Rate of Phytoremediation and Distribution of Arsenic Within *Pteris vittata*

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ABSTRACT

Pteris vittata is a standard choice of conducting phytoremediation in the field with arsenic contaminated soil because it has been identified as an arsenic hyperaccumulator, which can extract, translocate, and tolerate arsenic. This field-based study investigated the rate of arsenic uptake in *Pteris vittata* fronds over one growing season and the distribution of arsenic within *P. vittata* fronds. *P. vittata* grew in arsenic-contaminated soils in the Richmond Field Station amended with lime. Fronds were tagged and dated when they emerged as fiddleheads, and harvested at 5, 10, 15, and 20 weeks of growth. Ground plant tissue samples were analyzed for arsenic concentration using Inductively Coupled Plasma-Optical Emission Spectrometry after digestion with nitric acid and hydrogen peroxide. Results showed that arsenic concentrations within *P. vittata* fronds peaked at five weeks after the fiddlehead emergence and decreased over time. Furthermore, the total As within fronds declined after week 10. For the second objective, results demonstrated that the highest As concentrations were found at the fronds base, but the total As was distributed similarly to all parts of the frond. Therefore, in order to optimize As phytoextraction by *P. vittata*, researchers should harvest fern fronds by ten weeks of growth. Finally, because arsenic sequestration in pinnae is not based on pinnae location or pinnae size, our results suggest other factors drive arsenic sequestration, possibly including As transport within the fern and/or As availability in soil.

KEYWORDS

Soil, Phytoremediation, Hyperaccumulation, Chinese Brake Fern

INTRODUCTION

With rapid urbanization and industrialization, soil contamination has become an increasingly prevalent problem all over the world. This is of great concern, as pollutants can easily enter the food chain if contaminated soils are used for food production and cause serious health issues for humans (Dudka *et al*., 1999). Arsenic (As) has gained great attention due to its natural abundance, a substantial increase due to anthropogenic activities, and toxicity to human beings. Anthropogenic activities that lead to soil arsenic contamination include mining, pesticides, and timber preservation, which can lead to serious contamination in food chains and water supplies (Frankenberger and Arshad, 2002). Arsenic contamination has become a world-wide problem affecting an estimated 100 million people globally through exposure to contaminated drinking water and foods (Uddin and Huda, 2011). Acute arsenic poisoning can cause nausea, abdominal pain and severe diarrhea and chronic arsenic toxicity result in multisystem disease (Ratnaike *et al*., 2003). Therefore, effective and economical methods of arsenic remediation are urgently needed.

Although physical and chemical approaches can be effective methods in cleaning arsenic from the contaminated site, researchers have been searching for alternative solutions to reduce negative consequences for the soil. Biological approaches, which involve the use of plants to decontaminate soil or water by inactivating metals in the rhizosphere or translocating them in the aerial parts, have started gaining popularity (Lone *et al*., 2008); this process is called phytoremediation. Phytoremediation has been suggested as an effective and low-cost method to clean up contaminated soils (Salt *et al*., 1998). In the case of arsenic contamination, *Pteris vittata* (Chinese brake fern) is a standard choice because it is an arsenic hyperaccumulator, which can accumulate up to 22,630 mg As/kg in the frond, and the ratio of shoot As concentration to soil As concentration can exceed 10 (Ma *et al*., 2001). This plant has a great capacity to extract, translocate, and tolerate As (Zhao *et al*., 2002), and it can be applied to both organic and inorganic pollutants present in the soil and water (McGrath and Zhao, 1998). Although this method is economical and eco-friendly, it is time-consuming and requires long-term maintenance of the remediation site. Further research needs to be conducted on how to implement a more efficient solution.

Due to the usual high concentration level of arsenic in the contaminated soil, it usually takes several harvests of *P. vittata* over several years to remove the target amount of arsenic from the soil depending on the initial As level in the soil (Tu *et al*., 2004). Therefore, a better

understanding of the optimal harvest time for fern fronds is important for effectively remediating arsenic. Nowadays, the most common harvest intervals for the fern fronds is every six months, as this is the time until full maturation of the fronds (Lessl *et al*., 2013). However, in 2011, other researchers suggested that fronds should grow until they reach their maximum arsenic accumulation capacity (Natarajan *et al*., 2009). These two experiments are both conducted in greenhouse pot experiments, which are easier to control and might not be representative of what happens in a real situation.

Furthermore, to optimize phytoremediation, a better understanding of the mechanism of the phytoextraction process of the plant is indispensable (Danh *et al*., 2014). One of the most important factors is to understand how arsenic distributes in the *P. vittata* fronds over time because it can help researchers determine and predict the potential peak arsenic uptake by the *P. vittata* (Salido *et al*., 2003). Moreover, an understanding of the arsenic distribution within the fern fronds can help researchers further their comprehension of the physiology and function of the *P. vittata* as an arsenic hyperaccumulator (Zhang *et al*., 2002). Previous studies have found conflicting results. Some researchers found that arsenic concentration was the highest in the apical pinnae apex and decreased progressively in pinnae at lower positions in the same frond and was higher at the edge in the single pinnae (Sakai *et al.,* 2010). However, another study showed that the analysis of the different pinnae along each frond midrib proved that the concentration of As was the highest in the basal pairs of pinnae (Lombi *et al*., 2002). If arsenic is stored preferentially in a certain part of the frond, with storage in other parts increasing over time, this could suggest that the pinnae receiving arsenic initially reach capacity, and that arsenic storage in the frond is driven by overall storage capacity.

The overarching goal of this study is to optimize the As phytoextraction by *P. vittata*. Specifically, this study will 1) Determine the rate of As uptake in *P. vittata* fronds over one growing season; 2) Investigate the distribution of As within *P. vittata* fronds.

METHODS

Site description

My fieldwork was conducted over a 24-week period in the Richmond Field Station (RFS), which is a part of the Berkeley Global Campus located in Richmond, California. It is located at 1301 S 46th St, Richmond, CA 94804 (37.9170° N, 122.3298° W). Researchers from UC Berkeley, led by Professor Céline Pallud and Ph.D. student Sarick Matzen, have been conducting a field-scale phytoremediation research project using *P. vittata* at the arsenic-contaminated field site there and initial testing found that the arsenic contamination of the soil is higher than 100 mg/kg. Arsenic is found in Richmond Field Station soil due to the dumping of chemical waste from a former chemical plant on the adjacent property. The field site had been split into 36 small research plots (1.5m× 1.5m) with twenty-five *P. vittata* planted in each plot after application of lime (3.6 kg/plot, the equivalent of 16 tons/ha). My research was conducted in the six control plots in the field site, which has no application of any soil treatment. The *P. vittata* used in this research were purchased from Edenspace Systems Corporation by the Pallud Lab at UC Berkeley approximately six months before this study, and they were conserved in the Oxford Tract Greenhouse before planting.

Sample Tagging

To observe the full growth cycle of the fern fronds, I initiated my research in the middle of June 2017, which is the time when new fiddleheads emerge from the root system most substantially. During the tagging process, I selected three ferns that have at least three emerging fiddleheads from each plot as my samples and tagged these fiddleheads with the date of selection. I tagged all the newly emerging fronds (2~5 cm) from a total of 30 ferns on July 30th, 2017. I then labeled these fronds with hand-written waterproof paper tags (Plot, Fern, Frond#, Date, Height) (Example: 12 D 01, 170730, 3.3cm). Finally, I used green flora wire to tie the tags on the emerging fronds, and I received a total of 42 fiddleheads as my samples. After tagging, I performed weekly observations on the selected fiddleheads to make sure that they are in healthy conditions.

Sample Harvesting

To analyze the time effects on the rate of phytoextraction and the distribution of accumulated arsenic within the fern fronds, four sampling sessions were conducted every five weeks. After five weeks of observation, the first sampling session was conducted on August 30th. A total of eight healthy fern fronds were selected from six plots, four for each objective. Fronds were selected based on being healthy with no insect or other damage, and at a similar growth stage at that timepoint. They were carefully harvested with clean scissors from the bottom without damaging the roots or fronds and were put into separate bags, one sample per bag. Three other sampling sessions were also conducted on Oct. 5th, Nov. 15th, and Dec. 18th using the same method. Out of the 42 tagged fronds, only 32 fronds were selected as samples for this research. These fronds were dried in the drying room (30 C) at the UC Berkeley Oxford Tract, at 1751 Walnut Street, Berkeley, California 94720.

Plant Tissue Grinding

After drying for three weeks, dried samples were moved to the Pallud Lab in Hilgard Hall at UC Berkeley for physical property measurements and chemical analysis preparation. First, dry weights for each sample were measured using the electric balance in the Pallud Lab. Then, the collected sample fronds were separated into two groups based on their different research objectives. For the first research objective, sixteen whole fronds were ground into plant tissue powders using a Wiley plant tissue mill (20 mesh screen) and stored into sixteen 50 ml plastic vials with clear sample ID labels. For the second research objective, I focused on the first three sampling timepoints (5, 10, and 15 weeks after fiddlehead emergence) to figure out the changes in the distributions of accumulated arsenic within the fronds over time. The top six pairs of pinnae on each frond were identified as the top part, middle three pairs as the middle part, and the bottom six pairs as the bottom part. Also, based on the previous research that the accumulated arsenic was mostly stored in the pinnae but not in the stipe part of the frond, so for the second objective, we only ground the pinnae for each part using an agate mortar and pestle and stored the tissue powder in 4810 ml glass scintillation vials.

Plant Tissue Digestion

For plant tissue powder from the first research objective, approximately 1 g powdered subsample per sample was shipped to the Brookside Laboratories in New Bremen, Ohio and digested using EPA method 3050b.

Due to limited sample mass (< 0.5 g), samples for the second research objective were digested in-house for chemical analysis. To prepare the solution for chemical analysis using the Inductively Coupled Plasma method, a wet chemical digestion process is needed to dissolve the plant tissue and move the mineral and remaining organic materials into solution. This step was conducted in the Room 218 in the Archaeological Research Facility (ARF) at UC Berkeley. For samples from the second research objective, I first weighed approximately 100 mg of plant tissue powder from each glass vial using the weighing paper and electric balance in the ARF and then poured each sample into a clean 50 ml Teflon microwave pressure vessel. To each vessel, 10 ml of re-distilled 70% nitric acid (Veritas GFS Chemical) was added using a 20 ml syringe, and 4 ml of 30% reagent grade hydrogen peroxide (Fisher) was added using a 10 ml pipette. For nitric acid, the portion to be used was transferred into a clean 100 ml beaker, and for hydrogen peroxide, the portion to be used was collected directly from a small polyethylene bottle covered with foil. All work with open chemicals was conducted in a fume hood. This digestion also included three replicates of plant tissue samples (with replicate samples selected based on enough sample mass), three known standard samples that had been previously analyzed by Brookside Laboratories and three blank vessels, which were used to check the validity of the results.

After at least 40 min of cold digestion, during which vessels were lightly covered with stopper/caps, the pressure was released from each vessel in the fume hood. Each vessel was then sealed tightly and placed in the Microwave Accelerated Reaction System (MARS) carousel according to the recommended pattern. The digestion took place according to the following method: Max Power 1200W, 75% power for 13-20 vessels or 100% power for >20 vessels, Ramp 15:00 min, Temperature 200 ℃, Hold 15:00 minutes. A four-minute cool down period occurred programmed as part of the method, and vessels were let to cool an additional 30 min to 1 hour until they were safe to handle (30-40 C). Letting vessels cool sufficiently has the advantage of allowing volatilized components to re-condense. After the vessels cooled down, they were removed from the MARS carousel and opened under the fume hood. Then I transferred the digestion solution in

each vessel into a clean 50 ml plastic vial. I rinsed each vessel using Milli-Q water three times and transferred rinse water into the plastic vials. Finally, I sealed these vials and took them back to the Pallud Lab. To make each digestion solution to volume, under a fume hood, I quantitatively transferred the solution into a 100 ml volumetric flask and then added Milli-Q water to make the total volume up to exactly 100 ml and inverted the flask twenty times to make sure that the solution was fully mixed, resulting in an approximately 2% nitric acid solution. I then transferred 10 ml of the well-mixed diluted solution to a 10 ml syringe with a filter attached (0.45 μm), which I filtered into a 15 ml polyethylene tube for ICP analysis.

Arsenic Concentration Analysis with Inductively Coupled Plasma-Optical Emission Spectroscopy

For plant tissue powder from the first research objective, samples were analyzed for arsenic concentrations at Brookside Laboratories in New Bremen, Ohio using EPA method 6010. For the second objective, samples were analyzed for arsenic content using a Perkin Elmer ICP-OES with the help of Sarick Matzen. Samples prepared for ICP analysis were placed in the ICP autosampler, along with solutions representing a 5-point standard curve (also prepared with 2% nitric acid). During the arsenic concentration analysis with ICP-OES, sample counts were collected between 5 to 20 seconds with a delay time of 60 seconds and three replicate measurements per sample. The purge gas flow was high for 15 minutes before it ran back to normal and the sample flow rate was at 1 ml per minute with 5 seconds flush time. The sample rod was rinsed between samples in 2% nitric acid for 120 seconds. Arsenic counts were compared to scandium used as an internal standard. Each sample took approximately 5 minutes to run. Check standards and blanks were run every ten samples. Arsenic counts were converted into digest concentrations using the standard curve ($R^2 = 1$ and 0.9999 for the two ICP runs), and digest concentrations were converted into plant tissue concentrations by multiplying by the digest volume (100 ml) and dividing by the precise sample mass (about 0.1000 g). Replicates were within 0.2 to 8%, and the known plant tissue sample was 7 - 17% lower than values reported by Brookside. The total arsenic content of each frond (for the first objective) or each set of pinnae (for the second objective) was calculated by multiplying the plant tissue concentration by the respective biomass.

Statistical analyses

Statistically significant differences in arsenic concentration by biomass and total arsenic (both over time) were measured using single- or multi-factor Analysis of Variance (ANOVA) conducted on linear models estimating effects of time since fiddlehead emergence, arsenic location within the fern, and individual fern on 1) arsenic concentrations (mg/kg), 2) biomass (g) and 3) total arsenic (mg) contained in the ferns, as appropriate for each objective. Individual fern was not shown to affect results and was not included in subsequent analyses. Independent factors including time for objective 1 and time and location for objective 2. The dependent continuous variable is arsenic concentration, biomass, or total arsenic. Effects were considered significant at $P < 0.05$. Analyses were conducted in R 3.3.2 GUI 1.68 (Mavericks build) statistical computing package used with RStudio (Version 1.0.136).

RESULTS

The Rate of arsenic uptake over time and the optimal harvesting interval

The As concentrations within the fern fronds in the samples for this study decreased over time (Figure 1). The concentration reached its peak value at 2320 mg/kg after five weeks of growth since the fiddleheads emerged. Then, it decreased to 2018 mg/kg, 1243 mg/kg, and 779 mg/kg after 10, 15, and 20 weeks of growth. In an analysis of variance conducted on a linear model testing the effects of time on the As concentration, time significantly affects As concentration ($P=0.043$).

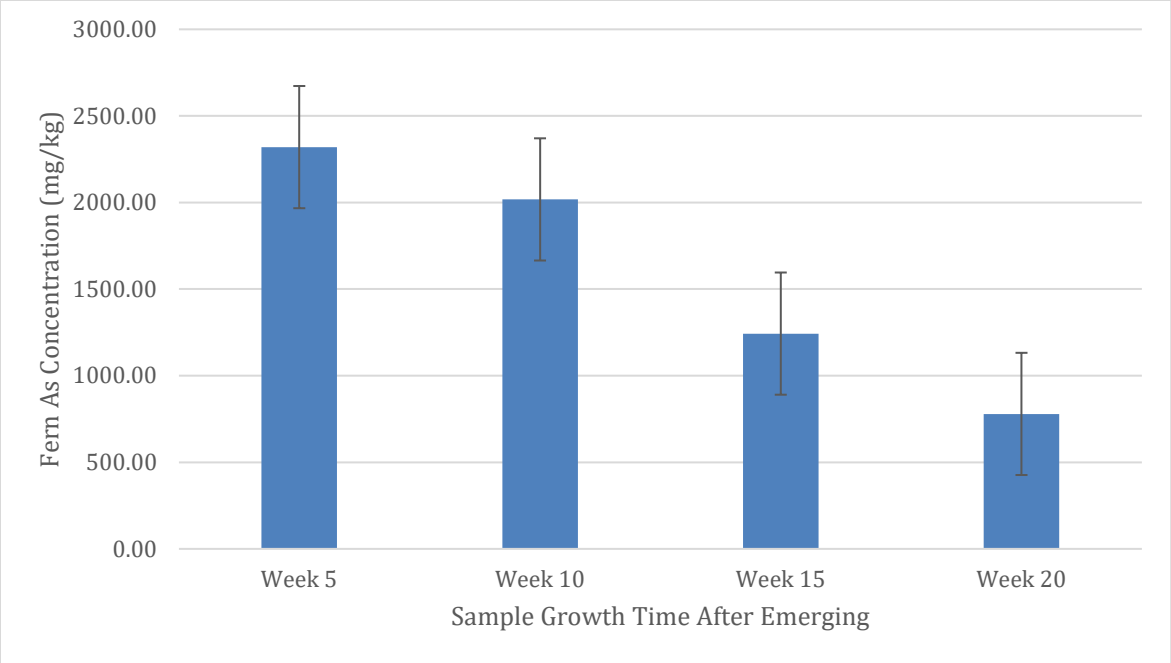

Figure 1. Fern As concentration over time. I used four datasets from Week 5 to Week 20 to determine the trend in As concentration within *P. vittata* fronds over time.

For phytoremediation, we not only care about the As concentration but also the total amount of As accumulated. The dry biomass data of fronds in these four time points show that the biomass increases slightly but not significantly over time (P= 0.404).

After multiplying the As concentration to the biomass, we received the total As in each sample frond (Figure 3). The result shows that the total As within fern fronds is the highest at five and ten weeks after fiddlehead emergence and started to decrease after week 10. In an analysis of variance, conducted on a linear model testing the effects of time on total As, time significantly affect total As content (P=0.034).

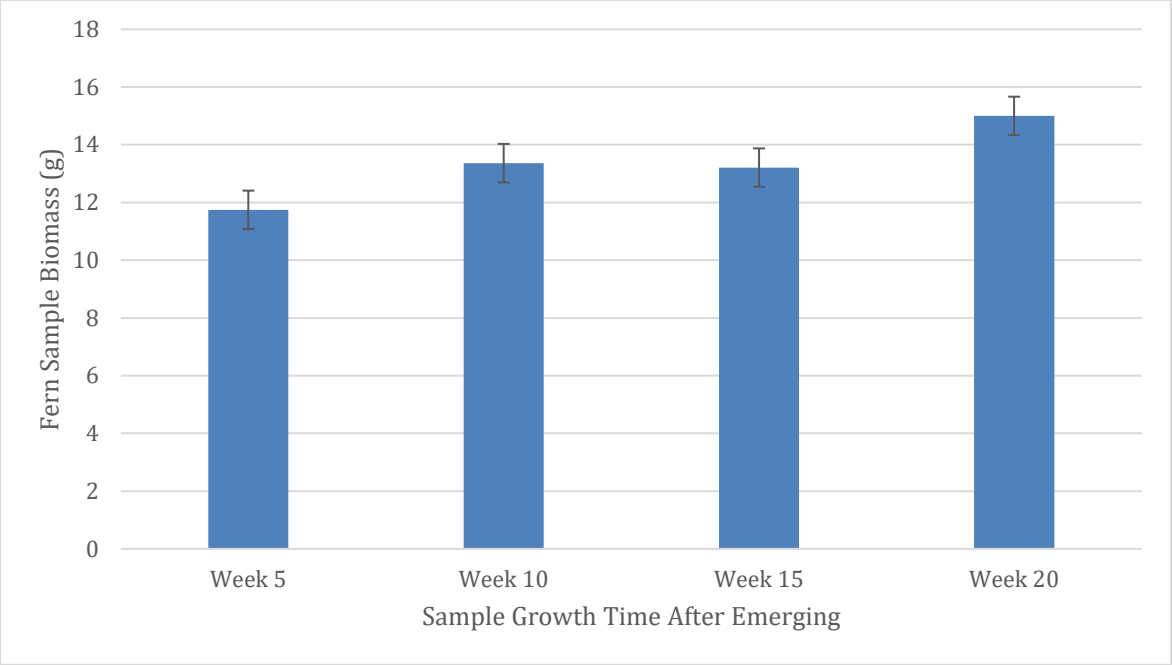

Figure 2. Fern Biomass over time. I used four datasets from Week 5 to Week 20 to demonstrate the trend in biomass of *P. vittata* fronds over time.

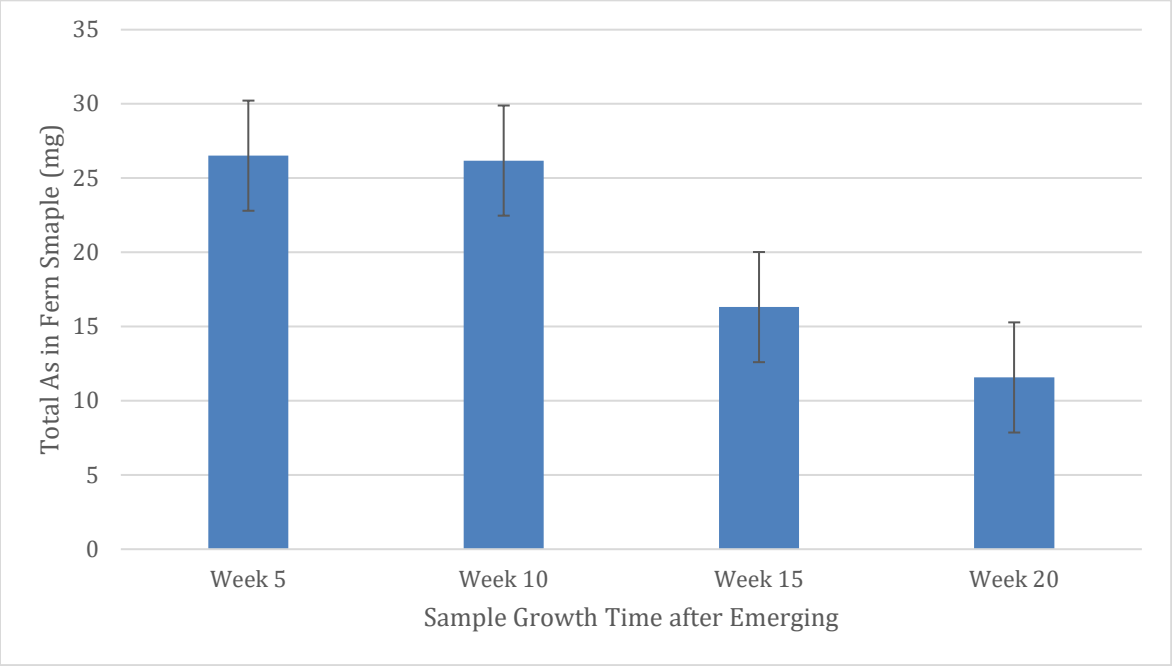

Figure 3. Total As in fern samples over time. I used four datasets from Week 5 to Week 20 to determine the trend in the total amount of As within *P. vittata* fronds over time.

The distribution of arsenic within *P. vittata* fronds and the movement of arsenic accumulated in the *P. vittata* over time

The As concentrations within the fern fronds are highest at the frond base (Figure 4). The As concentration was always the highest at the bottom parts and lowest at the top part over the 15 weeks of growth time. In an analysis of variance, conducted on a linear model testing the effects of time and location within the frond on As concentration, both time ($P=1.13\times 10^{-5}$) and location (P=0.00131) significantly affect As concentration.

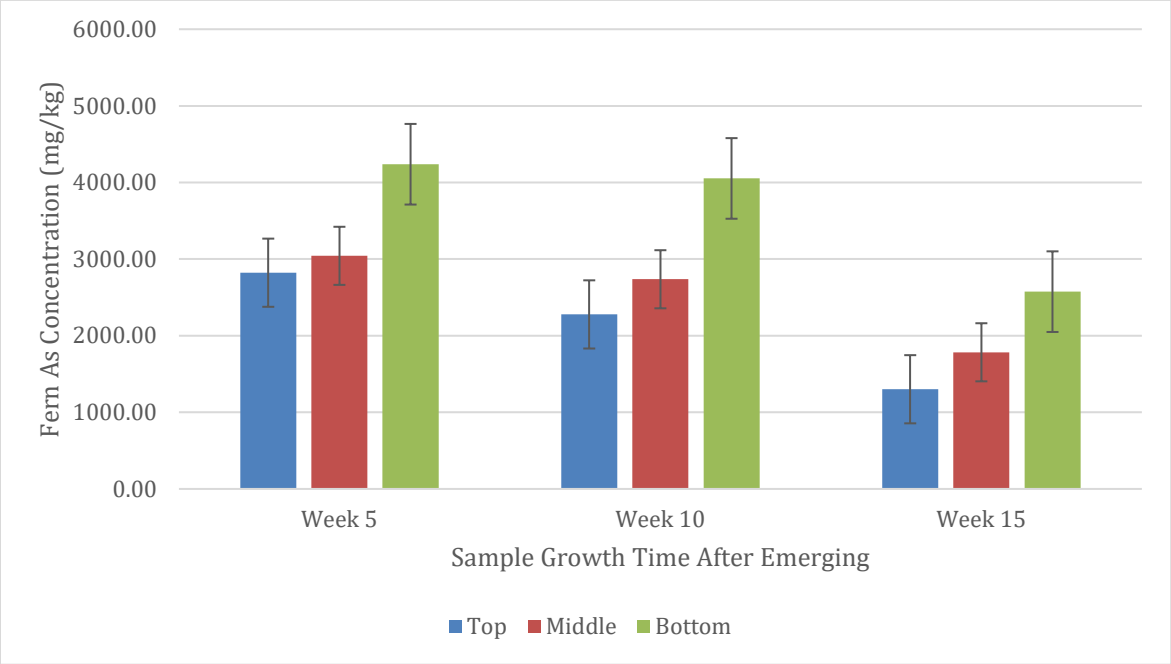

Figure 4. As concentration distribution within fern fronds over time. I used three datasets from Week 5 to Week 15 to determine the changes in As concentration distribution within *P. vittata* fronds over time. The blue column represents the top six pairs of pinnae, red column represents the middle three pairs of pinnae, and the green column represents the bottom six pairs of pinnae of each frond.

Then, after multiplying the As concentration to the biomass, we received the total As in each part of the frond (Figure 5). In an analysis of variance, conducted on a linear model testing the effects of time and location on total As concentration, the time (P=0.000503) significantly affects total As content. However, the location (P=0.714) does not significantly affect total As content. The As was distributed similarly to all parts of the frond at each time point analyzed, and concentrations were lower in the top 6 pinnae because pinnae are larger at the top of the frond, diluting concentrations.

Figure 5. Pinnae Sample Biomass over time. I used three datasets from Week 5 to Week 15 to demonstrate the trend in biomass of the selected *P. vittata* pinnae over time. The blue column represents the top six pairs of pinnae, red column represents the middle three pairs of pinnae, and the green column represents the bottom six pairs of pinnae of each frond.

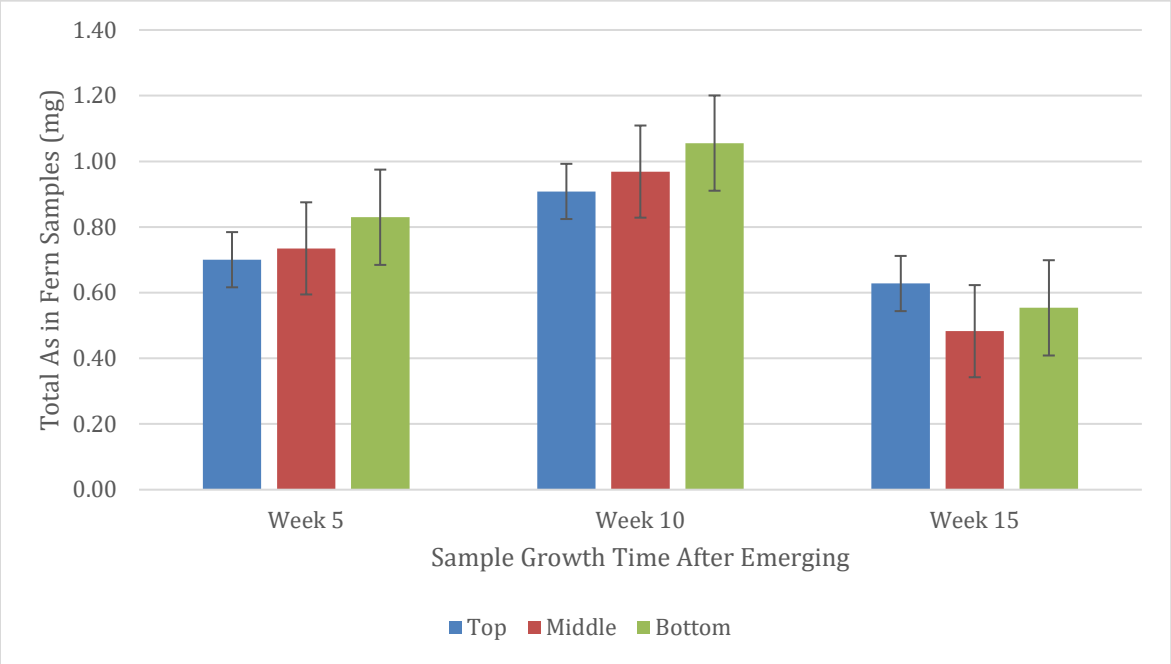

Figure 6. Total As distribution within fern fronds over time. I used three datasets from Week 5 to Week 15 to determine the changes in how did the total amount of As distribute within *P. vittata* fronds over time. The blue column represents the top six pairs of pinnae, red column represents the middle three pairs of pinnae, and the green column represents the bottom six pairs of pinnae of each frond.

DISCUSSION

My results showed that *P. vittata* was able to hyperaccumulate As from contaminated soils. The concentration levels of As within the *P. vittata* fronds decreased over time since week five after fiddlehead emergence, and the total amount of As within fronds decreased after week 10. Therefore, the optimal harvesting interval to remove soil arsenic contamination most effectively is by five to ten weeks of growth. Moreover, accumulated arsenic was primarily stored at the bottom part of the *P. vittata* fronds consistently across all three timepoints (Lombi *et al*., 2002). However, if we take into consideration the total amount of As within the fronds, we concluded that As was distributed to all parts of the frond similarly and As storage is based not on pinnae location or size, but be controlled first by other factors (Zhao *et al*., 2002).

Changes in the As level within the frond over time

My results suggest that the most efficient fern fronds harvesting interval to remove arsenic from contaminated soil by *P. vittata* is five to ten weeks after the fiddlehead emergence. Because it shows that the As concentrations within fronds decrease over time since week five, with total As within fronds not increasing after week five and declining after week ten. Previous studies have shown different conclusions on this topic in which some have mentioned that the optimal harvesting interval is much longer than my results suggest, every six months, which is also the most common harvest intervals for the fern fronds (Lessl *et al*., 2013). In 2009, another researcher suggested that we should let the fern grow until they reach their maximum arsenic accumulation capacity, which is >12 weeks of growth (Natarajan *et al*., 2011). These two experiments are both conducted in the greenhouse pot experiment conditions, which are easier to control and might not be representative of what happens in a field situation. Different from what I originally expected, the As concentration and the total amount of As within the fronts did not increase over time as the fronds reach full maturation. My hypothesis to this unexpected result is that the accumulated arsenic within the fronds went through a re-translocation from older fronds to the newly emerging fronds at a certain level. Although previous study has not shown any evidence on As re-translocation within *P. vittata*, in 1996, Mimura *et al*. have found some occasions that inorganic phosphate was re-translocated from the older barley leaves to the young leaves. In the case of

arsenic, a good chemical analog of phosphate is As(V), but evidence suggests that As is stored in fronds as As(III) (Wang *et al*., 2002). It is also possible that while young and maturing (pre-spore producing) fronds tolerate higher amounts of As, the production of spores (which begins on average at 14.5 weeks of growth (Benavides Quesada, 2015) lowers tolerance for As and causes As to be relocated to other, younger fronds. Finally, As could be effluxed from vacuoles through the epidermis and leached out of pinnae through accumulated moisture. Even though ferns in this field study were watered by drip irrigation and not by overhead watering, leaf surfaces could have accumulated moisture from the high relative humidity in the hoop house (up to 100%).

Distribution of accumulated arsenic within fronds

My results show that the highest concentration of accumulated arsenic occurs at the basal pairs of the fronds over time. However, when we compared the total amount of As within fronds, the difference in total As at different locations within the frond was not significant, showing the total amount of As was distributed similarly to all parts of the frond over time. Previous studies have found conflicting results on this topic. Some researchers found that arsenic concentration was highest in the apical pinnae apex and decreased progressively in pinnae at lower positions in the same frond (Sakai *et al*., 2010). However, another study showed that the analysis of the different pinnae along each frond midrib proved that the concentration of As was highest in the basal pairs of pinnae (Lombi *et al*., 2002), similarly to my results. However, neither previous study considered the total arsenic in addition to the concentration. My hypothesis to explain my finding that arsenic is distributed equally throughout the frond is that the As storage is based not on pinnae location or pinnae size, but could be controlled first by other factors including As transport in the xylem, As availability in soil, and/or uptake into the root. If As were stored first, for example, in bottom pinnae, with As increasing over time in middle and upper parts of the frond, we could identify an upper threshold for As sequestration in pinnae. In this case, with As allocated equally to all parts of the frond, arsenic sequestration in the frond could still approach capacity on a whole-frond basis. Capacity could decrease over time, since total As does not increase after Week 5 and decreases by Week 15. Additionally, factors outside the frond, like transport with in the fern and As availability in the soil, could control the increase in As content from fiddlehead emergence up to Week 5 and

then the plateau to Week 10, while factors inside the frond (for example, storage capacity and toxicity) could cause the decrease in As content after Week 10.

Effects of sample harvesting interval on fern fronds biomass

In this study, four sampling sessions were conducted in every five weeks to receive enough samples to study the time effects of the arsenic phytoextraction by *P. vittata*. My hypothesis is that the frond harvesting has significant effects on the growth of other fronds, which might hinder the increase in biomass of other fronds and leads to the result shown in Figure 2 that the biomass increases slightly but not significantly over time. Former ES student Benavides Quesada has demonstrated in her senior thesis that harvesting too often can cause detrimental effects to the fern and there is a statistically significant difference in the fern biomass between harvesting every 12 weeks and harvesting every 26 weeks (Benavides Quesada, 2015). Previous studies have also shown this hindering effect of harvesting. In 2013, Lessl and Ma studied the effects of phosphate rock on the growth of *P. vittata* and showed that smaller harvesting interval can decrease the effects of photosynthesis within the fern, which leads to the decrease in the whole fern. In 2008, Gonzaga *et al*. found a significant reduction in the frond biomass from the second harvest due to the drastic used in the first harvest, which made it difficult for plants to regenerate.

Limitations and Future Directions

This study could benefit from a stronger experimental design including random selection of tagged fronds since selecting only similarly-aged fronds to sample could have led to a sampling bias. Additionally, control of ferns planted in non-contaminated soil would have been useful for understanding As uptake in the contaminated soil. Future analyses should consider the effects of soil As concentrations within each plot on As uptake. Moreover, the fieldwork of this study is conducted from August 2017 to December 2017 at Richmond, California with Mediterranean climate, which is characterized by rainy winters and dry to hot summers. Therefore, the climate can be an important factor that leads to the similar frond biomass over time as the fronds grow much faster during summer than in winter. Furthermore, my research is conducted based on the performance of individual fronds. However, in a real-world situation, it is hard for farmers to keep

tracking each frond during phytoremediation. Therefore, future researchers should conduct research based on the effects of the whole fern over time or formulate a useful model which can predict the effects of the fern based on the performance of the fronds. Finally, in the previous section, I have mentioned that accumulated arsenic within *P. vittata* fronds might experience a re-translocation from older fronds to younger fronds as what happened to inorganic phosphate in barley leaves. The study of re-translocation of arsenic within *P. vittata* fronds can be a promising topic as this is important for researchers to better understand the mechanism of arsenic phytoextraction by *P. vittata*.

Broader Implications and Conclusion

My results show that to optimize As phytoextraction, we should harvest fronds by ten weeks of growth. Moreover, the highest As concentrations are found at the frond base, but As is distributed similarly to all parts of the frond. This represents that the As storage is based not on pinnae location or pinnae size, but on the As transport in the xylem. This study is important because nowadays, the usual harvesting interval is every six months, which is the period when the fern starts its fiddlehead emergence until the end of it. However, my study shows that this is not the most effective interval to remove soil arsenic contamination. This study has also proven that arsenic is distributed equally to all parts of the frond, so future work must consider other drivers of arsenic uptake and sequestration.

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