

**THE ROLE OF IRON PLAQUES IN IMMOBILIZING ARSENIC  
IN THE RICE-ROOT ENVIRONMENT**

by

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## ABSTRACT

Geogenic arsenic (As) causes illness to millions of people in South and Southeast Asia via the consumption of rice grown in contaminated water. Arsenic is absorbed by rice roots, which is transported through the shoots and delivered to the edible grains. Researchers have found that iron (Fe) plaques, consisting mainly of amorphous iron oxides, grow on the surfaces of the rice roots. Iron plaques sorb As, immobilizing the toxin, preventing it from absorption into the plant body. Observational studies have been conducted on the occurrences and locations of iron plaques, but their characterization and association with As are only beginning to be studied. The goal of this study is to elucidate the sorption mechanism and capacity of As to sorb to the Fe-oxide, as well as to determine whether variable plaque formation is due to variable O<sub>2</sub> exudation. We conducted both synchrotron-based bulk and micro-XANES and EXAFS studies, as well as bench-top extractions. We set up two Fe-treatments to investigate whether increased Fe concentration promotes increased plaque growth, immobilizing more As. The primary oxidation state of As found in plaques was As(V). The molar ratio of sorbed Fe:As did not significantly depend on the Fe-concentration. This signifies a specific molecular ordering of the plaque—more plaques sorb more As, but not at an increasing rate. Plants can immobilize 0.86-0.93 mg of As per gram of dried root, and approximately 10% of the Fe-oxide plaque itself consists of As. Variable plaque formation is due to O<sub>2</sub> exudation patterns. Knowing the potential amount of As sorption to Fe-plaques supports the farming practice of composting rice straw—a low-cost and natural way of adding iron to paddy soil to promote the growth of Fe-plaques on rice roots.

## Chapter 1

### BACKGROUND AND RATIONALE

#### 1.1 Arsenic in the Environment

The contamination of groundwater in South and Southeast Asia by arsenic (As) is the largest human poisoning event in human history endangering the lives of over 150 million people (Smith et al, 2000; Nriagu, 2002). Though geogenic As exists throughout the Earth's crust, populations in Bangladesh and the West Bengal Valley of India are exposed to As through the consumption of well-water as well as rice irrigated with As-contaminated water (Nordstrom, 2002; Gordon et al, 2004; Roberts et al, 2007). Though As has also been introduced to the environment anthropogenically, research must be focused on understanding the cycling of geogenic As. As in the food chain affects millions of impoverished people whose survival depends on farming and clean water (Bissen and Frimmel, 2003).

To understand the toxicity and mobility of arsenic, it is paramount to understand the speciation (form) of As, as well as its sorption mechanism to its sorbent (Cullen and Reimer, 1989). Arsenic is found mostly in two inorganic forms in soils and water (Bissen and Frimmel, 2003). The more toxic form, arsenite, hereafter referred to as As(III), occurs as  $\text{As(OH)}_3$ , and is more mobile in the environment because of its neutral charge over most of the pH range. The less toxic and less mobile arsenate, referred to as As(V), occurs as  $\text{H}_2\text{AsO}_4^-$  or  $\text{HAsO}_4^{2-}$  (Cullen and Reimer, 1989). Over most pH ranges found in nature, As(V) is negatively charged and thus sorbs to positively-charged oxide minerals, making it less labile than As(III).



As(III) is believed to be more toxic than As(V) to humans and other organisms as it binds to essential enzymes, preventing them from functioning (WHO, Environmental Health Criteria, 2001). As(V), however, is toxic nonetheless because it decreases the production of adenosine triphosphate (ATP) by replacing phosphate (National Research Council (NRC), 2001).

Human exposure to geogenic As is a result of many biogeochemical and abiotic redox steps (Cullen and Reimer, 1989). In the Himalayas, As originates in sulfide minerals. Through weathering and exposure to atmospheric oxygen, the minerals oxidize and transform to secondary minerals, where As sorbs to smaller iron (Fe)-oxide sediments (Fendorf et al, 2010). Once As-containing Fe-oxides reach ground level, As enters the groundwater through the sub-surface microbial reduction of Fe(III)-oxides to ionic Fe(II), followed by the microbial reduction of As(V) to the more mobile As(III), freeing As to flow in groundwater (Islam et al, 2004; Tufano et al, 2008). For microbial metabolism (and thereby reduction) to occur, the system must be anaerobic. These environments prevail when decomposing organic carbon's demand for oxygen is more than the rate of infused oxygen (Fendorf et al, 2010). Thus, conditions that increase the release of As to groundwater exist in anaerobic saturated soils containing sufficient amounts of dissolved organic carbon (DOC) from decaying plant matter (Fendorf et al, 2010).

Globally, total As concentrations in surface soils vary from the ppm to ppt (trillion) range (Cullen and Reimer, 1989). In Southeast Asia, As speciation and concentrations are extremely varied even locally, most likely due to the range in size of groundwater recharge systems—from tens of meters to hundreds of kilometers (Fendorf et al, 2010). Arsenic speciation also changes as groundwater distribution

flows one direction and is reversed yearly due to the submergence and drainage of paddy soils (Fendorf et al, 2010). Aside from areas proximal to rice roots which release oxygen (discussed in following chapter), drained/aerated soils mostly contain As(V) and irrigated/submerged soils mostly contain As(III) (Cullen and Reimer, 1989; Seyfferth et al, 2010).

The temporal and spatial complexities of the Southeast Asian conditions and the toxicity of As described above emphasize the importance of studying As and its pathways to human exposure. Arsenic severely affects human health both through drinking water and food consumption in this part of the world, and more research is necessary to reduce illness of the poor.

## **1.2 Arsenic Toxicity to Humans**

Arsenic enters the human body through the consumption of contaminated water and food. Arsenic poisoning—arsenicosis—causes skin, lung, bladder, and kidney cancer, as well as the commonly observed skin thickening and pigmentation (Gordon et al, 2004). Chronic exposure causing these conditions means diagnoses will increase as populations continue to ingest As-contaminated food and water (Smith et al, 2000).

It is estimated that arsenicosis will kill 200,000 to 270,000 people in Bangladesh alone (Gordon et al, 2004). While the World Health Organization's guideline for As in drinking water is set at 10 µg/L, Bangladesh's maximum allowable limit is 50 µg/L, which was also the United States' previous limit (Smith et al, 2000). In some areas of Bangladesh, more than 75% of tube-wells contain water above 50 µg/L of As (Gordon et al, 2004).

In addition to drinking contaminated water, Southeast Asians are particularly at risk for arsenicosis because inhabitants of the region depend on wetland rice, which is irrigated with As-contaminated ground water (Panaullah et al, 2009). Rice is a relatively cheap staple— it comprises over 70% of the daily caloric value of most people (Bangladeshi Rice Foundation, 2011). Rice is irrigated with contaminated groundwater from the same sources of the tube-wells used for drinking during the dry season (Williams et al, 2006). However, the average As concentration of irrigation water in Bangladesh is  $397 \pm 7 \mu\text{g/L}$  (Roberts et al, 2007). This value is believed to be much higher than the amount of As in drinking water because As is retained by the soils year after year through sedimentation in paddy systems (Panaullah et al, 2009).

Arsenic is taken up into rice plants through the roots, where it is transported to the edible grains. In Bangladesh, the amount of arsenic that actually reaches the grain is disputed amongst researchers. While some studies report figures between 0.21-0.27  $\mu\text{g}$  of As per gram of dry weight (Williams et al, 2006), Zavala and Duxbury (2008) estimate the average value across publications to be 0.152 mg of As per kg of grain (mg/kg equivalent to  $\mu\text{g/g}$ ). The equivalent amount of contaminated rice that could be safely consumed according to the WHO's guideline for safe limits of drinking water (10  $\mu\text{g/L}$ ) is consuming rice with As levels up to .08  $\mu\text{g/g}$  (Williams et al, 2006). The average daily intake of As from rice for a Bangladeshi adult is approximately 100  $\mu\text{g}$  As (400 g dry weight  $\times$  0.25 mg As/kg), which is five times the 20  $\mu\text{g}$  As intake from consumption of two liters of water at the WHO limit of 10  $\mu\text{g/L}$  (Panaullah et al, 2009).

In addition, the majority of As found in water and the grains is inorganic As, which is far more toxic than methylated forms (Williams et al, 2006). Clearly, current As levels in rice grains are far above the allowable and safe amount, and thus remediation is of paramount concern.

The severity of arsenicosis reaching across Southeast Asia has worsened over the last 30 years. Though tube-wells were dug beginning in the 1940's, it was not until the 1980's when the United Nation's Children Fund (UNICEF) sponsored hundreds of thousands of wells to avoid the microbial pathogens appearing in surface waters (Smith et al, 2000; Fendorf et al, 2010). Preliminary tests did not screen for arsenic, as its threat was not known, and thus drinking from tube-wells was believed to be safe (Smith et al, 2000).

The threat of arsenicosis is severe for the poorest populations who do not have access to clean water. Research must be conducted to reduce the amount of As that travels through the biogeochemical and biological pathway to enter the human body.

### **1.3 Chemistry and Biochemistry of Paddy-soil Systems**

In Southeast Asia, rice is grown during the Aman (monsoon/rainy) season and the Boro (dry) season. Aman rice grows using rainwater, whereas Boro rice, which was developed during the 1970's to reduce widespread famine, uses groundwater for irrigation (Panaullah et al, 2009). Though the development of Boro rice has helped Bangladesh reduce its dependence on other countries for food, there are compounding consequences as a result of using As-contaminated groundwater for irrigation.

The overall Fe and As chemistries in the field are generally mandated by the water's exposure to oxygen and distance from the well (Dittmar et al, 2007; Roberts et al 2007; Panaullah et al, 2009). Arsenic concentrations in paddy soils generally decrease with distance from the irrigation well (Panaullah et al, 2009). As groundwater is exposed to the surface and travels along irrigation channels, it is equilibrated with atmospheric O<sub>2</sub>, causing Fe<sup>+2</sup> to oxidize and precipitate as amorphous Fe(OH)<sub>3</sub> (Panaullah et al, 2009; Fendorf et al, 2010). Arsenic and phosphorus concomitantly adsorb to and co-precipitate with these iron minerals, and become suspended in the soil sediment (Cornell and Schwertmann, 2007; Reddy and DeLaune, 2008). This adsorption is beneficial from the human perspective, as As compounds become temporarily unavailable to irrigation water.

However, reduction occurs under the soil-water interface, where Fe oxides are progressively reduced under submergence (Panaullah et al, 2009; Fendorf et al, 2010). This releases As to the pore-water, making it available for plant uptake. Thus As in pore water increases as the rice plant ages due to the increasing depth of the sediment as time passes (Panaullah et al, 2009). Though the As in pore-water is primarily As(V), the ratio of the more toxic As(III) increases as the rice plants mature (Panaullah et al, 2009).

Another complication to the system is the aerobic environment immediately around the rice roots, caused by the release of O<sub>2</sub> through aerenchyma channels (Kirk, 2004; Hu et al, 2005; Dittmar et al, 2007). Aerenchyma is a tissue in wetland plant roots that contain air-filled intercellular spaces that deliver O<sub>2</sub> to submerged root surfaces (Kludze et al, 1993). O<sub>2</sub> is exuded radially outwards to the root surface through diffusion, and downward to the root tip through pressure (Hodge

et al, 2009). Rhizospheres need O<sub>2</sub> to maintain aerobic metabolism of the roots and microbes, and to oxidize potentially toxic substances such as Fe<sup>+2</sup>, Mn<sup>+2</sup>, and H<sub>2</sub>S (Kludze et al, 1983; Amstrong and Armstrong, 1988). The oxidized environment in the rhizosphere promotes the growth of Fe(III)-oxides surrounding the root, forming a plaque that encapsulates portions of the root (Reddy and DeLaune, 2008). Arsenic, as well as phosphorus, have high affinities for these oxidized Fe-oxides, and as in soil sediments, As is withheld and immobilized by these Fe(III)-oxides (Meharg, 2004; Hu et al, 2005; Seyfferth et al, 2010).

Even withstanding Fe plaques, As is still transported into rice roots. The method of transportation into the root depends on the speciation of the As. As(V) is transported into the root through an arsenate-phosphate-transporter—a transporter that can transmit both As and phosphorous (Meharg, 2004; Seyfferth et al, 2010). As(III) is transported through an aquaporin transporter in the root plasma membrane, which also transports other small neutral molecules such as silicic acid (H<sub>4</sub>SiO<sub>4</sub>) (Hossain et al, 2009; Seyfferth et al, 2010).

Understanding the sorption mechanism of As on the Fe-oxide plaque will demonstrate the fragility and reversibility of the complex. Either way, the sorption mechanism will better explain the fate of As on the plaques. Investigating the chemical environment of As will also allude to its speciation, which is important in understanding its toxicity.

The sorption mechanism of As to Fe oxides in soils is widely published (Waychunas et al, 1993; Fendorf et al, 1997; Manning et al, 1998; Langer and Inskeep, 2000; Goldberg and Johnston, 2001; Cornell and Schwertmann, 2007), but only a few studies show the localization or mechanisms of As and Fe oxides of

plaques on root surfaces (Blute et al, 2004; Seyfferth et al, 2010). Arsenic retention on Fe-oxides may differ in a biological setting (compared to an abiotic soil-only system) because of constant O<sub>2</sub> exudation, among other factors. Though various sorption parameters such as the sorption maximum pH, PZC on Fe-oxides, inner- vs. outer-sphere complex, mono-vs. bi-dentate forming tendencies for As(III) and As(V) have been identified on paddy and rhizosphere soil using Fe  $\mu$ -XANES, none has been conducted on Fe-root plaque (Takahashi et al, 2004; Voegelin et al, 2007; Yamaguchi et al, 2011).

The goal of this study is to promote Fe plaque formation by adding Fe to the system in order to reduce As uptake into the root. If sufficient plaque encapsulates the root, less As will come into contact with the transport mechanisms, minimizing As transported into the root body.

Plaque formation along the length of the root is variable, yet aerenchyma grows internally down the entire length (Xu et al, 2008; Mei et al, 2009; Seyfferth et al, 2010). Studies have stated that O<sub>2</sub> exudation from aerenchyma is the main biotic factor determining plaque formation (Mendelsohn et al, 1995; Wu et al, 2011). This study seeks to understand the disparity between both the role and presence of aerenchyma and the variable visible Fe-oxides along the root surface.

#### **1.4 Research Objectives**

This study contributes to the study of As mobility, transportation, and speciation in paddy soils as it relates to human health in Southeast Asia. The focus of this study is the formation and characterization of Fe-plaques along rice roots and their relationship to As retention. The research employs two types of techniques to gain knowledge of the system from opposite ends of the spatial scale. Synchrotron-based

techniques will be used to gain co-localization data of As and Fe, the speciation of As, and the sorption mechanism, by collecting  $\mu$ -XANES and  $\mu$ -EXAFS spectra.

Sequential digestions in the laboratory will be used to elucidate trends in the amounts and locations of both Fe and As by analyzing many replicates. The specific research objectives of this research are as follows:

- Examine the chemical environment of Fe and As on plaques as a function of Fe concentration in nutrient solution.
- Investigate the role of Fe concentration in nutrient solution on the degree of formation of plaques.
- Characterize the differences in the plaque's variable formation across the entire length of the rice root of roots grown in different Fe treatments.

No study thus far combines molecular-based synchrotron techniques to understand arsenic sorption mechanisms on root plaques with macroscopic investigations on statistical quantitative and visual degree of plaque formation.  $\mu$ -XANES and  $\mu$ -EXAFS tools will be used to address the first objective, whereas bulk digests and methylene blue staining of  $O_2$  exudation will address the second and third objectives. Overall, this study provides understanding on the permanence of the As binding on Fe plaques via its sorption mechanism. The comparison of As concentrations in the plants grown in low and high Fe treatments will help to understand the possibility of plaque-growth enhancement via fertigation. This will aid remediation efforts, since less As is taken up into the plant body when Fe plaques are present on root surfaces (Hossain et al, 2009). Additionally, this is the first study to visualize oxygen exudation and relate it to plaque formation.

Though both synchrotron studies and digestion studies have been separately conducted on this Fe-As system in rice paddies, I will tie both methods



together by studying plants grown in the same field-based concentrations to gather a better picture of the natural system that is poisoning millions of people. By pairing these studies, this research will lend valuable microscopic knowledge of localization and mechanisms to scientists applying new knowledge to the rice paddy system on a larger scale in order to remediate As toxicity in humans.

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## Chapter 2

### FORMATION OF IRON PLAQUES AND ARSENIC SORPTION ON RICE ROOTS (ORYZA SATIVA)

#### 2.1 Abstract

Geogenic arsenic (As) causes illness to millions of people in South and Southeast Asia via the consumption of rice grown in contaminated water. Arsenic is absorbed by rice roots, which is transported through the shoots and delivered to the edible grains. Researchers have found that iron (Fe)-oxide plaques grow on the surfaces of the rice roots, which have the potential to sorb As, essentially immobilizing the toxin in the rhizosphere, and preventing its absorption into the plant body. Observational studies have been conducted on Fe-plaques, but their characterization and association with As are only beginning to be studied. The goal of this study is to elucidate the speciation and localization of As on root plaques formed under different initial Fe concentrations, conducting both macroscopic wet-chemical studies and synchrotron-based spectromicroscopic studies including X-ray absorption near-edge structure ( $\mu$ XANES) spectroscopy and Extended X-ray absorption fine structure ( $\mu$ EXAFS) spectroscopy. Variability in plaque formation as a function of root-O<sub>2</sub> exudation was also determined using methylene blue stain. Two Fe-treatments were used to investigate whether increased Fe concentration promotes plaque growth, thereby immobilizing more As. The oxidation state of As in plaques was 85% As(V). The sorption complex has 72.5% similarity to As(V) adsorbed onto ferrihydrite (FHY). The high Fe treatment provided a statistically significant increase

in plaque growth as evaluated by DCB extraction of roots; however, the Fe:As molar ratios were similar for both the low and high Fe treatments. The root plaques immobilized 0.86-0.93 mg As per gram of root tissue (dry weight), with Fe:As molar ratios of 15-18:1 in the plaque. Variability in plaque formation was paralleled by O<sub>2</sub> exudation patterns. Knowing the potential amount of As sorption to Fe-plaques may support composting rice straw as a farming practice—a low-cost and natural way of adding iron to paddy soils to promote the growth of Fe-plaques on rice roots.

## **2.2 Introduction**

Chronic arsenic (As) poisoning of millions of people in India and Bangladesh via the consumption of rice demands a better understanding of As in the rice-root environment (Smith et al, 2000). The mobility of As and its association with naturally forming iron (Fe)-oxide plaques on the outer surfaces of rice roots necessitates further study. When As is sorbed to the plaque, its mobility is reduced—potentially lessening the amount of As absorbed into the root tissue where the toxin may be delivered to the edible grains (Seyfferth et al, 2010).

Wetland rice (*Oryza sativa*) grown during the Boro (dry) season requires groundwater for irrigation, which is often contaminated with As (Panaullah et al, 2009). The majority of roots grow between 0-15 or 20 cm under water, where they are presumably under anoxic conditions (Kusnarta et al, 2004; Khan et al, 2010). However, rice plants grow aerenchyma channels, which deliver O<sub>2</sub> via diffusion to their roots, allowing for respiration of the rhizosphere (Kirk, 2004; Hu et al, 2005; Geng et al 2005; Dittmar et al, 2007). This exuded O<sub>2</sub> promotes the precipitation of plaques—the ambient Fe(II) oxidizes to Fe(III)-oxides that form on root surfaces (Wang and Peverly, 1999; Reddy and DeLaune, 2008). Ambient As in soil pore-water

sorbs to these Fe-oxide plaques, forming an As-Fe complex, immobilizing the As in the rhizosphere, and preventing it from absorption into the plant (Meharg, 2004; Hu et al, 2005; Seyfferth et al, 2010).

The speciation of As determines its degree of toxicity and mobility. In pore water, speciation is largely controlled by Eh and pH (Wang et al, 2006; Mohan and Pittman, 2007). In pH neutral waters, oxidized conditions encourage redox transformations to As(V), whereas reduced conditions encourage As(III). Arsenic speciation of Bangladeshi paddy fields is thus varied, depending on the depth of measurement and drainage conditions. However, As sorbed on rice plaques has mostly been As(V) (Liu et al, 2006; Seyfferth et al, 2010).

The question of plaque presence on the root tip proves interesting, as researchers have disagreed on the matter. Chen et al (1980) and Seyfferth et al (2010) found no plaques formed on root tips, though Liu et al (2006) found plaque. Biologically speaking, root tips should have plaque, since the Casparian strip is not yet developed, allowing O<sub>2</sub> to be freely released to form Fe(III)-oxides (Mei et al, 2009; Kotula and Steudle, 2009). The formation of plaque on root tips may depend on a certain threshold of Fe concentration. The root tip may be an area of future research, as essential nutrients, as well as As, enter the root body at this point.

The objectives of this research are: 1. To determine the speciation and localization of As on Fe(III)-oxide plaques as a function of Fe concentration in growth media, 2. To document whether variable plaque formation is a function of variable O<sub>2</sub> exudation, and 3. To determine whether more plaque forms with increasing Fe. Reported natural concentrations of As and Fe from Bangladeshi paddy soils were used in nutrient solutions while growing plants to best simulate field conditions.



Synchrotron-based  $\mu$ -X-ray fluorescence ( $\mu$ -XRF),  $\mu$ -X-ray absorption near-edge structure ( $\mu$ -XANES), and  $\mu$ -Extended X-ray absorption fine structure (EXAFS) spectroscopic experiments allowing for in-situ analyses were paired with bench digestion studies to understand the molecular environment and loading of As and Fe. Methylene blue staining was also employed to visualize areas of plaque formation via areas of O<sub>2</sub> exudation. The plaque's loading capacity of As was determined via digestion.

## 2.3 Materials and Methods

### 2.3.1 Plant growth, root samples, and digestions

Plant samples for synchrotron and bulk analyses were obtained from rice plants grown aseptically and hydroponically. Rice (*Oryza sativa*) of the Japonica variety Nipponbare were grown under growth-lights in the laboratory. Seeds were dehusked, sterilized, and germinated with distilled deionized water in Petri dishes for 7 days. Seedlings were transferred to aseptic plant-growth magenta boxes measuring 20.5 cm tall on day 8, where they were exposed to 25 mL of nutrient solutions consisting of various Fe and As concentrations. During the entire growth period, plants were under cool fluorescent lights at an intensity of 120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  under a 16:8 hr light/dark regime (Bains et al, 2009). The temperature under the lights was  $23 \pm 2^\circ \text{C}$  and humidity was uncontrolled.

A sterilized nutrient solution was prepared from salts. Basal nutrients were supplied as ( $\mu\text{M}$ ): NO<sub>3</sub><sup>-</sup>, 4916; NH<sub>4</sub><sup>+</sup>, 100; P, 80; K, 1080; Ca, 1900; Mg, 500; S, 500; Mn, 0.6; Zn, 8; Cu, 2; B, 10; Mo, 0.1; and Ni, 0.1. After macronutrients were added, the solution was autoclaved, and then micronutrients were added via a 22  $\mu\text{m}$  syringe filter. The basal nutrient solution also contained 20  $\mu\text{M}$  Fe from either FeCl<sub>3</sub>

(synchrotron-analyzed plants) or FeSO<sub>4</sub> (bulk digest-plants). To maintain trace-metal availability, 57.7 μM of EDTA were supplied, which is 27 μM higher than the sum of Mn, Zn, Cu, Ni, and Fe concentrations. The nutrient solution composition is slightly modified from that of Pedler et al (2000), Seyfferth and Parker (2007), and Reichman and Parker (2007). The solution was buffered at pH 6.2 with 2 mM MES.

Four nutrient solution treatments were prepared by adding various amounts of FeSO<sub>4</sub> and As (70% As(V) and 30% As(III)) to the basal nutrient solution mentioned as follows: 1. -Fe/-As (no additional Fe or As), 2. +Fe/-As (additional 100 μM of Fe), 3. +Fe/+As (additional 100 μM of Fe and 14 μM As), and 4. ++Fe/+As (additional 200 μM of Fe and 14 μM of As). Iron and As concentrations reflect those found naturally in paddy soils in Bangladesh (Roberts et al, 2007; Panaullah et al, 2009; Garnier et al, 2010). 25 mL of nutrient solution were added to each magenta box. Plants were harvested on the 29th day. The entire plant was rinsed three times in distilled de-ionized water.

Root samples analyzed at the synchrotron were cryosectioned to 30 μm thin sections via the Leica CM3050S Cryostat partially using the CryoJane Tape-Transfer System. Primary roots were selected for cryosectioning. Samples embedded in Tissue-Tek OCT (Optimal Cutting Temperature) were mounted on taut Ultralene film, which was adhered to a slice of PVC pipe. Roots were sliced at three distances from the root tip—0.1 cm, 0.3 cm, and 1.0 cm. Digital images of cryosections were taken, and using ImageJ software, both size and number of aerenchyma channels in each cryosection were recorded. Roots for the whole root μ-XRF scan were mounted onto Kapton tape.

After rinsing, plants for bulk digests were divided into five replicates within each treatment, separated into root and shoots. Shoots were immediately dried for 48 h at 60°C, whereas roots underwent dithionite citrate bicarbonate (DCB) extractions to release all the Fe-oxide plaque and any of its associated As (Taylor and Crowder, 1983; Liu et al, 2006). Plant samples were extracted for 3 hours, rinsed, and solutions were analyzed for Fe and As concentration via ICP-MS and ICP-OES. Roots were similarly dried after the extraction. Dry and wet masses of roots and shoots were measured and recorded.

### **2.3.2 Methylene blue staining**

O<sub>2</sub> exudation was visualized via methylene blue analysis (Armstrong et al, 1988; Shiono et al, 2011). Roots of the +Fe/+As and ++Fe/+As treatments were placed in vented flasks containing 150 mL of 0.1 % agar reduced methylene blue solution until their roots were submerged. Roots were photographed 60 min later.

### **2.3.3 $\mu$ -XRF Imaging of Roots, $\mu$ -XANES, $\mu$ -XRD, and $\mu$ -EXAFS**

Synchrotron analysis was conducted at the National Synchrotron Light Source (NSLS) microprobe beamline X27A.  $\mu$ -XRF images of the root cryosections and whole roots were collected at 12,500 eV in both step-scan and rapid fly-scan modes, and were used to visualize As and Fe co-localization, as well as determine coordinates for scanning of  $\mu$ -XANES spectra. Synchrotron-based  $\mu$ -XRD data were used to evaluate Fe mineralogy.

$\mu$ -XANES and  $\mu$ -EXAFS spectra were collected from root cryosections. Samples were collected in fluorescence mode and scanned from 11,757 to 12,167 eV, and  $\mu$ -EXAFS spectra were extended to 12,367 eV. Reference spectra comprising of

salts — $\text{NaAsO}_3$  (As(III)) and  $\text{Na}_2\text{HAsO}_4$  (As(V))— were collected for determination of oxidation state. Two minerals were synthesized in the lab—As(V) adsorbed to ferrihydrite and As(V) co-precipitated with ferrihydrite (hereafter referred to as ADS and CPT) (Schwertmann and Cornell, 1991; Waychunas et al, 1993; Jia et al, 2006). Minerals were synthesized with a specified Fe:As molar ratio based on data from bulk DCB digests to best mimic the plaques. Spectra from standards were collected in transmission mode at beamline X-11A at the NSLS. Minerals were characterized and their identity confirmed via bulk XRD and BET surface analysis.

#### **2.3.4 Data processing and analysis**

All X-ray microprobe data were viewed using IDL-based beamline software (x27a\_plot and Xmap\_plotter) developed by CARS (U. Chicago, Consortium for Advanced Radiation Sources) and NSLS Beamline X26A (software available at [http://www.bnl.gov/x26a/comp\\_download.shtml](http://www.bnl.gov/x26a/comp_download.shtml)). Co-localization of As and Fe were determined by forming x-y plots in x27a\_plot. Spectroscopy data were edge-step normalized in IFEFFIT Athena software (Ravel and Newville, 2005). Oxidation states of As at select points of interest from all root-tip distances and treatments were determined by comparing white-lines positions of the spectra with the two salt standards.  $\mu$ -EXAFS spectra from the root plaque and the As(V) on FHY reference material were compared by linear least squares fitting using software from ALS beamline 10.3.2 (software available at <http://xraysweb.lbl.gov/uxas/Beamline/Software/Software.htm>).

## 2.4 Results and Discussion

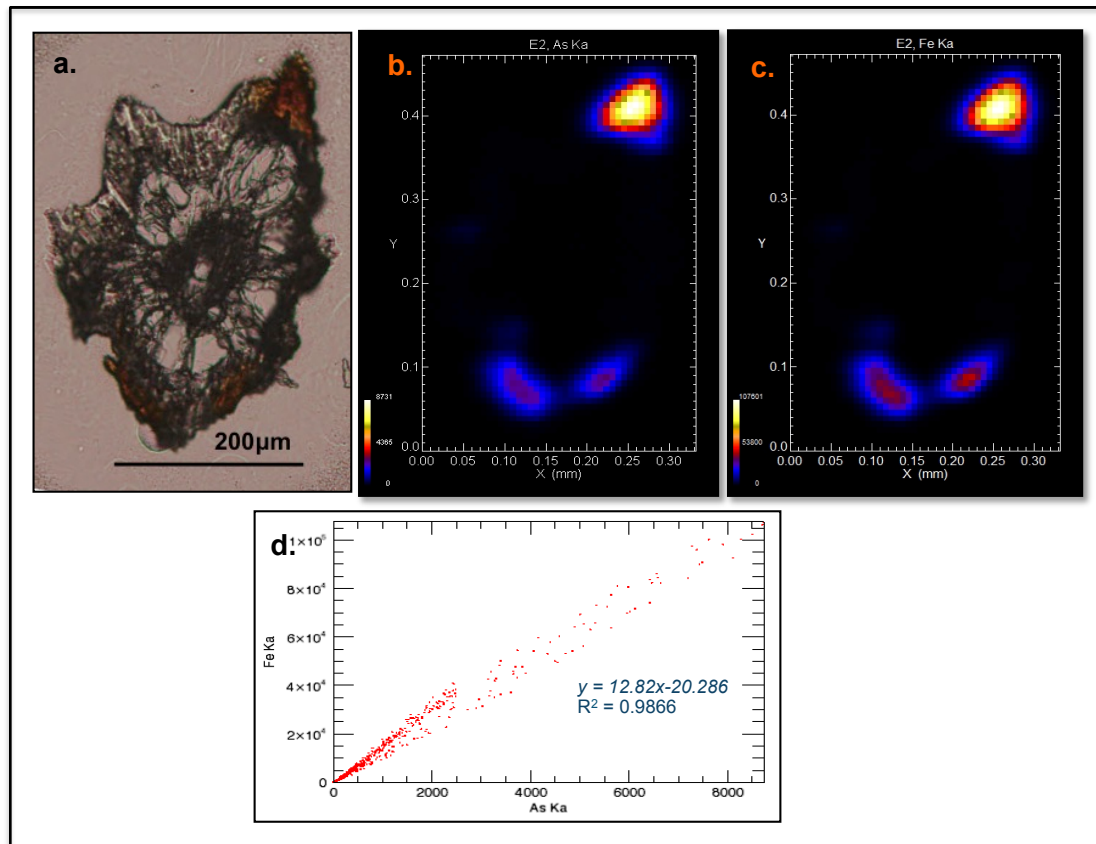
### 2.4.1 Nutrient solution chemistry

Although  $\text{Fe}^{+2}$  (ferrous) salts were added to nutrient solutions, they were quickly oxidized to  $\text{Fe}^{+3}$  (ferric) salts. The oxidation was visible by the change in color of the nutrient solution. A ferrozine spectroscopic analysis was conducted (Stookey, 1970), indicating 6% ferrous, and 94% ferric Fe in the nutrient solution after 24 hours of exposure to atmospheric  $\text{O}_2$ . The nutrient solution fed to plants likely contained mostly ferric Fe. The majority of previous publications studying plaques added ferrous Fe, and grew plants such that roots were presumably under reduced conditions, though they did not control  $\text{O}_2$  levels to maintain  $\text{Fe}^{+2}$  in solution and such redox or  $\text{Fe}^{+2}/\text{Fe}^{+3}$  ratios were not reported (Meharg, 2004; Liu et al, 2004; Rahman et al, 2008; Seyfferth et al, 2010). One hydroponic study added ferric Fe— $\text{Fe}(\text{OH})_3$ —and successfully formed plaque, not even mentioning redox processes or conditions (Zhang et al, 1999). This may be the second study demonstrating plaque formation in mostly oxidized media. In this study, ambient  $\text{Fe}(\text{OH})_3$  or  $\text{FeO}(\text{OH})$  may simply adsorb to the root surface, unlike previous theories of plaque formation whereby  $\text{Fe}^{+2}$  is oxidized via  $\text{O}_2$  exuded by aerenchyma, and sorbs immediately around the rice root, forming  $\text{Fe}^{+3}$  plaque (Meharg, 2004). Although oxidized media still produces plaque, measuring and maintaining the redox state of the growth media proves important, as this may affect the degree of plaque growth indirectly by controlling the amount of Fe in solution. Had the nutrient solution been more reduced, plaque formation would likely have been enhanced. The pH of nutrient solution at the end of plant growth had risen between 0.4 and 1.05 units from the initial pH 6.2.

#### 2.4.2 $\mu$ -XRD, $\mu$ -XRF images and $\mu$ -XANES spectroscopy

$\mu$ -XRD data revealed that all probed regions of Fe-oxide plaques were X-ray amorphous. This negative result makes it possible to eliminate a number of other ferric oxides and (oxy)hydroxides, and may suggest that the root plaque mineralogy is dominated by a ferrihydrite-like mineral precipitate (hereafter referred to as FHY) with high abundance (i.e., weight percent) of As and Zn. Previous publications have found that the majority of plaques were amorphous, with only small percentages of crystalline material such as lepidocrocite or goethite (Hansel et al, 2001; Hansel et al, 2002; Liu et al, 2006; Zhao et al, 2010; Seyfferth et al, 2011).

All  $\mu$ -XRF maps showed that Fe and As were highly co-localized (**Fig. 2.1**). A total of 17  $\mu$ -XRF images of cryosections from treatments +Fe/+As and ++Fe/+As were mapped at the beamline, all of which indicated extensive co-localization of Fe and As, with an average  $R^2$  value of .9749.



**Fig. 2.1** Co-localization of Fe and As. (a.) light microscope photograph of a 30 μm thin root cryosection from ++Fe/+As treatment, cut 0.3 cm from root tip (b. and c.) synchrotron μ-XRF map showing As and Fe concentration, respectively, from the root shown in (a.) (d.) correlation plot of As and Fe, with an  $R^2$  value of .9868.

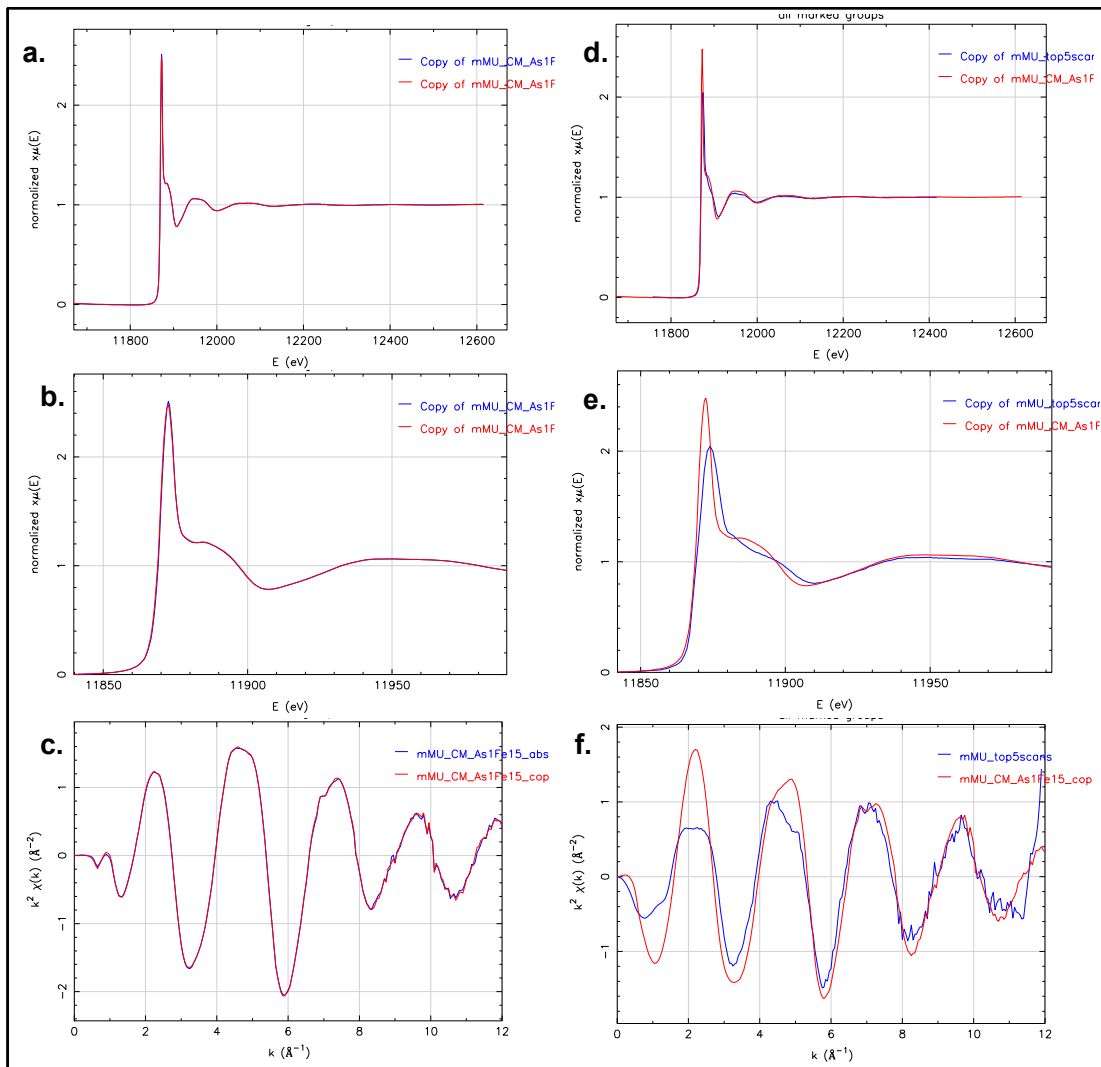
No correlation was found between distance from root tip (0.1, 0.3, and 1.0 cm) and amount of Fe or As sorbed on the root. The μ-XRF images of root cryosections all indicated Fe and As hotspots rather than a continuous surficial rind, but there was no pattern in their distribution as a function of distance from root tip. Defining the root tip may be more challenging than previously conceived. Although all cryosections from the root tip zone (less than 1.0 cm from tip) indicated plaque formation, μ-XRF cross-sectional maps cannot be used to evaluate As or Fe localization in the longitudinal direction.

Ninety-two As K-edge  $\mu$ -XANES spectra were collected from root plaque exposed in cross-section, and comparisons were made between the whitelines of salt-standards and unknown samples. 85% of spectra indicated As exclusively in the As(V) oxidation state, agreeing with many publications, where As(V) is the primary oxidation state of As found on plaques (Chen et al, 2005; Liu et al, 2006; Seyfferth et al, 2010). Oxidation state did not depend on Fe treatment or distance from root tip. At pH of 6.2 (below the PZC of FHY), the positive surface charge of FHY attracts  $\text{H}_2\text{AsO}_4^-$  As(V) ions (Raven et al, 1998). In addition, the pH and redox conditions of the rhizosphere environment promotes the stability of As(V) more so than As(III), explaining the dominance of As(V) in the plaques (Raven et al, 1998; Mohan and Pittman, 2007; Hossain et al, 2009).

Our efforts to determine the sorption mechanism of As(V) onto Fe(III)-oxide plaques proved more difficult and was beyond the scope of this study. A DCB extraction of roots indicated an average Fe:As molar ratio of 15:1 (+ Fe treatment), and thus the two presumed end-members—As sorbed to FHY (ADS) and As co-precipitated with FHY (CPT)—were synthesized with this ratio using the method described by Waychunas et al (1993). However, these two end-members showed identical  $\mu$ -XANES and  $\mu$ -EXAFS spectra (**Fig. 2.2**), suggesting that a CPT mineral may not form with initial Fe:As molar ratio < 15:1 and aging time < 2 days. The Fe:As ratio of Waychunas' CPT samples were 6.2 and 7.7, aging 1 and 3 weeks respectively, whereas in this study it was 16.7 (reflecting loading of As in plaques based on digestions), and samples were aged 2 weeks. Data for ADS and CPT minerals from Waychunas indicated the second shell As-Fe distances to be statistically indistinguishable, at 3.24 Å and 3.26 Å, respectively. Therefore, to distinguish



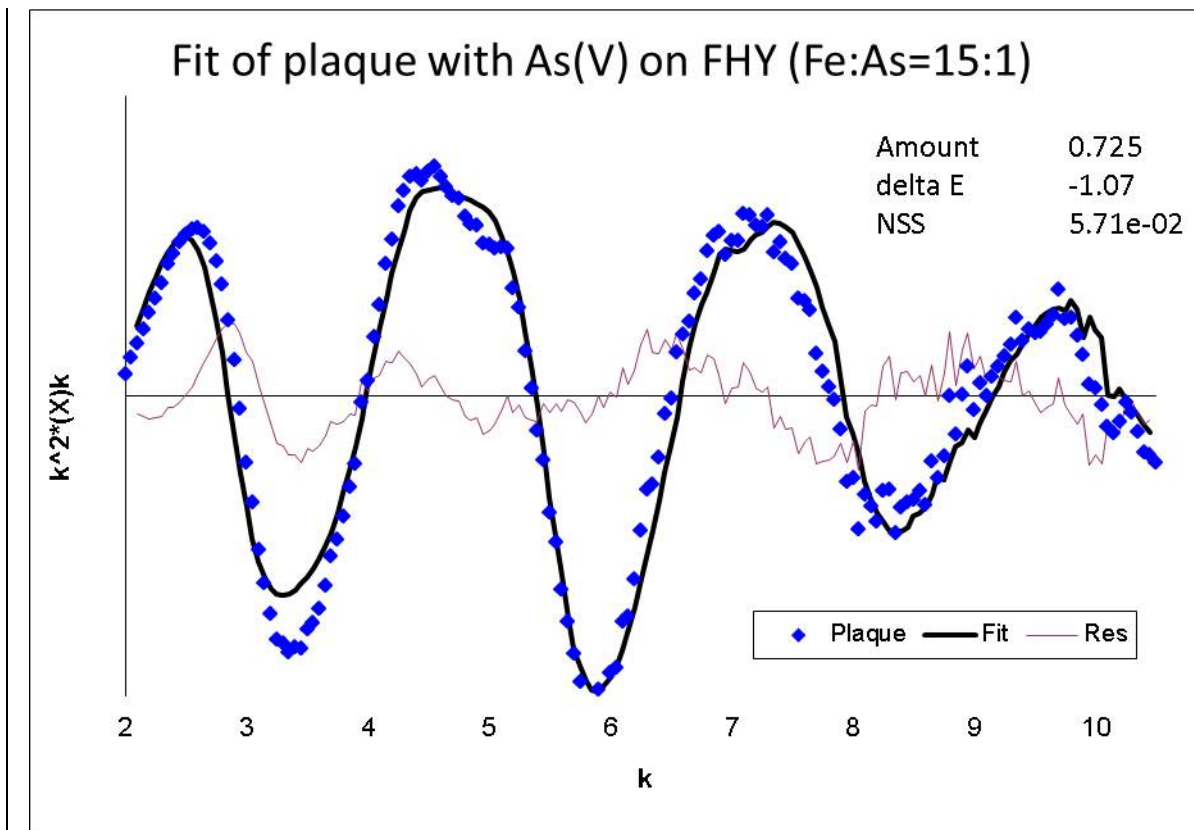
between the ADS and CPT samples using EXAFS , Waychunas et al. (1993) relied on the accurate evaluation of the second shell coordination number ( $CN_{As-Fe}$ ). One can accurately evaluate  $CN_{As-Fe}$  in a model system, but it is difficult to determine in a heterogeneous system with multiple metals due to mixed or overlapping atomic shells, weak backscatterers (e.g., Fe), interferences from As multiple scattering, and sub-optimal data quality. Thus it was not feasible to distinguish between adsorption and co-precipitation in the root plaque using  $\mu$ -EXAFS.



**Fig. 2.2.**  $\mu$ -EXAFS spectra of standards and unknowns. (a – c.) overlaid scans of ADS (blue) and CPT (red) standard minerals, plotted in (a.)  $\mu$ , (b.)  $\mu$ , focused on post-edge, (c.)  $k$ -space. (d. – f.) overlaid scans of unknown plaque sample (blue) and CPT (red) spectra, plotted the same way as in a. – c.

While the XAS spectra from root plaques do not match perfectly with the model compounds (Fig 2.2, panel d-f), linear least squares fitting (LLSF) using either model compound indicates root plaques consist of circa 72.5% As(V) on FHY (**Fig 2.3**); however, adequate standards to account for the fit residual were not discovered.

Despite the difficulties mentioned above to distinguish ADS and CPT forms, the binding mechanism of As(V) onto Fe(III)-oxide plaque can be inferred as inner-sphere adsorption, as the binding mechanism for As(V) on Fe(III)-oxides has been well established (Fendorf et al, 1997; Sherman and Randall, 2003).

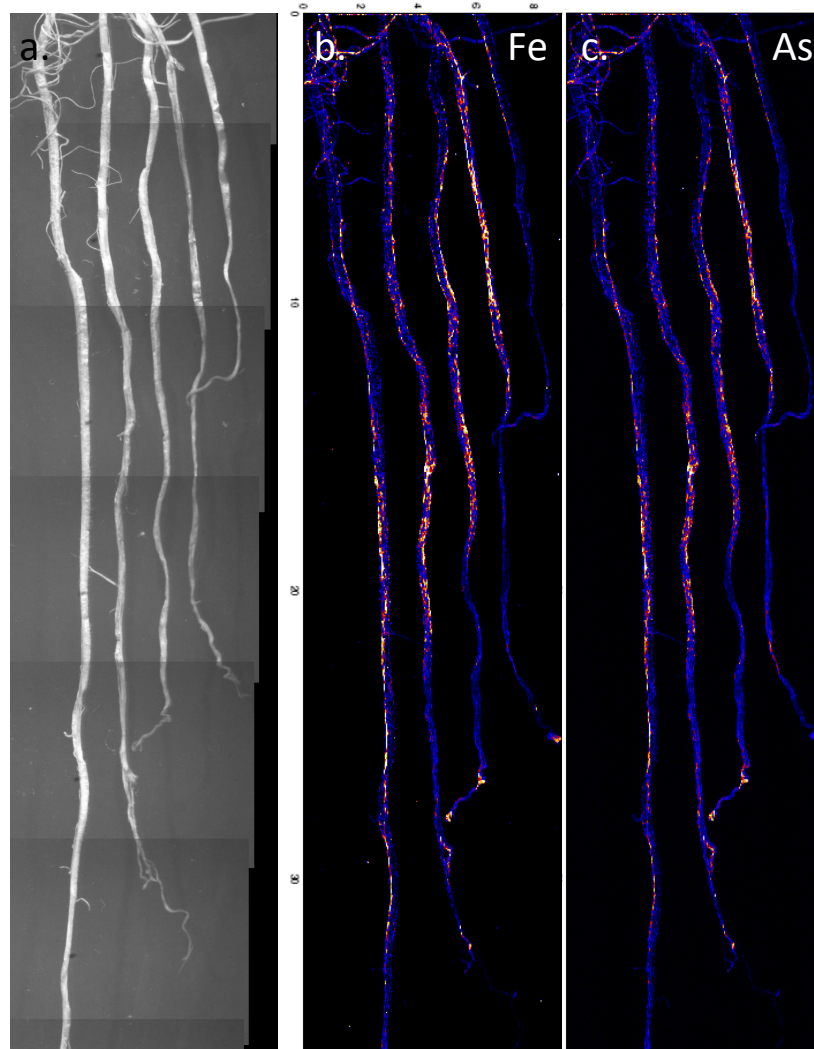


**Figure 2.3. One-component LLSF and fit residual of As K-edge  $\mu$ -EXAFS spectrum from root plaque. Model compound was As(V) on FHY (15:1 Fe:As molar ratio).**

### 2.4.3 Variable plaque formation and O<sub>2</sub> exudation

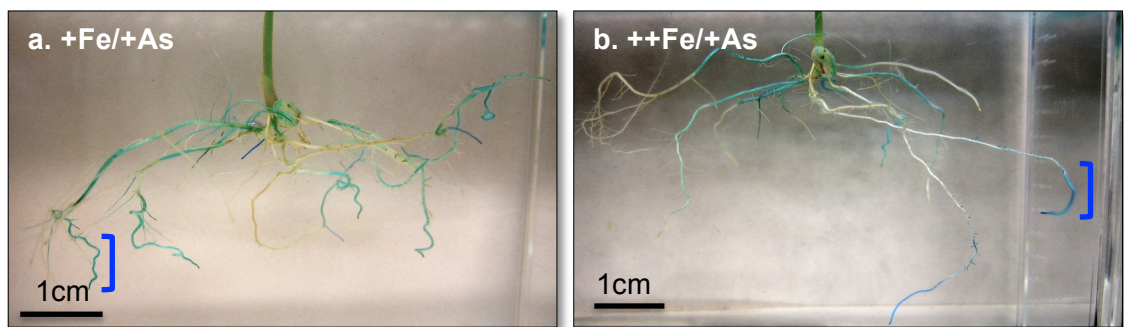
Variable plaque formation was documented on a group of roots from a single plant by a large-area  $\mu$ -XRF fly-scan map (**Fig. 2.4**). These roots were from the +Fe/+As treatment, and measured ~12 cm long. Areas of higher Fe and As

concentrations, or hotspots, are marked by warmer colors. Plaque formed in portions of the root, whereas some parts remained bare. Of the four root-tips imaged, two appear to have plaque at the very tip. Iron and As are also co-localized in the whole root  $\mu$ -XRF map, similar to root cryosections.



**Fig. 2.4**  $\mu$ -XRF map of roots from a single plant, from +Fe/+As treatment, measuring ~12 cm long. (a.) visible light microscope photograph of roots, (b.)  $\mu$ -XRF map of same roots showing Fe hotspots, (c.)  $\mu$ -XRF map of same roots showing As hotspots.

In order to determine O<sub>2</sub> exudation, methylene blue analysis was used. Blue staining on roots indicated O<sub>2</sub> exudation, whereas colorless portions did not release O<sub>2</sub> (**Fig. 2.5**). 58% of roots from +Fe/+As turned blue, whereas 50% of roots from ++Fe/+As turned blue. Overall color variation was equally evident in both treatments. Although these roots were only submerged in a nutrient solution ~1 cm deep, and were most likely not O<sub>2</sub>-depleted, they nevertheless formed aerenchyma and released O<sub>2</sub>. However, the majority of plants form aerenchyma and release O<sub>2</sub>, wetland or not, and thus the blue staining cannot be used as evidence of O<sub>2</sub>-stress in the root zone (Armstrong and Drew, 2002).



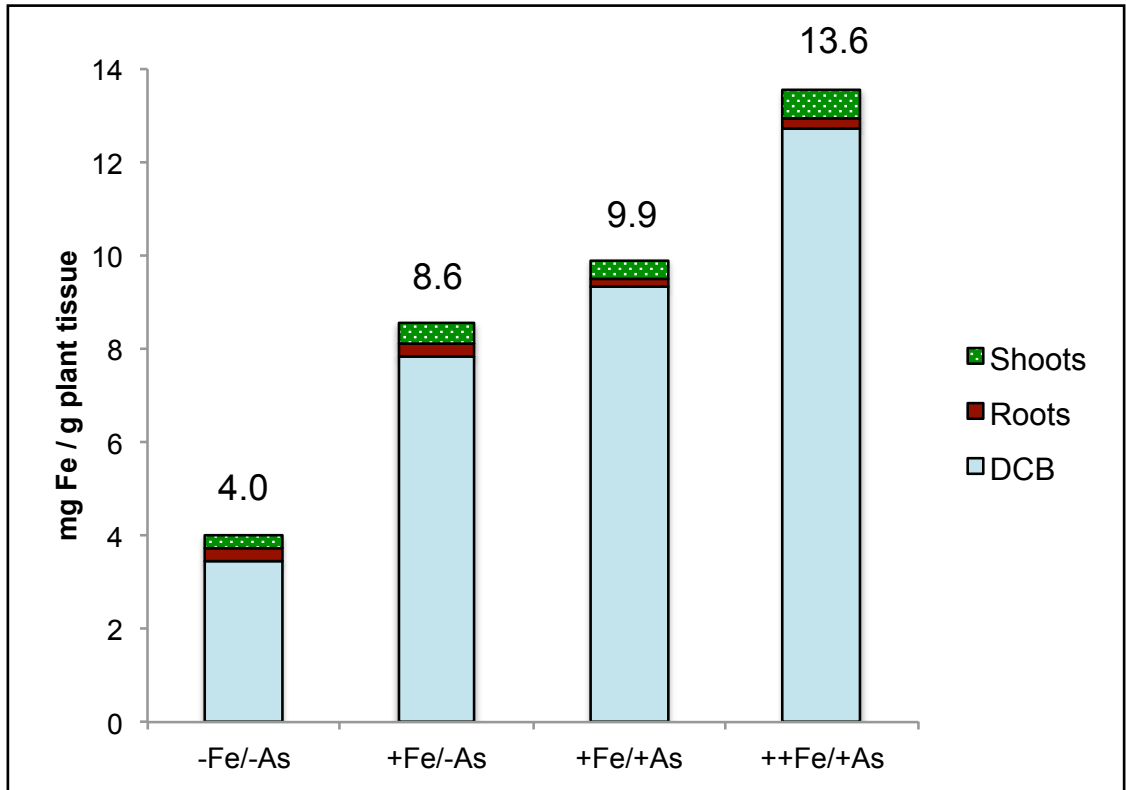
**Figure 2.5** Photographs of roots 1 hr after exposure to methylene blue solution. Both treatments show blue staining in some portions of roots, whereas other parts remain white. The bracket indicates the portion of root where cryosections were taken for synchrotron analysis.

Variable plaque formation down the length of roots may be accounted for by varied O<sub>2</sub> exudation, demonstrated by methylene blue. Though variable plaque formation has been documented previously, its reasons were unknown (Liu et al, 2006; Seyfferth et al, 2010). This may be the first study to demonstrate both variations in O<sub>2</sub> exudation and plaque formation.

#### 2.4.4 Bulk extraction and digestions

Amounts of Fe and As released to solution by the DCB extraction were used as a proxy for the amount of Fe and As constituting the plaque, since DCB extracts all forms of Fe-oxides and their associated species (Taylor and Crowder, 1983; Otte et al, 1991; Liu et al, 2005). A simple *t*-test revealed that the ++Fe treatment formed significantly more plaque than the two +Fe treatments combined (12.75 mg / g root and 8.59 mg / g root, respectively, p value = 0.02). This signifies that a higher concentration of Fe in nutrient solution promotes plaque formation. Holding all other variables constant, enhancing plaque is possible.

The amount of Fe plaque in the two +Fe treatments do not significantly differ. Thus the presence of As does not significantly affect the amount of plaque formation. Additionally, the +Fe treatments formed significantly more plaque than the -Fe treatment (p value << 0.001). Of the total Fe sequestered by the plants, 85-94% of the Fe was associated with the plaque and was removed by the DCB extraction—the remainder was contained in either the roots or shoots (**Fig. 2.6**).



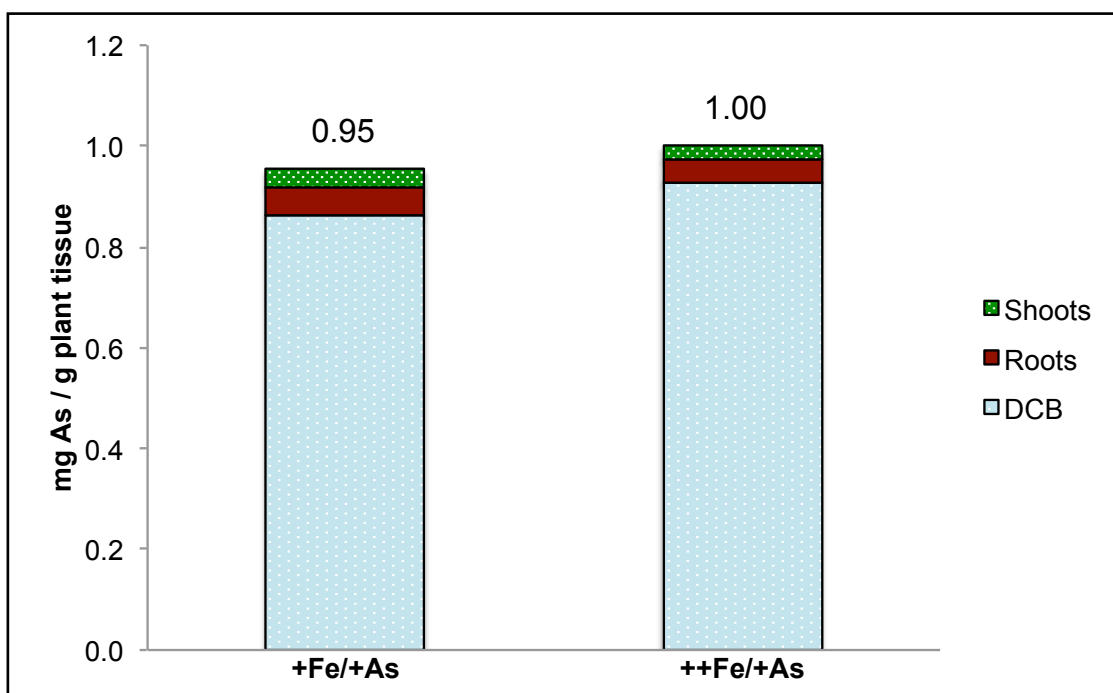
**Fig. 2.6** Amount of Fe sequestered by various portions of plant, as a function of treatment. Fe released during DCB extraction is a proxy for amount of Fe constituting plaques (Geng et al, 2005; Liu et al, 2007). Values above each bar represent sums of the 3 extracted portions. The two +Fe treatments formed significantly less plaque than the ++Fe treatment. -Fe contained 20  $\mu\text{M}$  Fe, +Fe contained 100  $\mu\text{M}$  Fe, and ++Fe contained 200  $\mu\text{M}$  Fe.

Although plaque formation can be enhanced, the amount of As sorbed per gram of root tissue (in plaque) did not significantly differ between the +Fe/+As and ++Fe/+As treatments (0.86 and 0.92 mg As / g root,  $p = 0.604$ ) (**Fig. 2.7**).

Although the ++Fe treatment forms more plaque, it did not sorb more As. This result indicates that the loading of As is not the same for +Fe and ++Fe treatments possibly because the initial Fe:As molar ratio was different by a factor of two—that the low +Fe treatment sorbs no more As per amount of plaque than does the ++Fe treatment.

This means when more Fe is present, Fe has a greater tendency to sorb to the root and form plaque—more than the ambient As.

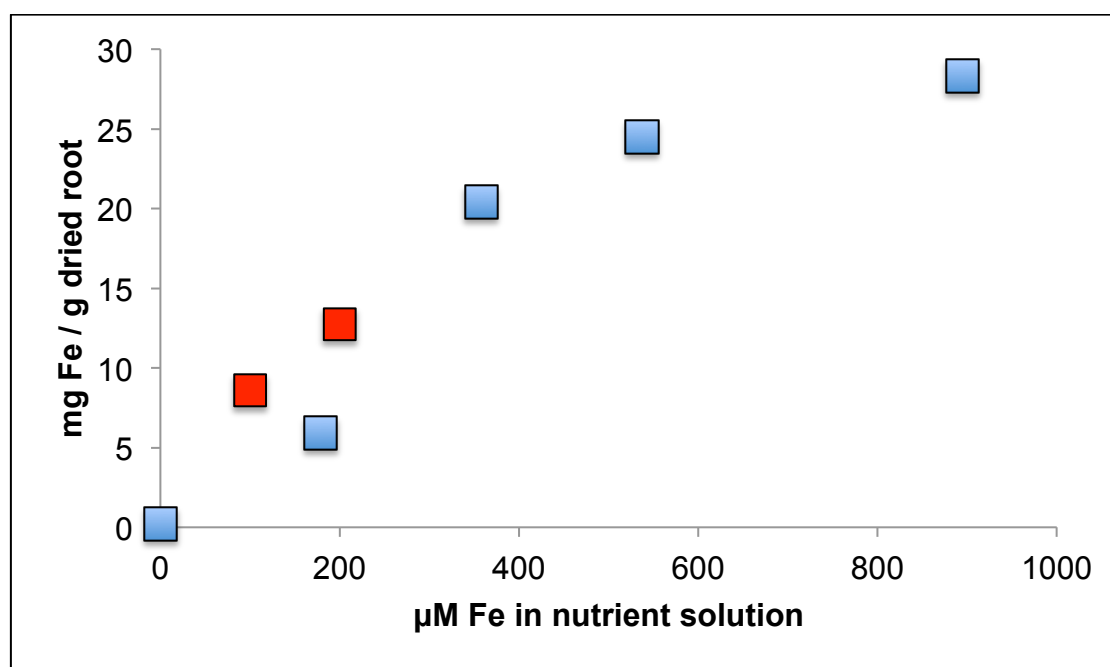
Plaque is an effective scavenger of arsenic. Of the total As sequestered by the plant, 90-92% was held in the plaque and hence removed by the DCB extraction. This indicates plaques sorb and immobilize As, minimizing As absorption into the plant, as previous studies have also suggested (Geng et al, 2005; Liu et al, 2006; Hossain et al, 2009).



**Figure 2.7** Amount of As sequestered by each of the three portions of the plant digested/extracted, measured in mg of As per gram of dried plant tissue. Values above each bar represent sums of the 3 extracted portions. The amounts of As sorbed by the two treatments did not significantly differ. Nutrient solutions both contained 14  $\mu\text{M}$  As, and 100  $\mu\text{M}$  Fe (+Fe) and 200  $\mu\text{M}$  Fe (++Fe).



The amount of extracted Fe in plaques is comparable with the other study that added ferric Fe to their hydroponic nutrient solution (Zhang et al, 1999) (Fig 2.8). Data from the current study is consistent with Zhang et al (1999) when comparing Fe concentration in nutrient solution and amount of Fe sorbed to the root surface (removed by DCB extraction). In fact, data from this study may even better fit the data of Zhang et al (1999) correcting for what looks like a data point inconsistent with their study, at an Fe concentration of 179  $\mu\text{M}$ .



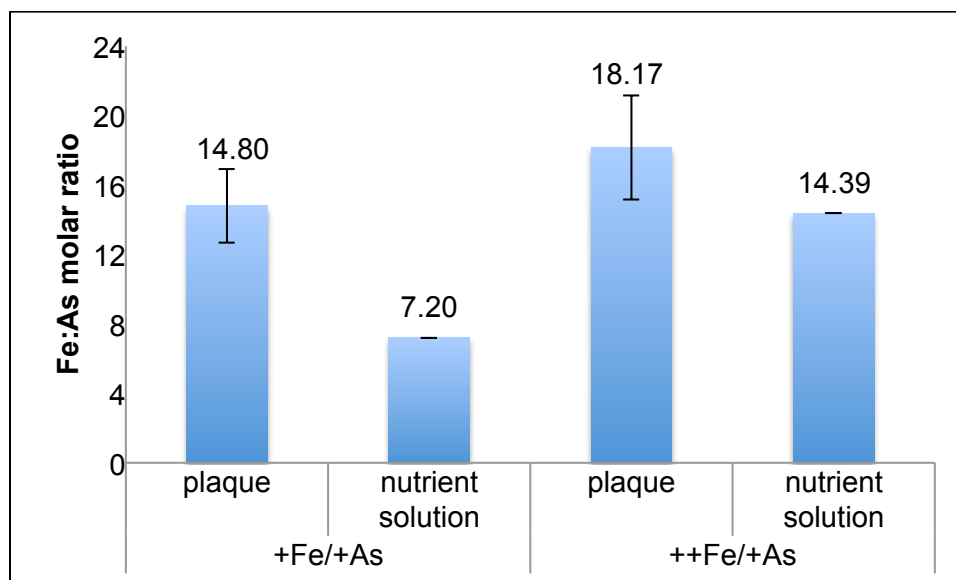
**Fig 2.8** Correlation between concentration of Fe in nutrient solution and amount of plaque formed on roots (mg Fe / g dried root). Data points in blue are from Zhang et al (1999) and points in red are from current study.

Zhang et al (1999) found a correlation between the amount of phosphorus (P) adsorbed to plaque and the amount of plaque. As(V) is a chemical analogue to

phosphorus, and many studies have found similarities in the behavior of P and As(V) (Liu et al, 2004; Chen et al, 2005). One reason why we may not have seen this similar behavior between the amount of As(V) adsorbed to plaque with the amount of plaque is because As concentrations were held constant in the two treatments, and thus in the ++Fe/+As treatment, As(V) and Fe(III) were competitors in sorbing to the root surface. Iron from the ++Fe treatment had a greater competitive advantage compared to Fe from the +Fe treatment simply due to its higher concentration.

The absolute values of extracted Fe and As in this study are also comparable and within the same order of magnitude as data from Geng et al (2005), Hu et al (2005), and Hossain et al (2009), although these studies supplied and allegedly maintained ferrous Fe. However, accurate comparisons cannot be made with the Hu et al (2005) and Hossain et al (2009) studies, as their plants were grown in soil, which distorts salt concentrations available to plants. The Geng et al (2005) study provided 10  $\mu\text{M}$  As and 100  $\mu\text{M}$   $\text{Fe}^{+2}$ , which closely resemble concentrations used in this study. They found  $\sim 5$  mg Fe/g and  $\sim 0.4$  mg As/g in the plaques, whereas we found 8.58 mg Fe/g and 0.95 mg As/g.

Although the Fe:As molar ratio of the plaques formed for the +Fe/+As and ++Fe/+As treatments did not significantly differ, that of ++Fe was slightly higher than +Fe (14.80 and 18.17, p value = 0.07) (**Fig. 2.9**). This indicates that the plaque from the ++Fe treatment has slightly more Fe per amount of As than plaque from the +Fe treatment. Overall, plaque is beneficial as it sorbs As, but since As concentrations were held constant, the added Fe competed with the As, making the ++Fe treatment no more efficient at sorbing As than the +Fe treatment.



**Fig. 2.9** Fe:As molar ratio of Fe and As found in plaques removed by dithionite citrate bicarbonate (DCB), and in originating nutrient solution for treatments +Fe/+As (100  $\mu$ M Fe and 14  $\mu$ M As) and ++Fe/+As (200  $\mu$ M and 14  $\mu$ M As).

In addition, Fig. 2.8 shows that molar ratios of Fe:As found in plaques are slightly higher than those found in the original nutrient solutions. The higher molar ratios in the plaques compared to each respective nutrient solution may also indicate Fe has a higher tendency or ability to sorb on roots and form plaque than As does.

## 2.5 Conclusion

This study serves to better understand plaque formation and its ability to sorb and immobilize As from the rice-root environment. Experimental conditions were chosen to mimic average field conditions. Though Fe-oxide plaque can be enhanced by adding Fe to nutrient solution, the amount of As(V) sorbed per gram of

root may not necessarily increase. Thus enhanced plaque formation may not be as beneficial as previously thought. Regardless, Fe-plaques do reduce As absorption into the plant root. Future studies should be focused on applying lab-based understandings to field studies where added variables create a more complex system. This is crucial before remediation efforts can be focused to alleviate this serious human health problem.

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### **Chapter 3**

#### **FUTURE RESEARCH**

The major implications of this study are 1) added Fe in nutrient solution can enhance Fe-plaque growth, and 2) As sorption may not increase despite increased plaque formation. Future research should determine the amounts of As in rice grains, when plants are grown in high and low Fe concentrations. These results would ultimately prove whether enhanced plaque reduces As delivery to the grain, which may be a potential solution to the human-health issue. Additionally, plants of the Indica variety can be grown, assuring that a local variety to Bangladesh would respond in the same way as the Nipponbare seeds used in the current study.

Plaque dissolution studies may also complement the current work. A time-series of plaques from various weeks may be set up and their roots may be digested to better understand changes in plaque formation as the plant ages. Additionally, when the rice plant is grown to maturity, roots could be submerged in large tubs measuring 30 cm deep, allowing for a more reduced rhizosphere, which will most likely enhance full plaque formation.

Finally, findings from the laboratory should be transferred to the field in Bangladesh, where large-scale fertigation experiments may be conducted. Success of these experiments may promote the practice of straw composting to add cheap Fe to paddy soils.