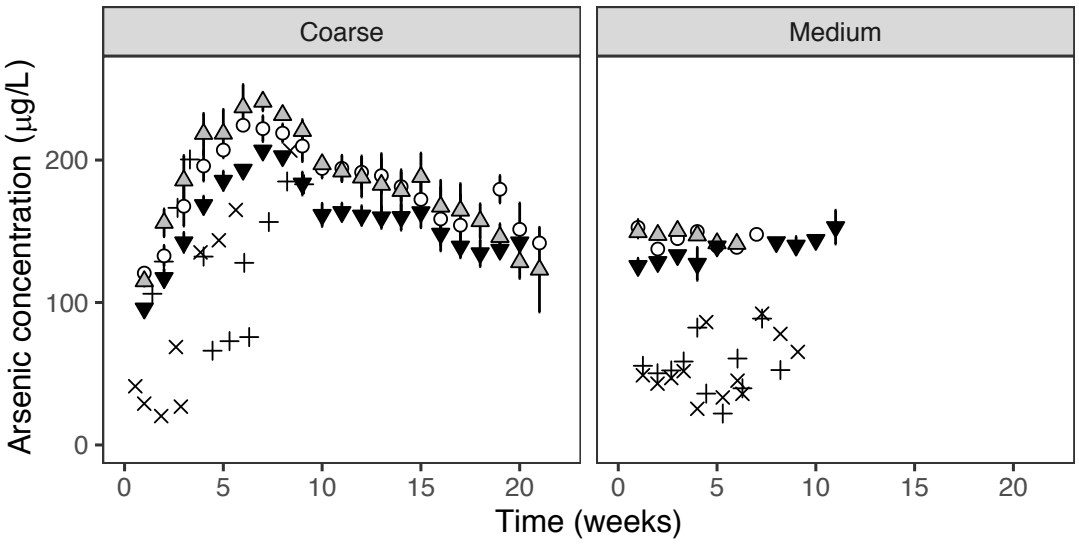

Figure 5. Arsenic concentrations in column effluent over 22 weeks of *P. vittata* growth (shapes) or over 10 weeks in the absence of *P. vittata* (crosses). Columns were filled with coarse- (left panel) or medium-textured soil (right panel). Points represent mean and standard error of 2-3 replicates (columns with ferns) or data of 1 replicate (columns without ferns).

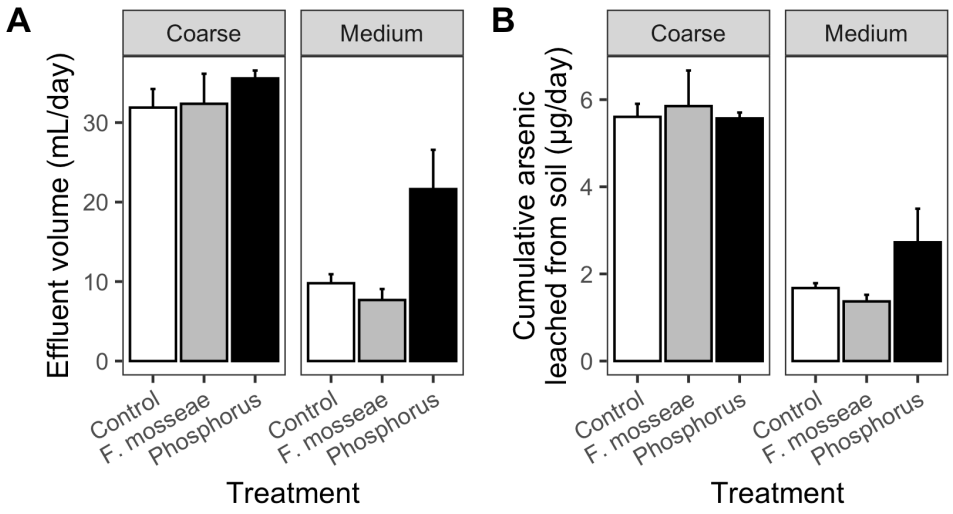

Figure 6. Effluent volume **(A)** and cumulative arsenic **(B)** and phosphorus **(C)** leached from soil, normalized to days of effluent, over 22 weeks of *P. vittata* growth in coarse- (left panels) or medium-textured soil (right panels). Points represent mean and standard error of 2-3 replicates.

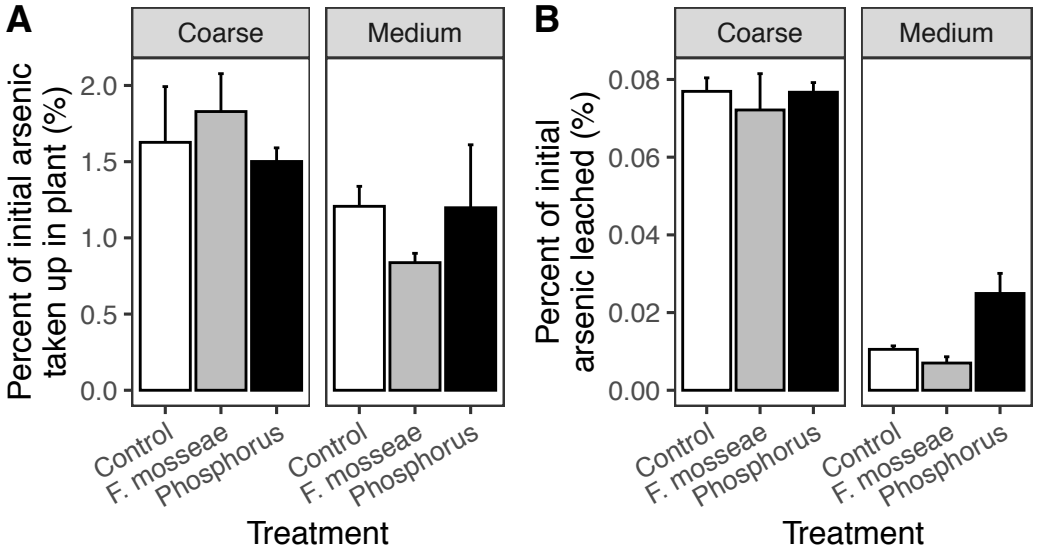

Figure 7. Percent of initial soil arsenic taken up in plant **(A)** or leached from soil **(B)**, over 22 weeks of *P. vittata* growth in coarse- (left panels) or medium-textured soil (right panels). Points represent mean and standard error of 2-3 replicates.

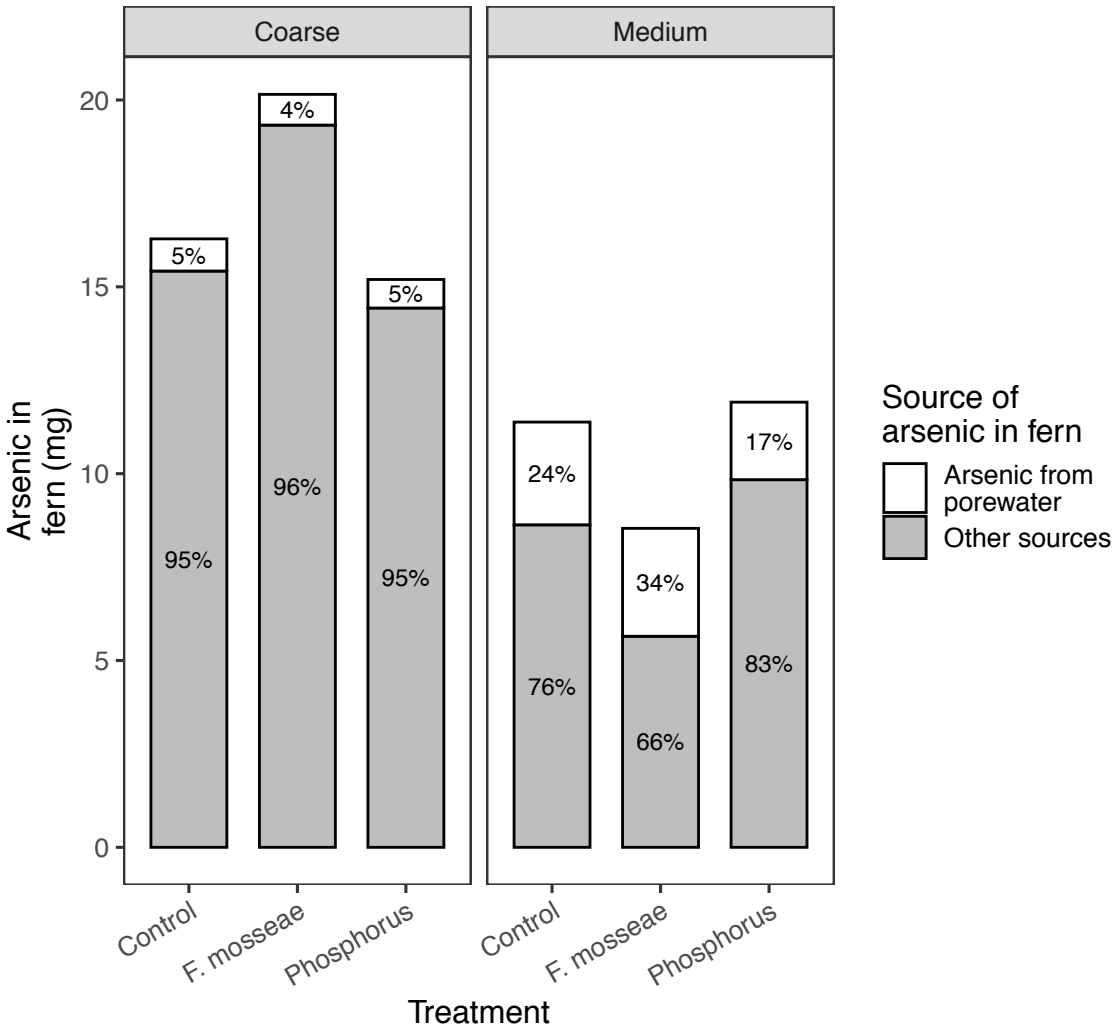

Figure 8. Mass and percent of arsenic in fern aboveground biomass contributed either from uptake of bulk porewater via transpiration flux, or from other sources, over 22 weeks of *P. vittata* growth in coarse- (left panel) or medium-textured soil (right panel). Points represent mean and standard error of 2-3 replicates.

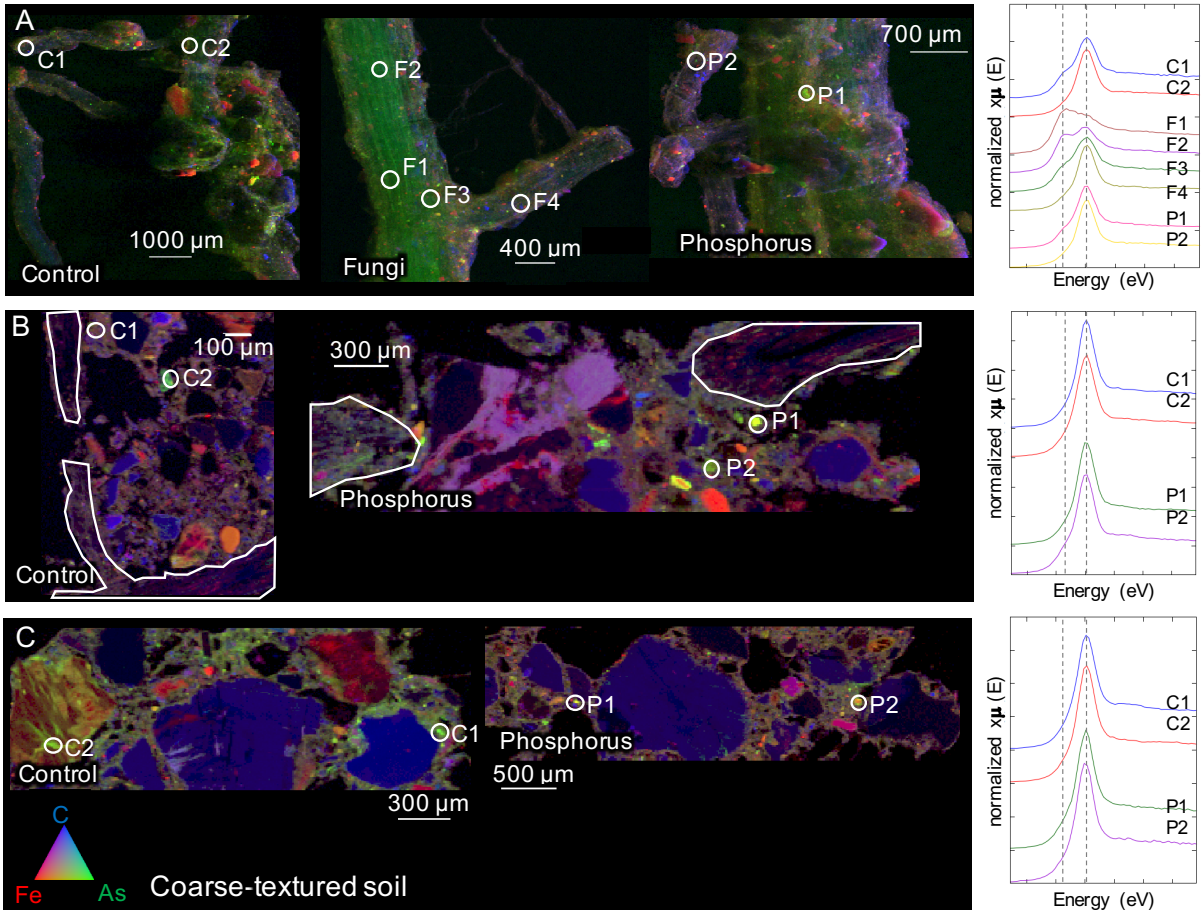

Figure 9. Microscale X-ray fluorescence tri-color images of As, Fe, and Ca in **(A)** whole roots with rhizospheric soil, **(B)** longitudinal thin sectioned roots with rhizospheric soil, and **(C)** thin sectioned soil aggregates, collected at 12keV. Roots (outlined in white as needed for clarity) and soil were sampled from ferns growing in coarse-textured soil and soil treatments are indicated in image labels. The tri-color shield indicates color in panels A, B, and C. Within an image, brighter colors indicate higher fluorescence signals from the imaged elements. In panel C, the edge of each aggregate is at the right margin and the aggregate cross-section center is near the left margin. Selected arsenic K-edge microscale X-ray absorption near-edge structure (XANES) spectra are reported for spots in images in panels to the left of each spectra plot. Vertical lines denote energies of 11872 and 11875 eV that were used to differentiate As(V) and As(III) species, respectively, and the X axis tick marks range from 11865 to 11890 eV. The complete library of standards is available in the Supplemental Information.

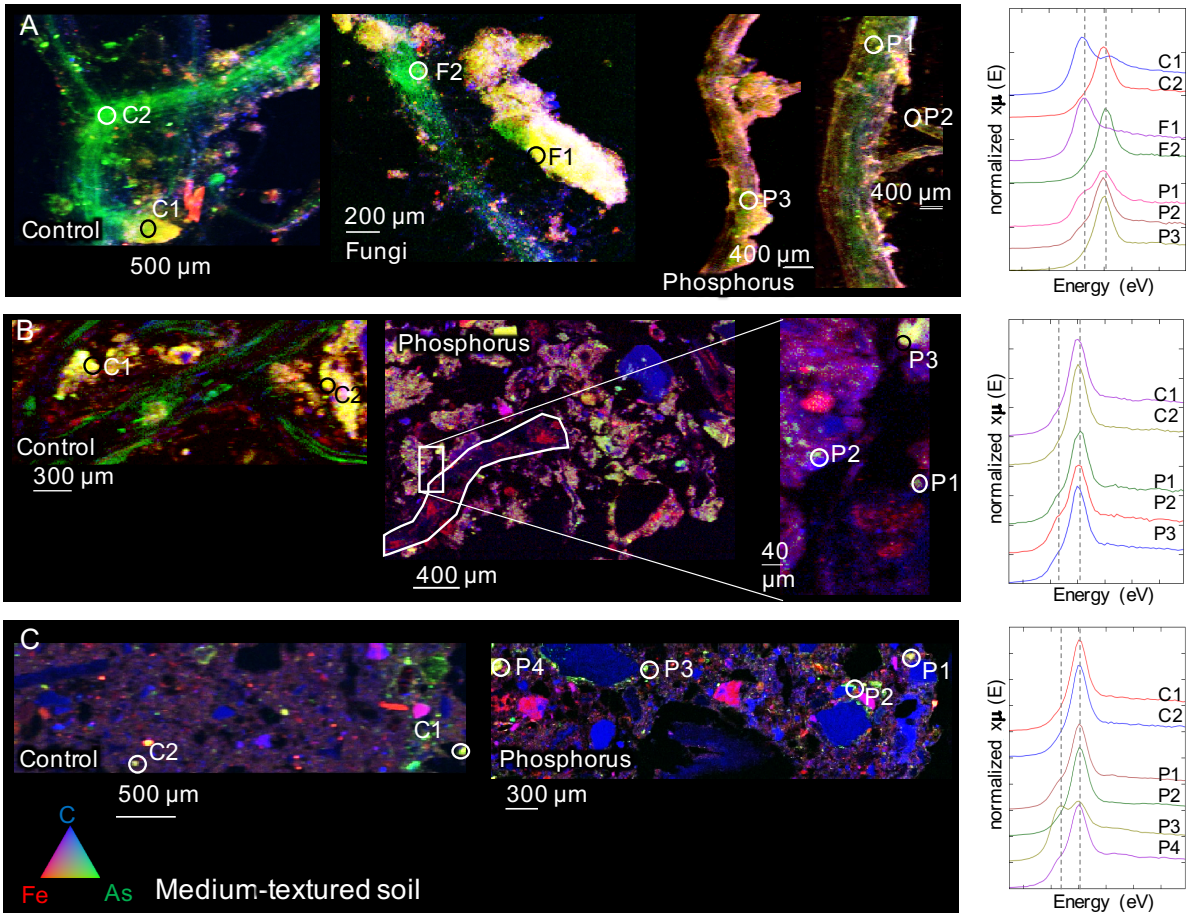

Figure 10. Microscale X-ray fluorescence tri-color images of As, Fe, and Ca in **(A)** whole roots with rhizospheric soil, **(B)** longitudinal thin sectioned roots with rhizospheric soil, and **(C)** thin sectioned soil aggregates, collected at 12keV. Roots (outlined in white as needed for clarity) and soil were sampled from ferns growing in coarse-textured soil and soil treatments are indicated in image labels. The tri-color shield indicates color in panels A, B, and C. Within an image, brighter colors indicate higher fluorescence signals from the imaged elements. In panel C, the edge of each aggregate is at the right margin and the aggregate cross-section center is near the left margin. Selected arsenic K-edge microscale X-ray absorption near-edge structure (XANES) spectra are reported for spots in images in panels to the left of each spectra plot. Vertical lines denote energies of 11872 and 11875 eV that were used to differentiate As(V) and As(III) species, respectively, and the X axis tick marks range from 11865 to 11890 eV. The complete library of standards is available in the Supplemental Information.

Chapter 4. Supplemental Information

Micro-focused and bulk X-ray spectromicroscopy

X-ray microprobe analyses were performed at the Advanced Light Source XFM beamline 10.3.2 (Marcus et al., 2004). Thirty micron-thick thin sections were mounted with silicon vacuum grease onto a Peltier-cooling stage. All data were collected in fluorescence mode at -20 ºC and ambient pressure using a Canberra 7-element Ge solid state detector and processed using a suite of LabVIEW custom software available at the beamline.

μXRF Mapping: Arsenic and iron spatial distribution in the samples was determined using micro-focused X-ray fluorescence (μXRF) elemental mapping with a 12 keV incident beam, using a 50 ms/pixel dwell time a 2 × 2.5 μm beam spot size, and a pixel size of 30 × 30 μm (Figure 9A Control), 9 × 9 μm (Figure 9A Fungi), 10 × 10 μm (Figure 9A Phosphorus), 10 × 10 μm (Figure 9B Control), 9 × 9 μm (Figure 9B Phosphorus), 8 × 8 μm (Figure 9C Control), 13 × 13 μm (Figure 9C Phosphorus), 10 × 10 μm (Figure 10A Control), 8 × 8 μm (Figure 10A Fungi), 20 × 20 μm (Figure 10A Phosphorus, both roots), 10 × 10 μm (Figure 10B Control), 8 × 8 μm (Figure 10B Phosphorus, 2 × 2 μm (Figure 10B Phosphorus inset), 9 × 9 μm (Figure 10C Control), 10 × 10 μm (Figure 10C Phosphorus). Maps were deadtime-corrected. Decontamination was not required.

μXANES: In sample regions of interest, arsenic and iron speciation were determined using arsenic K-edge and iron K-edge μX-ray absorption near edge structure (μXANES) spectroscopy. Spectra were calibrated using sodium arsenate (E0=11875 eV) and elemental iron (E0=7110.75 eV), respectively. All spectra recorded in the range 11770-12177 eV (As) and 7011-7416 eV (Fe) were deadtime-corrected, deglitched, calibrated, pre-edge background subtracted, and post-edge normalized using a suite of custom LabVIEW software available at the beamline. If counts were low, spectra were collected at 2 adjacent spots and merged.

Bulk XAS: Bulk XANES spectra were collected at SSRL beamline 7.3. Filter membranes were sealed and mounted on sample holders with Kapton tape. Data were collected in fluorescence mode using a cryostat sample holder at 10 K and a Canberra 30 element Ge solid state detector. Spectra collected in the range 11635-12877 eV (As) and 6880-8126 eV (Fe) were calibrated to a reference gold (E0=11919 eV) foil (As) or iron (E0=7112 eV) foil (Fe) in SixPak (Webb 2005), and normalized and plotted in Athena.

Least-square linear combination fitting: LSQ LC fitting of experimental XANES spectra (micro and bulk) was performed using custom LabVIEW software for arsenic in the range of 11770-12177 eV using a library of 68 standard arsenic compounds, and for iron in the range of 7011-7416 eV using a library of 79 standard iron compounds. The best LCF was obtained by minimizing the normalized sum of squares residuals (NSS=0=perfect fit), according to the formula $NSS = 100 \times \{\sum(\mu exp - \mu fit)^2 / \sum (\mu exp)^2\}$ where μ represents the normalized absorbance. Some spectra were corrected for over-absorption induced distortion using μ corrected = μ exp / (1 + a (1 - μ exp)) where the parameter a was adjusted. The error on the percentages of species found via LC fitting is estimated to ± 10%. 2D scatter valence plots were generated from Fe and As XANES data according to methods previously described in Marcus et al. (2008, Fe XAS database updated since) and a Matlab code described elsewhere (Fakra et al., 2018) and available at beamline 10.3.2. For iron valence plots, kappa and mu represent normalized absorption values at 7113 and 7117.5eV respectively. For arsenic valence plots, kappa and mu represent normalized absorption values at 11870.8 and 11889.9 eV respectively. Standards are plotted as black empty squares and sample data points are in color.

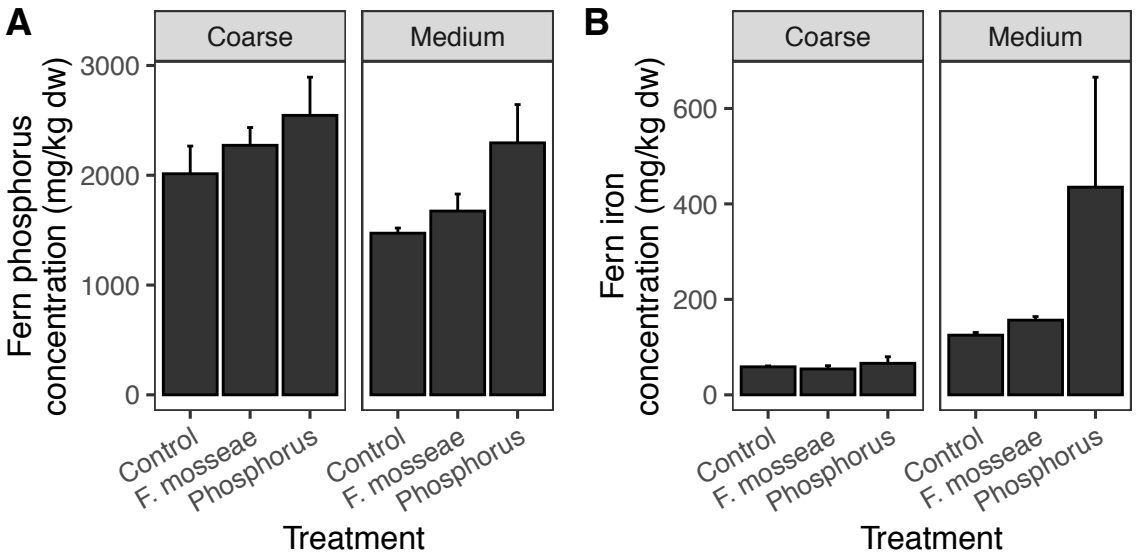

Figure SI-1. Whole aboveground biomass phosphorus concentrations **(A)** and iron concentrations **(B)** in ferns at final harvest at 22 weeks of growth, in coarse- (left panels) or medium-textured soil (right panels). Bars represent the mean and standard error of 2-3 replicates.

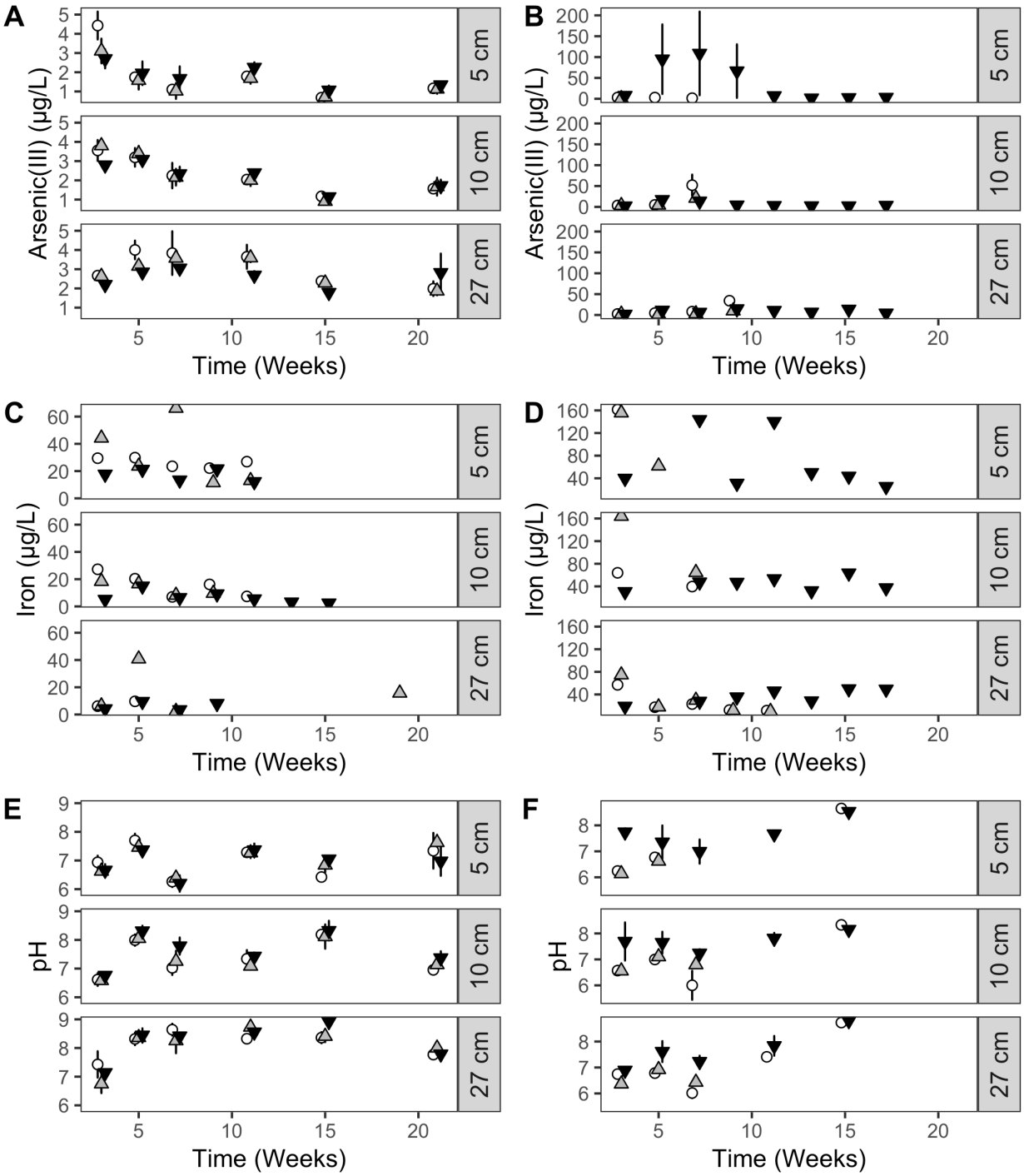

Figure SI-2. Concentrations of arsenic(III) and iron, and pH in porewater extracted from loamy sand columns (**A, C,** and **E,** respectively), or sandy loam columns (**B, D,** and **F,** respectively). Open circles represent control columns, grey triangles represent *F. mosseae* inoculated columns, and black triangles phosphorus-treated columns. Note the different y-axis scales. Points represent mean and standard error of porewater extracted from 2-3 replicates columns. In some cases, porewater could not be extracted from columns.

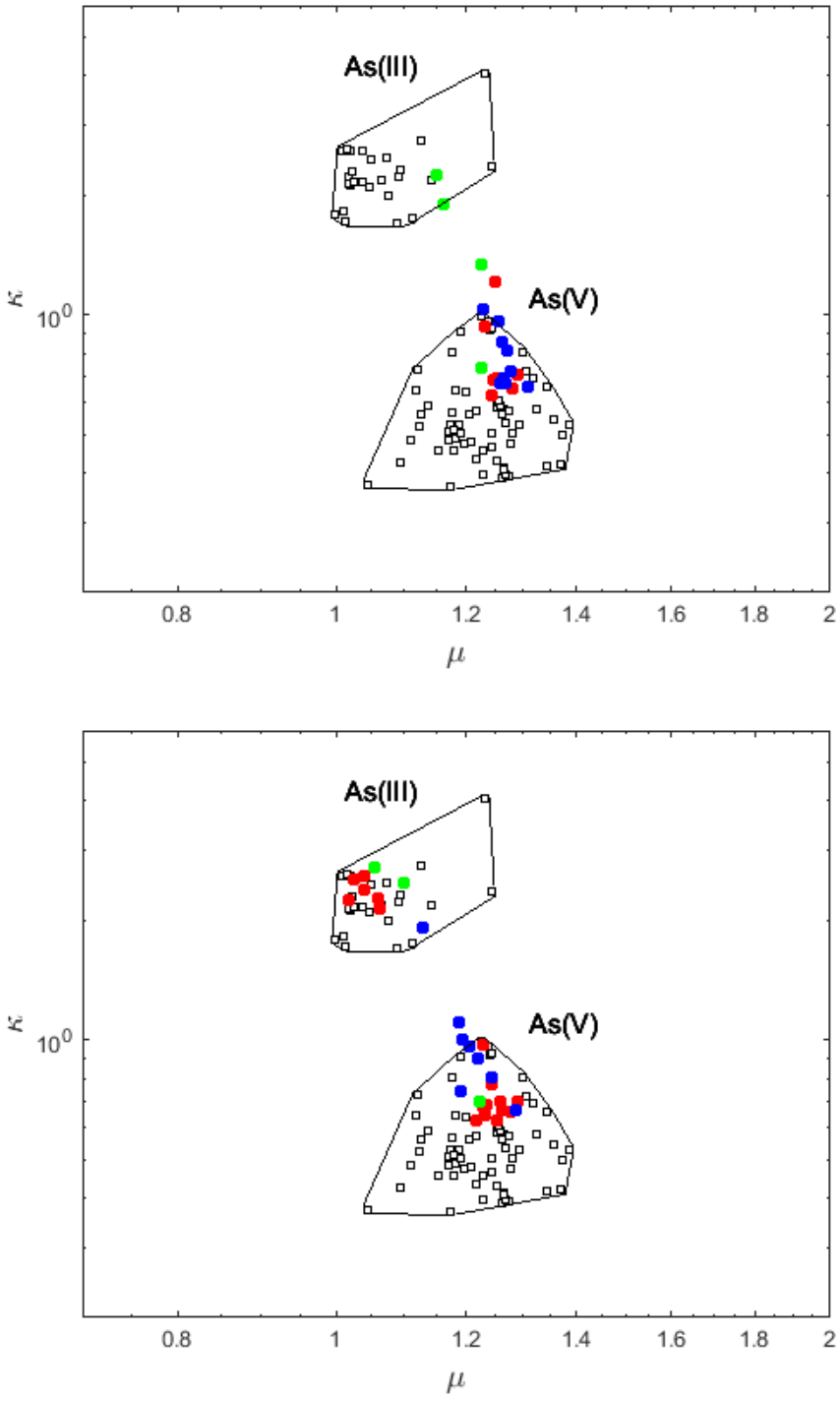

Figure SI-3. Valence plots of arsenic speciation in coarse- (top) and medium-textured soil (bottom). Sample data points are plotted in color by treatment (red=control, green=fungi, blue=phosphorus), across all sample types (whole roots, root thin sections, aggregate sections. Standards are plotted as black empty squares. Kappa and mu represent normalized absorption values at 11870.8 and 11889.9 eV respectively.

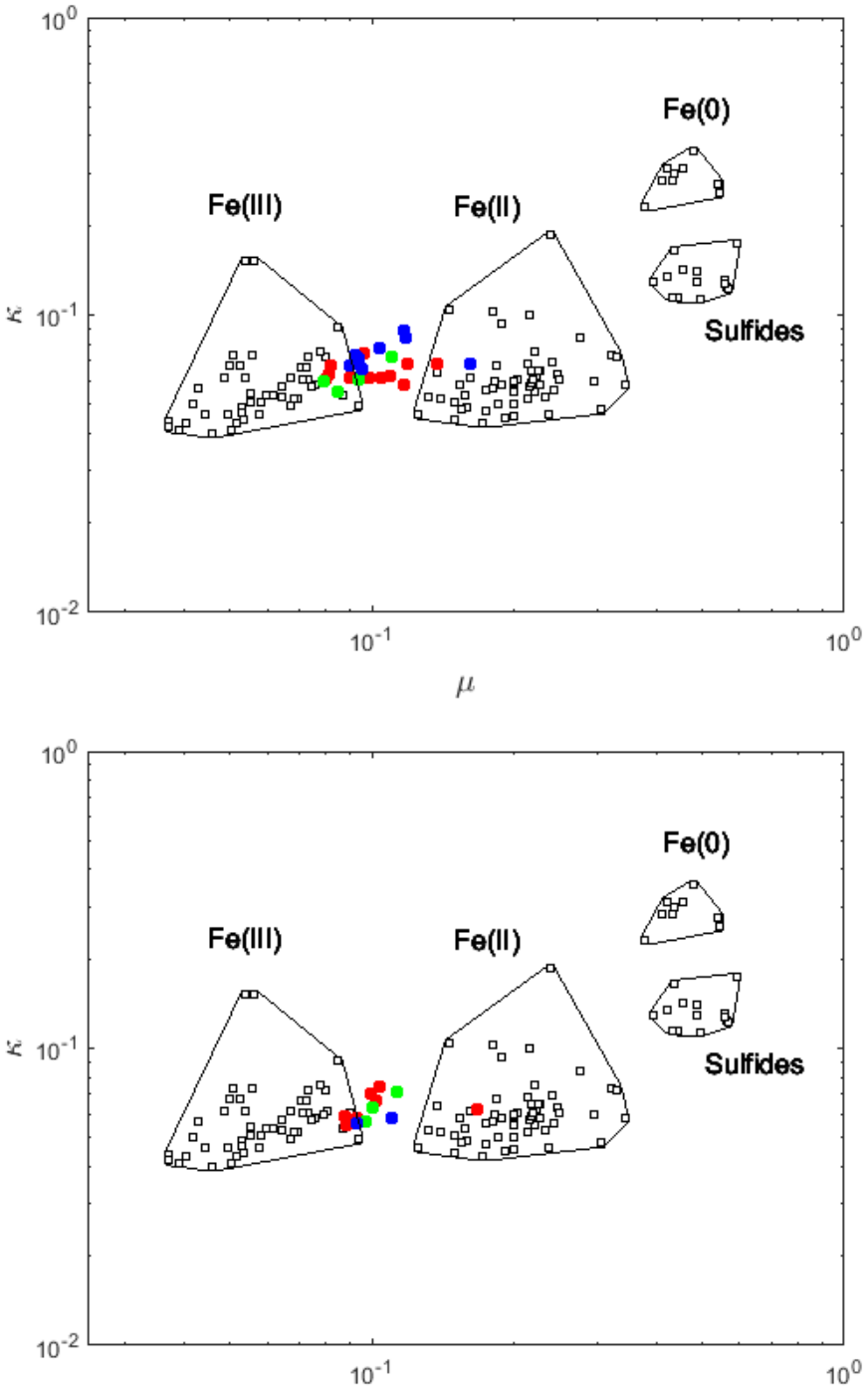

Figure SI-4. Valence plots of iron speciation in coarse- (top) and medium-textured soil (bottom). Sample data points are plotted in color by treatment (red=control, green=fungi, blue=phosphorus), across all sample types (whole roots, root thin sections, aggregate sections. Standards are plotted as black empty squares. Kappa and mu represent normalized absorption values at 7113 and 7117.5eV respectively.

Table SI-1. Linear combination fits for arsenic K-edge microXANES spots in Figure 9(A-C) and additional spots not shown. Samples collected from ferns growing in coarse-textured soil.

Sample type	Treatment	Spectra/Spot number	As(III) %	As(V) %	Normalized Sum of Squares
Whole root	Control	C1	28.5	83.0	5.99E-04
		C2	6.3	104.2	3.84E-04
		(not shown)	20.7	88.9	3.57E-04
	F. mosseae	F1	80.9	29.7	3.22E-04
		F2	63.0	46.6	3.24E-04
		F3	36.1	69.5	3.65E-04
		F4	6.3	99.9	3.38E-04
	Phosphorus	P1	20.0	90.4	1.93E-04
		P2	11.6	93.6	4.12E-04
		(not shown)	10.6	94.4	4.99E-04
		(not shown)	4.6	105.6	4.60E-04
		(not shown)	0.0	109.7	5.08E-04
Root thin section	Control	C1	2.5	107.1	4.91E-04
		C2	9.2	97.5	2.30E-04
		(not shown)	6.5	99.7	6.66E-04
	Phosphorus	P1	5.0	100.9	3.25E-04
		P2	21.5	89.4	1.99E-03
Aggregate thin section	Control	C1	7.4	103.1	1.77E-04
		C2	0.0	103.7	2.77E-04
	Phosphorus	P1	13.4	94.8	4.30E-04
		P2	15.9	94.6	4.66E-03

Table SI-2. Linear combination fits for arsenic K-edge microXANES spots in Figure 10(A-C) and additional spots not shown. Samples collected from ferns growing in medium-textured soil.

Sample type	Treatment	Spectra/Spot number	As(III) %	As(V) %	Normalized Sum of Squares
Whole root	Control	C1	99.8	11.4	1.34E-03
		C2	18.2	88.2	4.11E-04
		(not shown)	82.9	15.7	1.44E-03
		(not shown)	94.6	8.3	7.41E-04
		(not shown)	74.1	29.9	1.57E-03
		(not shown)	99.6	2.0	5.87E-04
		(not shown)	79.3	25.4	1.61E-03
	F. mosseae	F1	106.0	0.0	5.66E-04
		F2	3.5	98.8	4.68E-04
		(not shown)	97.7	13.0	5.85E-04
	Phosphorus	P1	44.8	59.6	1.21E-03
		P2	23.0	78.2	1.23E-03
		P3	8.2	93.6	7.78E-04
		(not shown)	13.8	88.9	6.93E-04
Root thin section	Control	C1	9.8	90.5	3.26E-04
		C2	7.2	97.3	2.93E-04
		(not shown)	6.3	101.4	6.61E-04
	Phosphorus	P1	12.3	88.5	6.14E-04
		P2	26.5	79.5	4.66E-04
		P3	18.1	84.7	4.13E-04
		(not shown)	44.5	64.0	3.62E-04
		(not shown)	18.0	85.7	4.31E-04
		(not shown)	7.1	99.3	4.99E-04
		(not shown)	29.5	78.2	4.51E-04
Aggregate thin section	Control	C1	7.4	96.6	4.58E-04
		C2	6.2	100.6	4.01E-04
		(not shown)	8.2	95.2	2.72E-04
		(not shown)	0.0	105.6	3.28E-04
		(not shown)	0.0	107.5	2.18E-03
		(not shown)	0.0	110.9	6.92E-04
		(not shown)	0.0	107.6	4.84E-04
	Phosphorus	P1	19.7	85.1	4.86E-04
		P2	0.0	103.9	1.99E-04
		P3	68.7	36.8	2.01E-04
		P4	23.5	79.3	3.01E-04
		(not shown)	25.6	80.0	3.74E-04
		(not shown)	12.2	95.8	2.58E-04

Table SI-3. Linear combination fits for iron K-edge microXANES spots in Figure 9(A-C) and additional spots not shown. Samples collected from ferns growing in coarse-textured soil.

Sample type	Treatment	Spot number	%	Component 1	%	Component 2	%	Component 3	Normalized Sum of Squares
Whole root	Control	C1	7.7	Iron (II-III) silicate	21.3	Iron (II) silicate	70.6	Iron (III) silicate	6.00E-05
		C2	61.9	Iron (II-III) silicate	18.7	Iron (II) silicate	18.9	Iron (III) oxide	7.29E-05
	F. mosseae	F1	(Fe spectra not collected)						
		F2	25.5	Iron (III) silicate	28.1	Iron (III) oxide	46.3	Iron (III) silicate	7.19E-05
		F3	10.6	Iron (II-III) silicate	71.5	Iron (III) oxyhydroxide	19.3	Iron (III) silicate	1.05E-04
		F4	58.3	Iron (II-III) silicate	29.3	Iron (II) silicate	13.8	Iron (II-III) silicate	8.13E-05
	Phosphorus	P1	12.2	Elemental iron	56	Iron (II-III) silicate	31.2	Iron (II) sulfate	1.83E-04
		P2	60.6	Iron (II-III) silicate	26.7	Iron (II-III) silicate	14.3	Iron (II) sulfate	4.26E-05
Root thin section	Control	C1	(Fe spectra not collected)						
		C2	14.1	Iron (III) silicate	24.5	Iron (II-III) silicate	61.2	Iron (III) silicate	5.71E-05
		C3	7.6	Iron (III) phosphate	26.6	Iron (II-III) silicate	65.2	Iron (III) oxyhydroxide	2.85E-05
	Phosphorus	P1	10.2	Iron (II-III) silicate	85.2	Iron (III) oxyhydroxide	4.9	Iron (II) silicate	6.19E-05
		P2	8.0	Elemental iron	72.1	Iron (III) oxyhydroxide	20.2	Iron (II) sulfate	9.30E-05
Aggregate thin section	Control	C1	20.5	Iron (III) silicate	46.0	Iron (II-III) silicate	33.2	Iron (III) silicate	4.65E-05
		C2	11.8	Iron (II-III) silicate	63.5	Iron (II-III) silicate	24.4	Iron (II) sulfate	1.01E-04
	Phosphorus	P1	10.9	Iron (II-III) silicate	33.9	Iron (II-III) silicate	54.9	Iron (III) oxyhydroxide	3.02E-04
		P2	7.8	Iron (II-III) silicate	86.4	Iron (III) oxyhydroxide	6.3	Iron (II) sulfate	3.40E-04

Table SI-4. Linear combination fits for iron K-edge microXANES spots in Figure 10(A-C) and additional spots not shown. Samples collected from ferns growing in medium-textured soil.

Sample type	Treatment	Spectra/ Spot number	%	Component 1	%	Component 2	%	Component 3	Normalized Sum of Squares
Whole root	Control	C1	7.0	Iron (II-III) silicate	16.5	Iron (II) silicate	76.1	Iron (III) silicate	4.00E-05
		C2	16.0	Iron (III) silicate	68.1	Iron (III) silicate	14.6	Iron (III) oxyhydroxide	7.06E-05
	F. mosseae	F1	28.2	Iron (II) silicate	26.9	Iron (III) oxide	48.1	Iron (III) silicate	9.23E-05
		F2	12.3	Iron (II-III) silicate	15.1	Iron (II) sulfate	75.5	Iron (III) silicate	9.54E-05
	Phosphorus	P1	18.5	Iron (II) silicate	13.0	Iron (II-III) silicate	69.0	Iron (III) silicate	4.91E-05
		P2							
		P3	33.0	Iron (II) silicate	12.2	Iron (II-III) silicate	55.7	Iron (III) silicate	4.53E-05
Root thin section	Control	C1	52.7	Iron (II) silicate	38.2	Iron (II-III) silicate	15.2	Iron (III) phosphate	1.14E-04
		C2	6.4	Iron (II-III) silicate	77.4	Iron (III) oxyhydroxide	21.8	Iron (II) sulfate	2.20E-04
	Phosphorus	P1	12.1	Elemental iron	28.6	Iron (II) sulfate	61.8	Iron (III) silicate	2.37E-04
		P2	(Fe spectra not collected)						
		P3	54.6	Iron (III) oxyhydroxide	30.8	Iron (III) oxyhydroxide	19.6	Iron (II) silicate	1.46E-04
Aggregate thin section	Control	C1	46.6	Iron (II-III) silicate	22.7	Iron (II) silicate	27.7	Iron (III) oxide	6.06E-05
		C2	18.2	Iron (III) silicate	70.7	Iron (II-III) silicate	7.9	Iron (II) carbonate	5.59E-05
	Phosphorus	P1	11.1	Iron (II-III) silicate	57.6	Iron (III) oxyhydroxide	35.3	Iron (II) sulfate	9.79E-05
		P2	6.1	Iron (III) silicate	5.3	Elemental iron	96.5	Iron (III) oxyhydroxide	8.30E-04
		P3	11.2	Iron (III) silicate	51.9	Iron (III) silicate	39.6	Iron (II) sulfate	9.24E-05
		P4	18.5	Iron (III) oxyhydroxide	18.2	Iron (II) silicate	66.6	Iron (III) silicate	8.47E-05

Table SI-5. Linear combination fits for arsenic K-edge bulk XANES spectra.

Texture	Treatment	Sample type	As(III) %	As(V) %	Normalized Sum of Squares
Coarse	Control	Bulk soil	10.1	92.3	6.96E-04
		Rhizosphere soil	33.2	70.0	5.37E-04
		Root	18.8	84.1	4.93E-04
	Phosphorus	Bulk soil	15.1	86.8	4.42E-04
		Rhizosphere soil	41.3	64.7	3.15E-04
		Root	26.6	77.1	3.32E-04
Fine	Control	Bulk soil	0	100.1	6.02E-04
		Rhizosphere soil	7.9	96.1	6.03E-04
		Root	19.2	81.5	3.63E-03
	Phosphorus	Bulk soil	10.5	93.7	5.79E-04
		Rhizosphere soil	18.7	83.8	4.26E-04
		Root	18.1	84.3	5.29E-04

Table SI-6. Linear combination fits for iron K-edge bulk XANES spectra.

Texture	Treatment	Sample type	%	Component 1	%	Component 2	%	Component 3	Normalized Sum of Squares
Coarse	Control	Bulk soil	12.5	Iron (II) silicate	17.4	Iron (III) silicate	69.9	Iron (III) oxyhydroxide	4.46E-05
		Rhizosphere soil	15.4	Iron (II) silicate	14.5	Iron (III) silicate	69.9	Iron (III) oxyhydroxide	3.63E-05
		Root	14.1	Iron (III) silicate	52.5	Iron (III) oxyhydroxide	34.2	Iron (III) silicate	3.96E-05
	Phosphorus	Bulk soil	14.2	Iron (III) silicate	76.6	Iron (III) oxyhydroxide	8.9	Iron (II) silicate	3.70E-05
		Rhizosphere soil	9.7	Iron (II) silicate	19.5	Iron (III) silicate	70.3	Iron (III) oxyhydroxide	3.81E-05
		Root	11.5	Iron (III) silicate	25.1	Iron (III) silicate	61.5	Iron (III) oxyhydroxide	4.78E-05
Fine	Control	Bulk soil	18.6	Iron (III) silicate	41.3	Iron (III) oxyhydroxide	39.8	Iron (II-III) silicate	4.92E-05
		Rhizosphere soil	20.6	Iron (III) silicate	34.0	Iron (III) oxyhydroxide	45.1	Iron (II-III) silicate	4.45E-05
		Root	6.6	Iron (II-III) silicate	50.2	Iron (II-III) silicate	41.2	Iron (III) oxyhydroxide	6.98E-05
	Phosphorus	Bulk soil	19.8	Iron (III) silicate	40.6	Iron (III) oxyhydroxide	38.1	Iron (II-III) silicate	4.16E-05
		Rhizosphere soil	21.5	Iron (III) silicate	36.3	Iron (II-III) silicate	41.0	Iron (II-III) silicate	5.15E-05
		Root	10.7	Iron (II-III) silicate	44.4	Iron (III) silicate	44.9	Iron (III) oxyhydroxide	4.87E-05

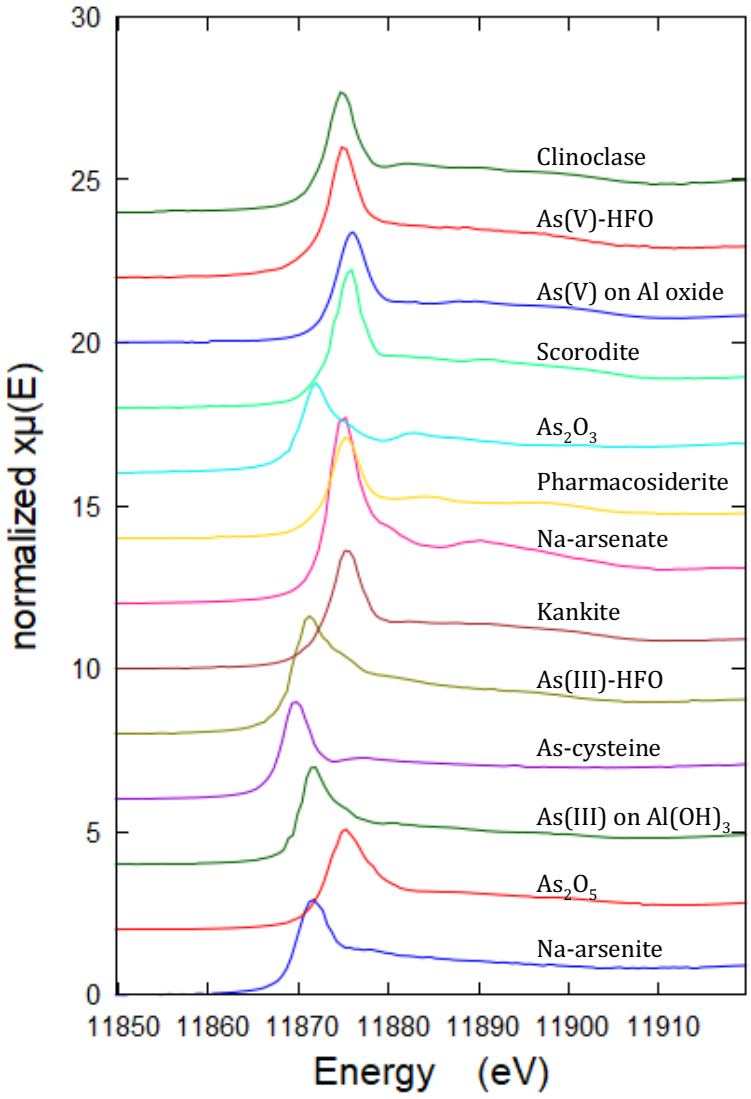

Figure SI-5. Standard spectra used in linear combination fitting of arsenic K-edge spectra in Figures 9-10 and Tables SI-1, SI-2, and SI-5.

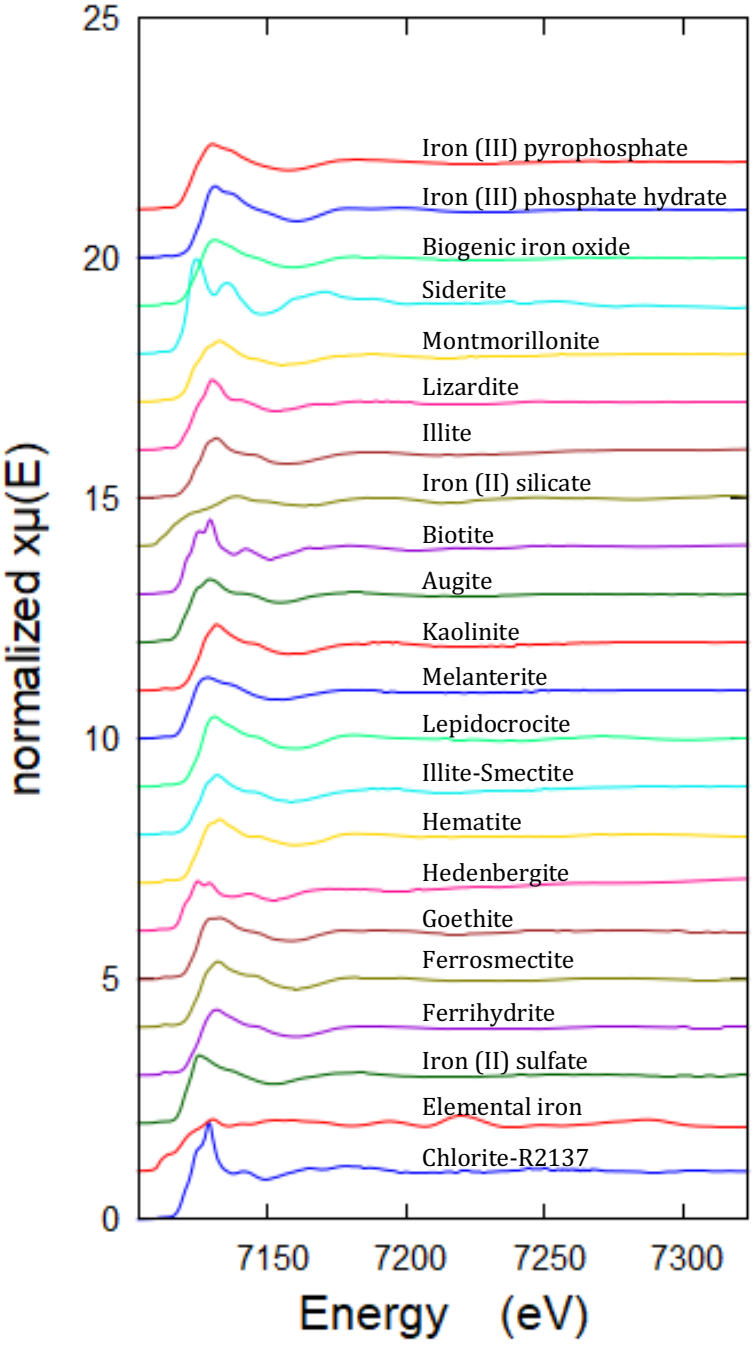

Figure SI-6. Standard spectra used in linear combination fitting of iron K-edge spectra in Tables SI-3, SI-4, and SI-6.

Conclusion

Motivation

The original motivation of this dissertation was to determine if arsenic phytoextraction is a viable method to remediate urban agricultural soils. The answer is no. The primary barrier is that remediation rates are far too slow to be appropriate for urban agricultural settings. Using the rates I measured, I estimated it would take decades to millennia to remediate soils moderately contaminated with arsenic. A secondary, practical barrier is the hazardous nature of arsenic phytoextraction. If phytoextraction is working, it produces large volumes of hazardous waste. Safely cultivating *P. vittata* for phytoextraction requires strict adherence to rigorous safety protocols. Phytoextraction, in my experience, would not be applicable to community-based urban agriculture settings.

Key findings

My findings can be summarized in four main points. First, I found that arsenic removal rates were slow and variable, ranging from 0.02±NA to 28.9±11.1 kg As/ha/yr. Nutrient application did not increase remediation rates. Second, *P. vittata* was not hardy under field conditions in a Mediterranean climate. However, compost application, frost protection, and greenhouse enclosures increased survival in *in situ* conditions, and mycorrhizal fungi inoculant appeared to increase fern tolerance to field stress. Third, the increase in surface soil arsenic concentrations, coupled with the greater depletion of arsenic from lower soil depth intervals, indicated the arsenic cycle during phytoextraction is complex. The cycle likely includes phytoenrichment of surface soil and arsenic leaching, in addition to sequestration and subsequent removal of arsenic in harvested plant tissue. Notably, I found fern uptake was only a small portion of total arsenic loss from soil under field conditions, necessitating that we investigate other loss processes beyond phytoextraction. Fourth, my results suggest that *P. vittata* arsenic uptake is limited to rhizosphere soil, though leaching can occur from rhizosphere and bulk soil. Critical work is needed to quantify how arsenic is depleted from soil over time *via P. vittata* arsenic uptake and leaching, given root growth and turnover.

Interrogating the *P. vittata*-soil-water arsenic cycle

Planting *P. vittata* sets in motion a leaky arsenic cycle. Arsenic is 1) mobilized in soil *via* rhizosphere geochemical processes, then either 2) leached from rhizosphere and bulk soils or 3) taken up into roots and sequestered in fronds, where it is then 4) removed from the site during frond harvest or 5) returned from *P. vittata* biomass back to surface soil through phytoenrichment. Theoretically, arresting this cycle at the right moment provides beneficial outcomes. However, the nature of plant growth creates multiple opportunities for arsenic loss that undermine these beneficial outcomes. Arsenic loss must be investigated on a site-specific basis to ascertain that environmental damage is not incurred during remediation.

Considering just the loss processes of arsenic phytoextraction and leaching, we would expect greater loss of arsenic from the root zone compared to lower depth intervals. Indeed, we must consider why greater depletion of arsenic from the lower depth interval was observed under field conditions in Chapters 2 and 3, considering the greater root density in the 0-10 cm depth, as observed anecdotally in field studies here and by Liao et al. (2004). I hypothesized that, in addition to arsenic, DOC leaches from the root zone to exchange with and release even more arsenic from bulk soil. But would this not happen in the upper as well as lower depth interval, constituting a

second loss process in addition to fern uptake of arsenic, leading to greater arsenic depletion from surface depth intervals?

For net loss to be lower in surface soil depth intervals, there must be an input of arsenic to this depth interval. I proposed this input was leaching of arsenic from living and senescent foliage and litter back to the soil surface. Enrichment likely occurs through surface deposition of arsenic and subsequent arsenic sorption and/or leaching of deposited arsenic to enrich arsenic over smaller depth intervals (e.g. 0-3 cm) than measured here. Future work should investigate arsenic concentrations in the soil profile at fine resolutions during long-term phytoextraction.

My findings suggest phytoenrichment is particular to long-term growth of *P. vittata* with large aboveground biomass and/or high frond arsenic concentrations. Phytoenrichment was not observed in ferns growing in fine-textured soil, which had lower biomass and arsenic accumulation (Chapter 2), nor in the shorter-term mesocosm study (Chapter 3). In the mesocosm study, aboveground biomass was smaller than in field settings and the soil surface was covered, allowing quantification of arsenic leaching without phytoenrichment.

Another difference between the mesocosm study (Chapter 4) and the 1-year, high resolution field study (Chapter 3) is the balance of fern arsenic accumulation compared to leaching. In the field study, arsenic depletion was greater than fern arsenic accumulation, while the reverse was true under controlled conditions. Under field conditions, larger fern aboveground and, likely, root biomass could promote leaching through greater release of root exudates. Furthermore, under field conditions, field site flooding could increase arsenic leaching. Field site flooding is especially likely in Mediterranean and monsoon climates in sites with fine textured soils, as in Chapter 2 (fine textured soil 0-30 cm) and Chapter 3 (clay pan below 20 cm).

I also found the effects of mycorrhizal fungi inoculation varied with study, which speaks to the complex interactions of soil microbial communities with the *P. vittata*-soil arsenic cycle. In chapter 3, under field conditions, *F. mosseae* inoculation increased *P. vittata* aboveground biomass, slightly but not significantly increased the mass of arsenic accumulated in the fern, and significantly decreased remediation times. In contrast, in Chapter 4, under mesocosm conditions, *F. mosseae* inoculation did not affect biomass or arsenic accumulation in the fern, but did lead to greater arsenic depletion from soil. The discrepancy is likely related to the different stressors of field compared to soil column growth conditions on *P. vittata* and *F. mosseae*. Indeed, in Chapter 2, the possible role that mycorrhizal fungi played in phosphorus and nitrogen uptake, coupled with the positive effect of compost application on fern survival, points to the importance of soil microbial communities to supporting *P. vittata* growth in the presence of environmental stressors. Yet, due to the inherent heterogeneity in soil, plant, and climate processes, field-scale phytoextraction remains highly unpredictable and therefore outcome predictions are highly unreliable, even if stress impacts are mitigated through soil microbial community interactions.

Broader impacts

Beyond phytoextraction, my findings have important broader implications. My results highlight the importance of system-scale approaches to metal(loid) cycling that relate rhizosphere to bulk soil processes, consider losses, and investigate deeper soil horizons. In a novel approach, I linked field, meso-, and micro-scale investigations to elucidate such processes. In particular, my work shows the strengths of coupling synchrotron-based spectromicroscopy to larger-scale studies to elucidate mechanisms controlling system-scale elemental cycling. My findings advance our understanding of metal(loid) cycling in hyperaccumulator-influenced ecosystems, and provide

support for the phosphorus starvation theory as a framework for understanding why hyperaccumulation occurs.

My work also speaks to the challenges and opportunities of community-based science. First, in all my studies I found evidence that arsenic leaches during phytoextraction, which begs the question, did this research project contaminate the local environment, particularly groundwater? I think the answer is most likely no. Indeed, monitoring of groundwater at the Richmond Field Station field site (Chapter 3) indicated no arsenic in groundwater above actionable thresholds prior to, during, and after the study. At both field sites, arsenic mobilized in this study most likely sorbed to clay layers immediately below the soil depths investigated here.

Second, this dissertation research, specifically the 4-year field study, took place on a public property in a community where I lived for 20 years. Through this field study, I cultivated new interpersonal relationships and community-university partnerships. I experienced broader positive outcomes of research investment in urban agriculture, and leveraged these relationships to benefit the community and university even when the original dissertation goals, remediation of urban agricultural soil, failed. Furthermore, I want to acknowledge that conducting research in close proximity to people's homes will cause inconveniences as well as engender positive benefits.

Locally, this project led to university support of urban agriculture groups including Berkeley Community Gardening Collaborative and Urban Tilth. As an outgrowth of work at my first field site, I worked with one of my community collaborators, Corinne Haskins, Project Director, Berkeley Community Gardening Collaborative, to start an on-going environmental justice program for African-American and Latinx high school students, bringing students to the field site for paid, hands-on community science work. I also worked with my colleague Joshua Arnold and farm executive director Doria Robinson on the Urban Tilth soil building team, which enabled high school apprentices in a paid internship program to conduct their own soil sampling on a new 4-acre farm.

Advancing diversity, inclusion, and equity is the common thread to my work off and on campus. I prioritized experience with environmental justice as I selected over 60 research assistants, primarily students of color and women, to participate in my research. I designed and taught a 4-semester special field course, the Sustainable Soils Research Incubator, to mentor twelve students in 2 cohorts, with 75% from underrepresented minorities in STEM, along with first generation and LGBTQ students. This experience was particularly meaningful for me. While I had previously come out as transgender to academic peers on an individual basis, it was the first time I was out to a group of students, using my own story to facilitate discussions on our collective experiences, and how these motivate our work, as students and researchers from groups decentered in STEM.

I want to continue to build a future where we take seriously anti-oppressive and sustainable science, work with and not against soil biogeochemical processes, and put tools back in the hands of communities. Overall, I seek to support equitable and sustainable processes to generate and apply knowledge, to make life livable.

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