IMPACT OF TWO WATER MANAGEMENT SYSTEMS ON ARSENIC SPECIATION AND MICROBIAL POPULATIONS IN RICE RHIZOSPHERE

A Dissertation

by

ANIL KUMAR C. SOMENAHALLY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Soil Science

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Approved by:

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	Richard Loeppert
Committee Members,	Scott Senseman
	Robin Autenrieth
	Paul DeLaune
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ABSTRACT

Impact of Two Water Management Systems on Arsenic Speciation and Microbial Populations in Rice Rhizosphere. (December 2010)

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Arsenic (As) is a problem with rice production systems throughout the world as high As concentrations are reported in rice grains originating from several parts of the world. This characteristic is mainly due to the flooded conditions utilized in rice culture. We hypothesized that the soluble As concentrations in the rice rhizosphere can be decreased by growing rice more aerobically through intermittent flooding. Intermittent water management practices might also change microbial populations in the rice rhizosphere that might potentially impact As chemistry and bioavailability. Two field-scale experiments were conducted over two years to study the impact of intermittent and continuous flooding on As speciation and microbial populations in the rice rhizosphere. As levels and speciation in the rhizosphere soil, root-plaque and pore-water were determined using a high performance liquid chromatography-inductively coupled plasma-mass spectroscopy (HPLC-ICP-MS). The microbial populations were assessed from the rhizosphere soil and root-plaque samples using quantitative polymerase chain reaction (qPCR) and 16S rRNA sequencing. Pore-water and root-plaque total-As concentrations significantly decreased in the intermittent compared to the continuous flood plots. Inorganic arsenite (iAs^{III}) was predominant in pore-water and inorganic arsenate (iAs^{V}) in root-plaque and soil. Root-

plaque sequestered significantly higher levels of As (almost tenfold higher) than the adjacent rhizosphere soil. Grain As concentrations also decreased by 35 to 45% in the intermittent compared to the continuously flooded plots. Organic As species, monomethyl and dimethyl arsenate were detected in the rhizosphere with relative increases and decreases among the treatments. *Bacteria* were the predominant group (91 to 94% and 48 to 78% of total community in root-plaque and rhizosphere soils, respectively). *Archaea* were also a major component of rhizosphere soil with their populations being higher under continuous flooding. The relative abundance of iron-reducing bacteria was around 3 to 6 % of the total community in root-plaque and around 6 to 6% in soil, with significantly lower abundance in the intermittent compared to the continuously flooded plots. Results of these studies demonstrated that intermittent flooding could be a potential management option to reduce grain As in rice cultivated on fields with moderate to high As concentrations.

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CHAPTER I

GENERAL INTRODUCTION

1.1 Problem Statement

Arsenic (As) is a naturally occurring toxic metalloid, found in many geological minerals such as arsenopyrite (FeAsS), orpiment (As_2S_3), realgar (α-As_4S_4) and others. Natural As contamination is reported throughout the world with more than 50 million people exposed to a higher level of As than the World Health Organization limit of 20 µg day^{-1} in drinking water for the average adult, either through drinking water or diet (BGS and DPHE, 2001; Duxbury et al., 2003). In the South-Central USA, arsenic in the form of sodium hydroxymethylarsinate (commonly known as monosodium methane-arsonate (MSMA)) and disodium methyl-dioxido-oxoarsorane (commonly known as disodium methane-arsonate (DSMA)), which were popular defoliants and pesticides, were extensively used in cotton production for several decades. This practice has resulted in widespread As-contamination of these soils (Woolson, 1977). Currently, rice is grown on these soils, many with moderate to high residual soil-As concentrations, and rice is also extensively cultivated on As-contaminated fields in South and Central Asia. Growing rice on As- contaminated soils has recently garnered much attention, as several studies reported high As concentrations in rice grain originating from different parts of the world including rice grown in the South-Central USA (Meharg et al., 2009; Zavala et al., 2008). As a result, the consumption of As-impacted rice, apart from drinking water, can be an additional exposure route for millions of people worldwide (Mondal and Polya, 2008; Ohno et al., 2007; Williams et al., 2007a).

This dissertation follows the style of Soil Science Society of America Journal.

1.2 Motivation

Rice generally accumulates higher As concentrations compared to that by other cereals (Williams et al., 2007b), which is mostly due to the flooded conditions utilized for rice production. Moreover, growing rice in soils with high As concentrations may result in the development of straighthead (SH), a physiological disorder characterized by sterility of the florets and significant yield decreases (Gilmour and Wells, 1980; Yan et al., 2005). Hence, there has been a greater need to switch to alternative water-management practices to decrease As concentrations in rice grain as a means of reducing total As exposure. Several recent studies have shown that As concentrations in rice grain can be decreased by growing rice more aerobically, since bioavailable As concentrations have been found to decrease in the root zone (Li et al., 2009a; Xu et al., 2008). This trend is attributable to the higher solubility of As under reduced conditions. The accurate determination of soil-As species in a field-scale study is essential to understand these trends.

Soil microorganisms are capable of transforming As in the environment, thus impacting its solubility, mobility and bioavailability. Arsenic is toxic to most forms of life, including microbes. As^{V} is chemically similar to phosphate and can inhibit oxidative phosphorylation when present within a microbial cell (Oremland and Stolz, 2003). As^{III} binds to sulfhydral groups of amino acids and can interfere with the function of sulfur-containing proteins (Abernathy et al., 1999). As^{V} enters microbial cells through phosphate transporters and As^{III} through aqua-glyceroporins (Rosen, 2002). Many microbes have evolved As detoxification and resistance through an As^{III} efflux mechanism that involves the arsenate reductase enzyme (*arsC*) that reduces As^{V} to As^{III} (Oremland and Stolz, 2003). Some microbes can methylate inorganic forms of As to MMAs/DMAs/TMAs as a detoxification process, since organic-As compounds are less

toxic than the inorganic-As species (Hall et al., 1997). A separate group of microbes known as dissimilatory arsenate-reducing prokaryotes (DARP), which are mostly prevalent in anoxic environments, are capable of utilizing As^V as an energy source (Oremland and Stolz, 2003). Thus, microbes play a significant role in controlling As speciation and As cycling in the environment.

The adverse effects of heavy metals on the soil microbial community have been demonstrated in several studies (Khan and Scullion, 2000; Roane and Kellogg, 1996), showing that toxic metals can result in decreased microbial biomass (Fliessbach et al., 1994). Previous studies have also shown that total microbial biomass and fungal community populations declined proportionally with increasing As contamination (Bardgett et al., 1994; Edvantoro et al., 2003). Arsenic contamination could also lead to a proliferation of As-resistant microbes, thus shifting microbial community composition (Turpeinen et al., 2004).

Microorganisms are an important component in the rhizosphere and strongly impact the cycling and bioavailability of metals. Microbial communities may also be affected by different water-management practices due to changes in soil-redox conditions (Zhou et al., 2002). Continuous flooding might select for facultative or obligate anaerobes, whereas, resilient aerobes and facultative anaerobic microorganisms might dominate in intermittently flooded soils due to the alternating wet/dry cycles (Xiang et al., 2008). Anoxic conditions over time can also lead to a proliferation of iron/sulfate reducers and methanogens (Himmelheber et al., 2009), which could be expected to dominate in continuously flooded rice. Very few studies have been conducted to investigate the response of microbial communities to long-term As contamination under different water-management systems. Thus, it is also important to understand the rhizosphere microbial population response to changes in water management.

The research objectives were:

- To standardize a method for As speciation in rice soils by extraction and HPLC-ICP-MS.
- To study the impact of continuous- vs. intermittent-flood irrigation on As speciation in the rice rhizosphere at low and high soil-As concentrations.
- To study microbial communities and selected populations in continuous flooding vs. intermittent flooding at low and high soil-As concentrations.

In Chapter II, several chemical reagents were evaluated for total As recovery from the rice paddy soil in order to select a suitable method for As-species quantification using a HPLC-ICP-MS system. The soil As was extracted from different soil textures using 5 different ligand-based extraction procedures. The selected method based on the As recovery results was evaluated for recovery of individual species through a spiking study.

In Chapter III field experiment results for As speciation in different compartments of the rice rhizosphere in response to two water management systems under two levels of soil As are discussed. Rhizosphere and grain As speciation relationships are explained, in order to evaluate the impact of water treatments on As solubility and uptake.

In Chapters IV and V microbial population dynamics in rhizosphere and root surface in response to water management practices are presented. Relationships between specific microbial populations and rhizosphere As concentrations are discussed.

CHAPTER II

METHOD FOR ARSENIC SPECIES EXTRACTION FROM RICE RHIZOPSHERE SOIL

2.1 Synopsis

Arsenic (As) occurs naturally in the environment, with contamination reported throughout the world. Arsenic can undergo several chemical and microbial transformations in soil, including oxidation/reduction, methylation/demethylation, and volatilization. As a result, several As species exist in the environment and accurate determination of these species is essential to understand its bioavailability to plants. This study was conducted to evaluate several chemical reagents for recover of As from rice paddy soils in order to standardize a method for quantification of As species using a high performance liquid chromatography-inductively coupled plasma-mass spectroscopy (HPLC-ICP-MS) system. Five different chemical reagents were used to extract As from two different soils. A chemical extraction method was selected based on the recovery of As, which was then evaluated for recovery of As species in a fortification study. Rice rhizosphere soil samples from a field experiment were obtained to evaluate the recovery of As using the selected method. Among all of the chemical reagents and sequential combinations of reagents tested, sequential extraction with 0.4 M H_3PO_4 + 0.4 M NaOH recovered the highest total As (around 84% of total As) and hence was selected for further evaluation. Results of the fortification study demonstrated that the extraction efficiency ranged from 73 to 93% in the order of $DMA^V > MMA^V > iAs^V$. The total recovery of As from the field experiment soils averaged approximately 77% with no statistically significant difference among the treatments. Appropriate quantification of iAs^{III}, iAs^V, MMA^V and DMA^V using HPLC-ICP-

MS system after extracting with 0.4 M H_3PO_4 + 0.4 M NaOH was possible, hence this method was adopted for quantifying As in the rice rhizosphere in later studies.

2.2 Introduction

Arsenic (As) is a naturally occurring toxic metalloid, with natural As contamination reported throughout the world. The common inorganic species are arsenite (iAs^{III}) and arsenate (iAs^{V}), organic forms are monomethyl arsenite ($MMAs^{III}$), monomethyl arsenate ($MMAs^{V}$), dimethyl arsenite ($DMAs^{III}$) and dimethyl arsenate ($DMAs^{V}$). The inorganic species are usually more prevalent in the environment than their organic counterparts that are mostly a product of microbial methylation (Cullen and Reimer, 1989) or in some cases attributed to the use of organic arsenicals as agricultural pesticides or defoliants (Woolson, 1977).

Inorganic arsenate, which is generally more prevalent under oxidized conditions, occurs under environmental conditions as a charged species (pK_a = 2.2) and forms strong bonds with iron oxides as both bidentate and monodentate inner-sphere complexes (Dixit and Hering, 2003). Thus, under oxidized conditions iAs^{V} is largely immobile in the environment. Arsenite (pK_a = 9.2) is more stable under anoxic conditions, is relatively less strongly adsorbed under reduced conditions, and as a result is more soluble and mobile in the environment under reduced conditions (Masscheleyn et al., 1991). Although transformations between iAs^{V} and iAs^{III} can occur chemically due to changes in redox conditions (Masscheleyn et al., 1991) and physicochemical surface processes (Bissen and Frimmel, 2003), the transformations are largely controlled by microbial processes (Campbell et al., 2006; Oremland and Stolz, 2003). Similar to iAs^{V}, $MMAs^{V}$ and $DMAs^{V}$ are relatively stable under oxidized conditions, and $MMAs^{V}$ (pKa = 4.2) is strongly adsorbed to iron oxides from pH 3 to 10, whereas $DMAs^{V}$ (pK_a = 6.1) forms strong bonds with iron oxides at only lower pH values (pH <7) (Lafferty and Loeppert, 2005).

Rice is extensively cultivated on As-contaminated soils in several parts of the world resulting in high grain-As concentrations (Meharg and Rahman, 2003). Precise As speciation in the rice rhizosphere in a field-scale study is critical for understanding As bioavailability in rice paddies. As speciation in paddy soils is a technically challenging task due to the high spatial and temporal variability and the analytical issues associated with As speciation.

In soil, both organic and inorganic species are strongly adsorbed to various soil minerals, thus quantitative extraction of As species from soils is very difficult (Jackson and Miller, 2000). Moreover, the desorption efficiencies of the various As species follow different trends with respect to pH (Lafferty and Loeppert, 2005). Thus, the use of multiple reagents with different pH values might be useful to improve the simultaneous extraction efficiencies of samples with both organic- and inorganic-As species; however, a strong reagent with very high or low pH values could lead to transformations between iAs^{V} and iAs^{III} species (PantsarKallio and Manninen, 1997). Several extraction methods have been tried using high and low pH reagents with a recovery percentage ranging from 40 to 99% (Jackson and Miller, 2000; Montperrus et al., 2002; Pizarro et al., 2003). The commonly used reagents for As-species extraction include phosphate and OH^{-}, phosphoric acid, acetic acid, and ammonium phosphate (Jackson and Miller, 2000; Kahakachchi et al., 2004; Martens and Suarez, 1997); however, there is not a published method for As speciation that could be readily implemented in our study as none of the published methods were able to produce consistently high rates of recovery. Thus, developing an efficient method for As speciation has become an essential prerequisite for our studies. The objective of this study was to standardize a method for As speciation in rice rhizosphere soils by extraction and analyzing by HPLC-ICP-MS analysis.

2.3 Material and Methods

2.3.1 Chemical Reagents

We evaluated five different reagents for total recovery of As from two soil texture; a sandy loam soil (montmorillonitic, thermic typic albaqualf) and a sandy clay loam soil (vertic hapludalf). The extracting reagents were: (1) 0.4 M H_3PO_4 (pH = 1.6); (2) 0.4 M NaOH (pH = 12.4); (3) ammonium oxalate at pH 3, in the dark; (4) 0.5 M sodium phosphate + 0.1 M ascorbic acid at pH 1.6; and (5) 0.5 M sodium phosphate + 0.1 M ascorbic acid at pH 1.6. In method (4) and (5) the pH was adjusted with 1 M HNO_3. Approximately 1g of soil was weighed into 50-mL polypropylene centrifuge tubes. Ten mL of the respective extracting solutions were added to the tubes, which were then agitated for 6 hr at 200 rpm on a reciprocating platform shaker. The samples were then centrifuged, and the supernate was decanted, filtered and diluted with deionized water (DIW) for subsequent As analysis. All samples were filtered with 0.2-µm membrane syringe filters before analysis.

Additionally, total As concentrations of the soil samples were determined, which enabled the determination of extraction efficiencies of extraction methods (1) to (5). The total As concentrations were determined following a open digestion method with HNO_3/H_2O_2 digestion (US-EPA, 2007). Approximately 1g of soil was weighed into 250-mL digestion vessels. Ten mL of 1:1 HNO_3 was added to the samples and were digested at 95^{o}C for 15 minutes with refluxing on a temperature-programmable 48-well, graphite-block digestion system (Digi Prep MS, SCP Science, Montreqal, Canada). The samples were cooled and then 5-mL of concentrated HNO_3 was added and digested for 30 minutes at 95^{o}C with refluxing. This step was repeated for 2 to 3 times till the brown fumes disappeared. The samples were maintained at this temperature till the sample volume was reduced to approximately 5 mL, which were then cooled. Samples were then

digested with 2 mL of water and 3 mL of 30 % H_2O_2 at 95^oC. Addition of 30 % H_2O_2 was continued at 1 mL till the effervescence was minimal. The samples were then cooled, diluted and filtered with 0.2 µ membrane using a syringe filter.

2.3.2 Fortification Study for As Species Recovery by Extraction Method

Based on extraction efficiency of methods (1) to (5) (see table on page 14), sequential extraction with 0.4 M H_3PO_4 + 0.4 M NaOH was selected for As species extraction from the experimental soils. We evaluated the efficiency of the 0.4 M H_3PO_4 + 0.4 M NaOH method for the recovery As species from sandy loam and sandy clay-loam soils. Solutions containing iAs^{III}, iAs^{V}, $DMAs^{V}$ and $MMAs^{V}$ at two concentrations (10 mg L^{-1} and 250 µg L^{-1}) were prepared. Five mL of each As solution was added to 1g of soil separately in triplicates and then the soil suspensions were incubated for 24 hr with mild agitation on a rotary shaker. Five mL of deionized water was added to the control samples. The soil solutions were then centrifuged, and the supernatent was decanted and analyzed for As concentration. The soil residue was then air-dried and the As species were extracted with 0.4 M H_3PO_4 and 0.4 M NaOH. Ten mL of 0.4 M H_3PO_4 (pH 1.6) were added, and the suspensions were agitated for 6 hr on a reciprocating platform shaker. The samples were then centrifuged at approximately 7500g-force for 5 minutes, and the supernatant was decanted. Ten milliliters of 0.4 M NaOH (pH~12.2) were added to the soil residue, and the suspensions were agitated for 6 hr on a reciprocating platform shaker. Equal amounts from the two supernatant solutions were diluted 100-fold using a solution of 2 mM HNO_3 + 0.5 mM EDTA. The final sample matrix of 4 mM H_3PO_4 + 4 mM NaOH + 2 mM HNO_3 + 0.5 mM EDTA at a pH ~3.0 was then analyzed for concentrations of As-species. The recovery of added As species by the phosphate + NaOH method was calculated after subtracting for native soil As.

2.3.3 As Recovery by Phosphate and Hydroxide Reagents

Rice-rhizosphere soil-samples were obtained from a field experiment conducted in research plots at US Department of Agriculture, Dale Bumpers National Rice Research Center, Agriculture Research Service, Stuttgart, AR for two years, in 2007 and 2008. The objective of the field experiment was to evaluate the impact of different water management practices on As speciation in the rice rhizosphere at two soil As concentrations. One of the experimental plots has been continuously amended with monosodium methane-arsonate (MSMA) in alternate years for more than twenty years. MSMA was applied to the surface soil before planting, at the rate of 6.7 kg $ha^{-1}yr^{-1}$. The adjacent native soils had not received any As-containing products for at least the last 20 years. The water treatments, which were superimposed on the native and MSMA soil treatments, included both intermittent and continuous flooding. Under intermittent flooding, the plots were flooded and allowed to dry until surface cracking initiated and then were re-flooded. The treatment combinations used in this study were (1) MSMA-flood (MF) (2) MSMA-intermittent (MI) (3) No-MSMA flood (NF) and (4) No-MSMA intermittent (NI). The soil in the experimental plots was a fine, montmorillonitic, thermic typic albaqualf (Crowley silt loam). Each treatment plots were distributed using a split-split plot design and each treatment contained four replicate plots randomly distributed within each treatment plots. Each replicate plots contained 9 rows with 0.2-m spacing between each row and were 1.5m long. The seeds were sown in the middle of April, and the first flood was introduced at four weeks following sowing, when the plants were about 30 cm tall. All of the other management practices are outlined elsewhere (Yan et al., 2005).

2.3.4 Sampling

In 2007, the rhizosphere soil samples were collected at planting (1 d after planting), one month after flooding (60 d after planting) and 3 weeks before harvest (120 d after planting). In 2008, samples were collected at 3 weeks before harvest (120 d). Three plants from each replicate plots were removed along with the adhering bulk soil with the aid of a shovel and shaken to remove the loose soil. Remaining non-rhizosphere soil was removed manually, leaving only a few millimeters of soil around the roots, which was then manually collected. Soil samples were air-dried in the lab and ground to <0.2 mm size and stored at room temperature until further analysis. The concentrations of As species were determined following the sequential extraction 0.4 M H_3PO_4 + 0.4 M NaOH method as explained in the previous section. The total As concentration in the soil samples was determined following digestion with HNO_3/H_2O_2 (US-EPA, 2007).

2.3.5 Instrumentation and Chemicals

The DMA^V and $MMAs^V$ were obtained from Chem Service (West Chester, PA, USA) as dimethyl arsinic acid and monosodium acid methanearsonate, respectively. Arsenate was obtained as sodium arsonate ($Na_2HAsO_4 \bullet 7H_2O$) from Sigma (St. Louis, MO, USA) and iAs^{III} as arsenite oxide (As_2O_3) from Alfa Aesar (Ward Hill, MA, USA). A PerkinElmer 200 HPLC system (Waltham, MA, USA) with a Dionex IonPac AG7 guard column (Dionex, Sunnyvale, CA, USA) and a Dionex IonPac AS7 anion-exchange column (Dionex, Sunnyvale, CA, USA) was used for separation of As species, which were then quantified by using a ICP-MS model DRC-ELAN II (Perkin Elmer Waltham, MA, USA). The HPLC instrument parameters are presented in Table 2.1. The post column addition of 3% methanol was used to offset ionization

problems due to variable C concentrations. The total As concentration in the soil samples was determined by ICP-MS.

Table 2.1. Instrument parameters used for speciation of arsenic in soil samples.

Instrument	HPLC-ICP-MS (Perkin Elmer model Elan DRCII)		
HPLC column	Ion exchange AS7 with AG7 guard column (Dionex, Sunnyvale, CA)		
Mobile phase	Eluent A:1mM HNO_3 (pH~3)		
	Eluent B: 50mM HNO_3 (pH~1.5)		
As species measured	iAs^{V}, iAs^{III}, $DMAs^{V}$ and $MMAs^{V}$		
Gradient elution program			
Time (min)	**A**	**B**	**Gradient**
5 (equilibration)	100%	0%	0
2.5	100%	0%	0
6.5	0%	100%	1

2.4 Results

2.4.1 Comparison of Chemical Reagents for As Recovery

The total As recovery by five extraction methods (1) to (5) averaged about 75% and there was no significant difference among the methods (Table 2.2). Phosphoric acid and ammonium oxalate methods extracted the highest quantity of total As from both of the samples. Since the extraction efficiency of individual reagents was not as high as we anticipated, several sequential extraction combinations of the methods were evaluated to get higher quantitative extraction. Sequential extraction with 0.4 M H_3PO_4 at pH 1.6 followed by 0.4 M NaOH at pH 12.0 yielded the highest recovery of total As compared to the other methods (85%) (Table 2.2). Thus we implemented this method for As species extraction from the experimental soils.

2.4.2 As Species Recovery by Phosphate and Hydroxide Method

Using H_3PO_4 + NaOH extraction it was also possible to accurately quantify all of the four As species (iAs^V, iAs^{III}, $DMAs^V$ and $MMAs^V$) from both soil types (Figure 2.1). The recovery by the H_3PO_4 + NaOH method varied significantly among the As species in the order; $DMAs^V > iAs^{III} > MMAs^V > iAs^V$ (Table 2.3). The average As recovery percent in the fortificqation study by H_3PO_4 + NaOH showed that both iAs^V and MMA^V were extracted approximately at 80% compared to iAs^{III} and DMA^V which extracted at approximately 90% (Table 2.3). The recovery of iAs^V species from the sandy loam soil was slightly higher than the sandy clay loam soil. Soil texture did not affect the recovery of other As species. $MMAs^V$ recovery was slightly higher in soils fortified with higher concentrations of $MMAs^V$ than the soils with lower $MMAs^V$ concentrations. All of the iAs^{III} was likely converted to iAs^V by the time of analysis as the soils were air dried before extraction. The average total-As recovery by the H_3PO_4 + NaOH method from the field experiment soils was around 77% (Table 2.4) which is less than the average total As recovered in the fortification study of approximately 86%. The As recovery percentage did not statistically differ between the samples from various treatments and years.

Table 2.2. Arsenic recovery by different extraction methods.

Method	Sandy loam	Sandy clay loam
	% recovered to total acid digested As	
0.4 M Phosphoric acid	81.7 (±7.02)	78.4 (±4.67)
0.4 M Sodium hydroxide	78.1 (±6.19)	75.4 (±5.12)
0.175 M Ammonium oxalate + 0.1 M oxalic acid in dark	80.8 (±8.01)	79.5 (±7.87)
0.5 M Dihydrogen phosphate + 0.1 M citric acid	75.3 (±7.79)	73.5 (±3.39)
0.5 M Dihydrogen phosphate + 0.1 M citric acid	76.8 (±9.26)	74.1 (±8.12)
0.4 M Phosphoric acid + 0.4 M Sodium hydroxide	85.7 (±8.12)	83.8 (±8.12)

Sandy loam soil collected from Stuttgart, AR (thermic typic albaqualf) and Sandy clay loam soil collected from Beaumont, TX (vertic hapludalf). Values in parenthesis are standard error of mean.

Table 2.3. Recovery of fortified As species by H_3PO_4 + NaOH extraction method.

Soil texture	As added / g of soil	As species added			
		iAs^V	iAs^{III}	MMA^V	DMA^V
		% Extracted			
Sandy loam	None	81.5 (±5.13)	ND¥	ND¥	ND¥
	1.25 µg	90.7 (±3.97)	ND*	79.0 (±3.04)	97.8 (±2.97)
	50 µg	83.0 (±5.62)	ND*	85.1 (±4.71)	95.5 (±3.19)
Sandy clay loam	None	79.3 (±8.23)	ND¥	ND¥	ND¥
	1.25 µg	72.3 (±8.69)	ND*	80.5 (±3.63)	95.7 (±4.76)
	50 µg	81.0 (±7.31)	ND*	86.3 (±6.06)	96.0 (±5.19)

¥ Not detected in native soil. * Not detected as all of the fortified iAs^{III} converted to iAs^V
Values in parenthesis are standard error of mean.

Table 2.4. Recovery of total As from the experimental field soil by H_3PO_4 + NaOH extraction method.

Treatments	Sampling Date			
	1 d	60 d	120 d	120 d-2008
	% recovery			
MSMA flood	75 (±7.17)	78.2 (±7.91)	78.5 (±8.57)	73.6 (±5.87)
MSMA intermittent	82.9 (±6.90)	79 (±9.08)	73.3 (±9.08)	70.4 (±8.11)
No-MSMA flood	87.1 (±7.91)	77.6 (±9.91)	74.1 (±8.23)	70.6 (±6.09)
No-MSMA intermittent	76.4 (±8.48)	84 (±7.03)	66.2 (±10.12)	80.1 (±6.56)

Note: The percent recovery calculated by comparing to total As extracted by acid digestion. Values in parenthesis are standard error of mean.

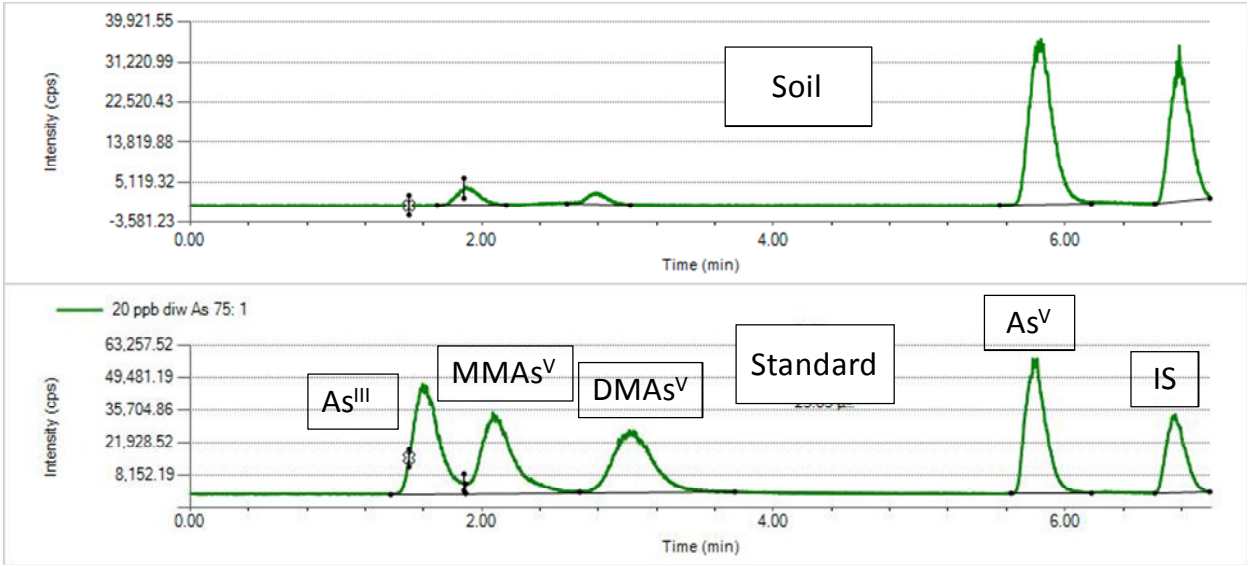

Figure 2.1. HPLC-ICP-MS chromatograms for As species from standard and soil samples. IS=Internal standard.

2.5 Discussion

All five of the reagents tested for recovery of As extracted less (~75%) of the total soil As than we had anticipated. Both inorganic and organic As species are strongly adsorbed to soil minerals which tend to desorb at low efficiency with ligand exchange reagents (Lafferty and Loeppert, 2005). The pKa values for iAs^{V}, MMA^{V} DMA^{V} and iAs^{III} ranges from 2.2 to 9.2 with desorption efficiency also ranging from low to high pH (Lafferty and Loeppert, 2005), thus it is very difficult to get quantitative extraction of all of the species with a single extractant (Jackson and Miller, 2000). Thus we tried sequential extraction with several combinations of all of the five reagents in order to get quantitative extraction and appropriate quantification using the HPLC-ICP-MS system. Among all of the combinations evaluated for recovery of iAs^{V}, iAs^{III}, MMA^{V} and DMA^{V} species, sequential extraction with 0.4M H_3PO_4 at pH 1.8 followed by 0.4M NaOH at pH 12.0 was the most efficient. This may be due to the lower pH of the H_3PO_4 extractant which may facilitate weakening of Fe-O-As bonds allowing phosphate to more efficiently replace As from iron oxides (Lafferty and Loeppert, 2005). Similarly at high pH, Fe-O-As bonds are weakened due to increased repulsive potential between negatively charged Fe oxide surface and the negatively charged iAs^{V} species. Also, OH^{-} competes with iAs^{V} for adsorption sites at the mineral surface (Raven et al., 1998). Moreover, the solubility of iron oxides is also greater at pH >9 (Lindsay, 1979) which increases quantitative extraction of As (Raven et al., 1998). Similarly, Jackson and Miller (2000) observed that PO_4 at low pH extracted a higher proportion of iAs^{III} compared to that by OH^{-1} at high pH which extracted a higher proportion of the total iAs^{V}, MMA^{V} and DMA^{V}.

The sequential extraction method was also most compatible with the instrument conditions mentioned in Table 2.1 for quantification of individual species using a HPLC-ICP-

MS system. The H_3PO_4 + NaOH method extracted lower iAs^V and MMA^V compared to iAs^{III} and DMA^V. This might be due to the tendency of iAs^V to form inner sphere complexes with iron oxides and be strongly adsorbed (Fendorf et al., 1997). MMA^V also forms inner sphere complexes with aluminum and iron oxides (Cox and Ghosh, 1994) and is strongly adsorbed to ferrihydrite and goethite between pH 4 – 7 (Lafferty and Loeppert, 2005).

As^{III} was the first species to elute followed by MMA^V, DMA and iAs^V (Figure 2.1). Arsenate was retained for a longer time in the column and eluted after 6.5 minutes when the gradient elution pH reached less than 2. It is common to see DMA^V eluting before MMA^V when a higher pH mobile phase is used (Pizarro et al., 2003). However, in our study MMA^V was eluted earlier than DMA^V due to lower pH eluent (pH ~ 3). An unidentified As species was consistently observed in most of the soil samples from MF treatment which eluted after iAs^V (Figure 2.1). This could be one of the thio-arsenate compounds, largely a mono-thioarsenate compound which is a charged species (Wallschlager and London, 2008).

2.6 Conclusions

Among all of the chemical reagents evaluated, the sequential extraction with 0.4M H_3PO_4 followed by 0.4M NaOH provided the highest recovery of As from the soils. The sequential extraction using the H_3PO_4 and NaOH reagents recovered appreciable quantities of As species from rice paddy soils. The extraction efficiency ranged from 73 to 93% in the order of $DMA^V > MMA^V > iAs^V$. These results indicate that it is possible to accurately quantify iAs^{III}, iAs^V, MMA^V and DMA^V from soil samples using HPLC-ICP-MS.

CHAPTER III

IMPACT OF TWO WATER MANAGEMENT SYTEMS ON ARSENIC SPECIATION

3.1 Synopsis

Rice cultivation on arsenic (As) contaminated-soils is of potential concern since rice usually accumulates higher As concentrations in grains compared to other cereals. This characteristic is mainly due to the flooded conditions utilized in rice culture. We hypothesized that the soluble As concentrations in the rice rhizosphere can be decreased by growing rice more aerobically through intermittent flooding. A field-scale experiment was conducted to study the impact of intermittent and continuous flooding on As speciation in different compartments of the rice rhizosphere. Several As species were extracted from the rhizosphere soil, root-plaque and pore-water, and were quantified using a HPLC-ICP-MS. The soil As concentrations were naturally higher in MSMA plots than the No-MSMA, but there was no significant difference between the water treatments. Pore-water and root-plaque total-As concentrations significantly decreased with the intermittent (85 to 86% in pore-water and 50 to 56% in root-plaque) compared to the continuous flood. Arsenite was predominant in pore-water, whereas iAs^{V} was predominant in root-plaque and soil. $MMAs^{V}$ was detected only in MSMA soils and $DMAs^{V}$ in pore-water and root-plaque samples from the continuously flooded plots. Total grain As concentrations also decreased by 35 to 45% in the intermittent compared to the continuously flooded plots and only $DMAs^{V}$ and iAs^{III} were detected in rice grains, and $DMAs^{V}$ concentrations also decreased in the intermittent plots. Results of this study demonstrated that intermittent flooding could be a potential management option to reduce soluble As

concentrations in the rice rhizosphere and grains in rice cultivated on fields with moderate to high As concentrations.

3.2 Introduction

Arsenic (As) is a toxic metalloid known to cause cancer in humans. Worldwide, more than 50 million people are exposed to higher levels of As than is recommended by World Health Organization (10 µg day^{-1} in drinking water), either through consumption of As-containing drinking water or food (BGS and DPHE, 2001; Duxbury et al., 2003). In Bangladesh alone, more than 30 million people are exposed to high levels of As. In South East Asia, the As contamination is mainly of geological origin. In other parts of the world the release of As from mining activities (Klumpp and Peterson, 1979), and the use of As-containing pesticides (Woolson, 1977) and wood preservatives (Townsend et al., 2003) has led to widespread contamination. Under natural conditions, soil-As concentrations are usually less than 5 mg kg^{-1}, but concentrations as high as 40 mg kg^{-1} have been reported in contaminated soils such as those irrigated with As-rich groundwater in Bangladesh (Meharg and Rahman, 2003; Panaullah et al., 2009). The most common inorganic species of As are arsenite (iAs^{III}) and arsenate (iAs^{V}) while common organic forms include monomethylarsonous acid ($MMAs^{III}$), monomethylarsonic acid ($MMAs^{V}$), dimethylarsinous acid ($DMAs^{III}$) and dimethylarsinic acid ($DMAs^{V}$). The inorganic species are usually more prevalent in the environment than their organic counterparts which are mostly a product of microbial methylation or in some cases attributed to the use of organic arsenicals as agricultural pesticides or defoliants.

Rice is extensively cultivated on As-impacted soils throughout the world, resulting in high grain-As concentrations (Meharg and Rahman, 2003). As a result, the consumption of As-impacted rice, apart from drinking water, can be an additional exposure route for millions of

people worldwide (Mondal and Polya, 2008; Ohno et al., 2007; Williams et al., 2007a). Arsenic in the form of sodium hydroxymethyl-arsinate (commonly known as monosodium methane-arsonate (MSMA)) and disodium methyl-dioxido-oxo-arsane (commonly known as disodium methane-arsonate (DSMA)), which are popular defoliants and pesticides, were extensively used in cotton production for several decades and are still used for turf production. This practice has resulted in widespread As-contamination of many soils (Woolson, 1977). Currently, rice is grown on many of these soils, often containing moderate to high residual soil-As concentrations. High soil-As concentrations in these soils have also been linked to the incidence of straighthead in rice, a physiological disorder characterized by sterility of the florets and significant yield decreases (Gilmour and Wells, 1980; Yan et al., 2005).

High grain-As concentrations are exacerbated by the practice of continuous flooding, which is commonly used for the cultivation of rice (Xu et al., 2008; Yan et al., 2005). This phenomenon is mainly attributable to the flooded conditions in which As is more readily released to pore-water by reductive dissolution of iron oxides and also increased conversion of iAs^{V} to the iAs^{III} form (Masscheleyn et al., 1991; Takahashi et al., 2004). Arsenite, a neutral species under normal paddy conditions (pH 4 to 8), is considered to be more readily bioavailable than iAs^{V}. In contrast, iAs^{V}, which is strongly adsorbed to iron and aluminum oxides (Lafferty and Loeppert, 2005; Raven et al., 1998), is more prevalent under oxidized conditions. Recent pot-scale studies have shown that both intermittent flooding with wet/dry cycles (Li et al., 2009a) and aerobic-rice cultivation (Xu et al., 2008) resulted in significantly reduced grain-As concentrations compared to that obtained with continuous flooding. Cultivation of rice with intermittent flooding appears to have significant potential as a management strategy to reduce grain-As accumulation; however there is very little data on the occurrence of the various As species in the rice rhizosphere of

intermittently flooded soil at the field scale. Rice can take up both iAs^{V} and iAs^{III} species (Meharg and Hartley-Whitaker, 2002), as well as organic species such as $MMAs^{V}$, $MMAs^{III}$, $DMAs^{V}$ and $DMAs^{III}$ (Li et al., 2009b). DMAs and MMAs have been detected in rice paddies (Takamatsu et al., 1982) and not much is known about the impact of intermittent cultivation on concentrations of organic-As species.

The rice rhizosphere is somewhat oxidized even under continuously flooded conditions, since with rice as with most other aquatic plants, oxygen is released through the root epidermal layer as a result of radial oxygen diffusion from the aerenchyma structure (Colmer, 2003). As a result, iron oxides precipitate on the root surface to form root-plaques. The root-plaques adsorb significant amounts of As, mostly in the form of iAs^{V} and iAs^{III}, and can impact the amount of As taken up by rice plants (Hossain et al., 2009; Liu et al., 2004a). Arsenic adsorption also depends on the amount of iron oxide plaque formed (Hossain et al., 2009; Zhang et al., 1998), which can vary with soil iron-oxide content and mineralogy and differences in soil-redox potential (Chen et al., 2008). Thus, As quantification and speciation in root-plaque samples is an important factor in understanding As availability to plants under different water-management practices. The objective of this study was to compare the impacts of continuous vs. intermittent flooding on the speciation of As in the rice rhizosphere and grains.

3.3 Material and Methods

3.3.1 Experimental Site and Treatments

Field experiments were conducted in research plots at the US Department of Agriculture, Agriculture Research Service, Dale Bumpers National Rice Research Center, Stuttgart, AR, for two years, in 2007 and 2008. One of the experimental plots was a straighthead testing plot of approximately 1 ha area, which has been continuously amended with MSMA in alternate years

for more than twenty years (Yan et al., 2005). This plot has been used for rating rice varieties for straighthead resistance. MSMA was applied to the surface soil immediately before planting, at the rate of 6.7 kg $ha^{-1}yr^{-1}$ (equivalent to 3.1 kg As $ha^{-1}yr^{-1}$). The adjacent No-MSMA soil had not received any As-containing products for at least the last 20 years. The water treatments, which were superimposed on the No-MSMA and MSMA soil treatments, included both intermittent and continuous flooding. Under intermittent flooding the plots were initially flooded, then allowed to dry until small, surface cracks were evident, at which point the plots were re-flooded. The treatment combinations used in this study were (1) MSMA flood (2) MSMA intermittent (3) No-MSMA flood and (4) No-MSMA intermittent. In intermittently flooded plots, the wet/dry cycles were continued throughout the rice growing season until 3 weeks before harvest, at which time all plots were allowed to dry. The soil type in the experimental plots was a fine, montmorillonitic, thermic Typic Albaqualf (Crowley silt loam). The rice varieties were Wells (yr 2007) and Cocodrie (yr 2008); both are popular high yielding varieties that are relatively susceptible to straighthead (Yan et al., 2005). The treatment plots were arranged in four completely randomized replicates. The seeds were sown in the middle of April, and the first flood was introduced at four weeks following sowing, when the plants were about 30 cm tall. All the other management practices were conducted as outlined by Yan and associates (Yan et al., 2005).

3.3.2 Sampling

The samples for As speciation included rhizosphere soil, rhizosphere pore-water, root-plaque and grains. The plants were removed along with the adhering bulk soil with the aid of a shovel and shaken to remove the loose soil. The remaining non-rhizosphere soil was removed manually, leaving only a few millimeters of rhizosphere soil around the roots, which was then

manually collected. Roots were then thoroughly washed with deionized water (DIW) to remove and collect rhizosphere soil adhering to the roots. After removal of the rhizosphere soil, the iron-oxide plaque remained firmly affixed to the roots, which taken together are hereafter referred to as root-plaque samples. In 2007, soil samples were collected at 1 d after sowing, 60 d after sowing (one month after first flooding) and 120 d after sowing (3 weeks before harvest). In 2008, samples were only collected at 120 d (3 weeks before harvest). The plots with continuously flood treatment were submerged at the time of sampling both at 60 d and 120 d, whereas the plots with intermittently flood treatment were saturated without any standing water. At the time of sampling during 60 d time-point the flood treatment-plots had been continually flooded for approximately 5 weeks and the intermittent flooded plots had been flooded for approximately 1 week. At the 120 d sampling time, the flood treatment-plots had been continually flooded for approximately 12 weeks and the intermittent flooded plots had been flooded for approximately 1 week. All soil and root-plaque subsamples were stored at 4 ^{O}C during transportation from field to the lab. Soil samples were air-dried in the lab and ground to <0.2 mm size and stored at room temperature until further analysis. The root-plaque subsamples were immediately extracted for As species and the extracted solutions were stored at 4 ^{O}C until further analysis.

In order to collect pore-water samples, bulk-soil samples from the 0 to 6-cm depth were collected from the non-rhizosphere area (between plants) using a 2.5-cm diameter corer with brass insert rings. The insert rings with soil sample were capped with polypropylene end caps, stored on ice during transport to the lab. The core samples were then vacuum filtered at a negative pressure of 138 kPa for 20 min to extract pore-water samples, which were subsequently acidified to pH 3 with 100 mM HNO_3 and preserved at 4 ^{O} C until further analysis.

The redox potential was measured in the rice rhizosphere at each sampling time using a platinum electrode inserted into the soil and a Ag/AgCl reference electrode placed in the flood water, and with rest of the methodology followed as outlined by Fiedler et al. (2007).

Grain samples were obtained during harvest from each treatment plot. Approximately 50 g of grain was dehulled, milled, ground to flour and stored at 4 $^{\circ}$C until further analysis. More information on grain analysis can be found in Pillai et al. (2010).

3.3.3 As Extraction

The total As concentrations were determined by inductively-coupled-plasma mass-spectrometry (ICP-MS) after following a open digestion method with HNO_3/H_2O_2 (US-EPA, 2007).

The concentrations of As species were determined by high performance liquid chromatography (HPLC)-ICP-MS following a sequential extraction with 0.4 M H_3PO_4 and 0.4 M NaOH. Approximately 1g of soil was accurately weighed into 50 mL polypropylene centrifuge tubes. Ten mL of 0.4 M H_3PO_4 (pH 1.6) was added, and the suspensions were agitated for 6 hr on a reciprocating platform shaker. The samples were then centrifuged at approximately 7500g-force for 5 min, and the supernatant was decanted. Ten mL of 0.4 M NaOH (pH~12.2) were added to the soil residue, and the suspensions were agitated for 6 hr on a reciprocating platform shaker. Equal amounts from the two supernatant solutions were diluted 100-fold using a solution of 2 mM HNO_3 + 0.5 mM EDTA. The final sample matrix of 4 mM H_3PO_4 + 4 mM NaOH + 2 mM HNO_3 + 0.5 mM EDTA at a pH ~3.0 was then analyzed for concentrations of various As-species. A similar extraction procedure was followed to extract As species from root-plaque samples with approximately 2.5 g of fresh roots.

The concentrations of As-species in rice grain were determined by HPLC-ICP-MS following triflouroacetic acid (TFA) extraction (Heitkemper et al., 2001). Two mL of TFA were added to approximately 0.5 g of rice flour in a Teflon digestion tube and incubated for 2 hr at room temperature. The mixture was then heated at 80^{o}C for 4 hr or until the solvent had evaporated to dryness. The residue was then dissolved in 15 mL deionized water and filtered using a 0.2 µm nominal pore size cellulose nitrate membrane in a syringe filter. The total grain-As concentration was determined following HNO_3/H_2O_2 digestion (Zavala and Duxbury, 2008).

3.3.4 Instrumentation and Chemicals

The DMA^V and $MMAs^V$ were obtained from Chem Service (West Chester, PA, USA) as dimethyl arsinic acid and monosodium acid methanearsonate, respectively. Arsenate was obtained as sodium arsonate ($Na_2HAsO_4 \bullet 7H_2O$) from Sigma (St. Louis, MO, USA) and iAs^{III} as arsenite oxide (As_2O_3) from Alfa Aesar (Ward Hill, MA, USA). A Perkin Elmer 200 HPLC system (Waltham, MA, USA) with a guard column (Dionex IonPac AG7, Sunnyvale, CA, USA) and an anion-exchange column (Dionex IonPac AS7) was used for separation of As species, which were then quantified by in-line ICP-MS using a Perkin Elmer DRC-ELAN II. The HPLC instrument parameters are presented in Table 3.1. The post column addition of 3% methanol was used to offset ionization problems due to variable C concentrations. The total As was measured by ICP-MS.

3.3.5 Statistical Analyses

Statistical analysis such as standard error calculation and mean comparison analysis were performed using SigmaPlot version 11.0 (Systat Software, 2007). The graphs were constructed using both Windows Excel and SigmaPlot software. The statistical significance was measured at 95% confidence interval (CI).

Table 3.1. Instrument parameters used for speciation of arsenic in soil samples.

Instrument	HPLC-ICP-MS (Perkin Elmer model Elan DRCII)		
HPLC column	Ion exchange AS7 with AG7 guard column (Dionex)		
Mobile phase	Eluent A:1mM HNO_3 (pH~3)		
	Eluent B: 50mM HNO_3 (pH~1.5)		
As species measured	iAs^{V}, iAs^{III}, $DMAs^{V}$ and $MMAs^{V}$		
Gradient elution program			
Time (min)	A	B	Gradient
5 (equilibration)	100%	0%	0
2.5	100%	0%	0
6.5	0%	100%	1

3.4 Results and Discussion

3.4.1 As Speciation in Rhizosphere Soil Samples

The total concentrations of acid-digested soil-As were significantly higher in As-amended plots compared to No-MSMA plots as a result of MSMA application for over 20 years. The total As concentrations averaged 21.9 and 6.9 mg kg^{-1} in MSMA and No-MSMA plots, respectively (Figure 3.1). The total-As concentrations did not demonstrate any apparent differences among the water treatments for the 1 d and 60 d samples (Figure 3.1); but were noticeably higher in the intermittent plots than the continuously-flooded plots for the 120 d samples both in MSMA and No-MSMA plots (Figure 3.2). This trend indicates that more As was lost from the bulk soil under continuously flooded compared to the intermittently flooded conditions by the end of growing season, which could be due to plant uptake, leaching and/or volatilization.

The total As extraction efficiency by sequential extraction with H_3PO_4 + NaOH extract was approximately around 85% in MSMA flood, 73% in MSMA intermittent and No-MSMA flood and 65% in No-MSMA intermittent samples when compared to the total acid-digested As (Figure 3.1). Inorganic As, in the form of iAs^V was the predominant As species observed in the H_3PO_4 + NaOH extract from each of the air-dried soil samples. In comparison, iAs^{III} was only detected in trace quantities. The soil-sample pretreatment (air drying and grinding) likely resulted in the transformation of iAs^{III} to iAs^V, thus in subsequent discussions, the iAs^V and iAs^{III} concentrations of bulk-soil samples are summed and presented as inorganic As. The inorganic-As concentrations were significantly higher in the MSMA soil, with concentrations ranging from 14.7 to 17.3 mg kg^{-1} compared to 4.9 to 5.3 mg kg^{-1} in the No-MSMA soil (Figure 3.1). There were no consistent differences in inorganic-As concentration for the continuous-flood versus the intermittent-flood treatments. During the growing season, inorganic soil-As concentrations increased over time in the MSMA-plots, which is likely due to the conversion of organic As to inorganic As by microbial demethylation of MSMA. The inorganic-As concentrations did not vary appreciably during the growing season in the No-MSMA plots. Although As had been applied only in the form of MSMA to the MSMA plots, a low quantity of $MMAs^V$, accounting for 3 to 8% of the total As, was detected. Neither $MMAs^V$ nor $DMAs^V$ was detected in the No-MSMA plots (Figure 3.1). The $MMAs^V$ concentration gradually decreased over time in the MSMA plots during the 2007-season (Figure 3.1). Most of the 2007-applied MSMA was lost within 3 to 4 months after application, but there was no apparent difference in the rates of loss between the two water treatments. The corresponding increases in inorganic-As concentration suggest that MMAs was demethylated by microbes. Microbial demethylation of As is prominent in the environment (Cullen and Reimer, 1989), with studies reporting up to 50% demethylation

of $MMAs^{V}$ during a 10 to 70 d incubation period (Dickens and Hiltbold, 1967; Gao and Burau, 1997). Anaerobic demethylation of $MMAs^{V}$ and $DMAs^{V}$ to reduced As species such as iAs^{III} and $MMAs^{III}$ has also been reported (Sierra-Alvarez et al., 2006).

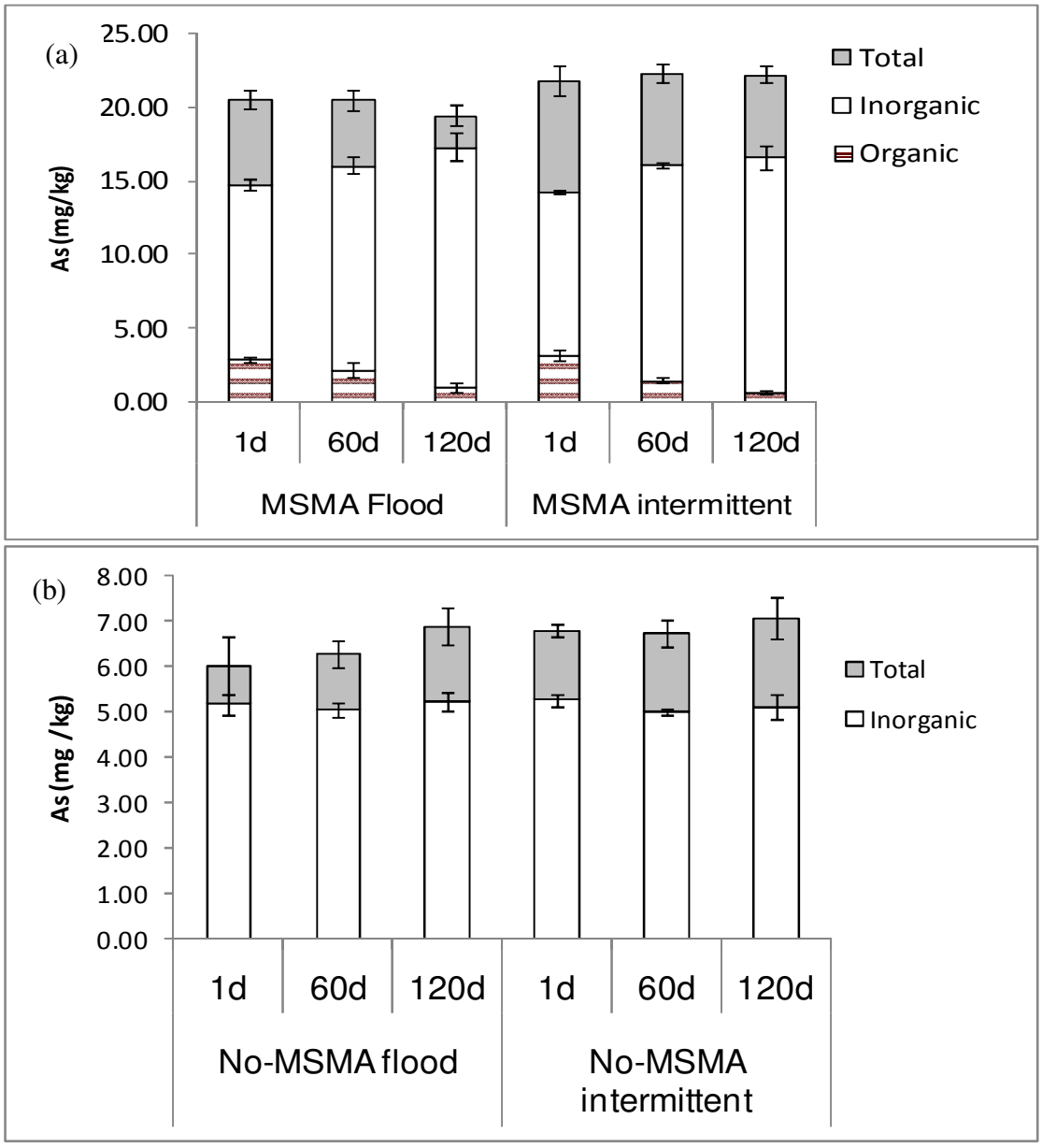

Figure 3.1. Concentrations of total, inorganic, and organic As species in MSMA (a) and No-MSMA (b) soil under continuous or intermittent flooding at 1 d after sowing (1 d), 4 weeks after flooding (60 d), and 3 weeks before harvest (120 d), in the 2007 experiment. Error bars indicate standard error of mean.

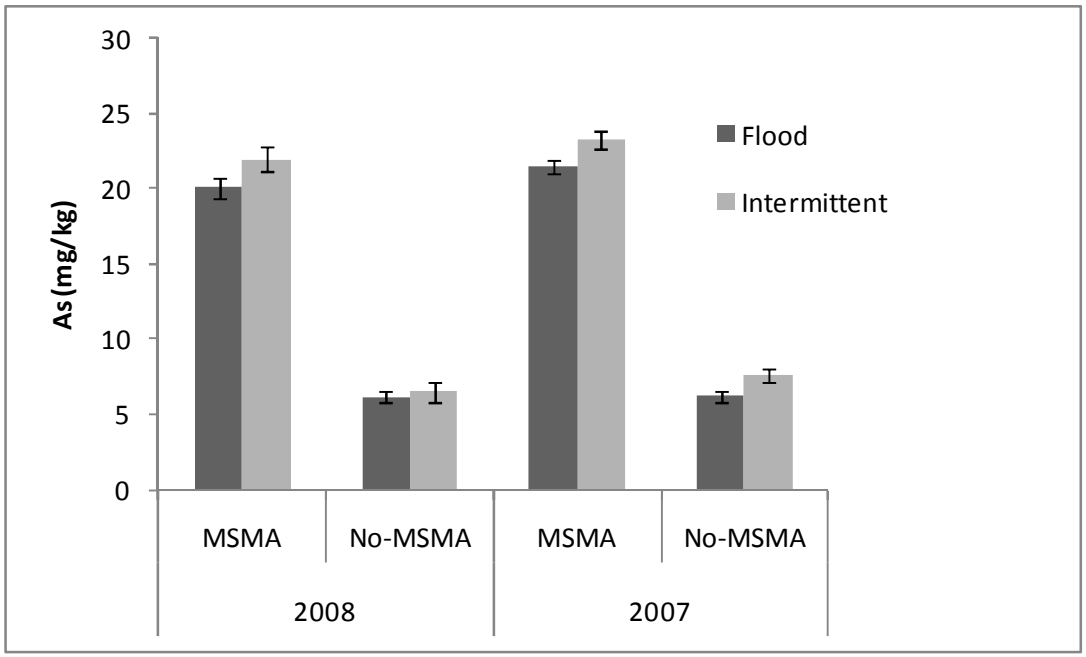

Figure 3.2. Concentrations of total acid digested As in rhizosphere soils at 120 d under different water management regimes and soil As concentrations. Error bars indicate standard error of mean.

3.4.2 As Speciation in Pore-water Samples

The average total-As concentrations of the soil pore-water samples ranged from 25.3 µg L^{-1} for continuous flood to 3.6 µg L^{-1} for intermittent plots (Figure 3.3). For the continuous-flood treatment, the total pore-water As concentrations were approximately 7 times higher than with the intermittent-flood treatment for both the MSMA and No-MSMA soils.

Arsenite was the predominant As species detected in pore-water with significantly higher concentrations detected in flood than the intermittent plots, with average concentrations of about 23.2 (±2.1) µg L^{-1} in MSMA flood to 5.0 (±1.6) in MSMA-intermittent plots and 14.1 (±1.3) µg L^{-1} in No-MSMA flood compared to 2.1 (±0.2) in No-MSMA intermittent (Figure 3.3). Arsenite accounted for 76% of the total As detected in the As-flood plots and 97 to 99 % of the total As detected with the other treatments. The intermittent flooding resulted in significant decreases in

pore-water iAs^{III} concentrations compared to that of the continuously flooded plots, with up to 78 and 85 % decrease in the MSMA and No-MSMA plots, respectively. Arsenite is a neutral and relatively more soluble As species at pH 7, especially under anaerobic conditions (Masscheleyn et al., 1991). It is interesting that the iAs^{III} concentration was higher in the No-MSMA-flood plots than the As-intermittent plots, indicating that water management had a greater impact than bulk-soil As concentration on the levels of dissolved iAs^{III} as well as total-dissolved As. The redox measurements taken at 60 d and 120 d sampling time-points indicated that the rice rhizosphere in the continuous-flood treatment was more highly reduced, with an average redox potential of -250 mv (±23) compared to -145 mv (±25) for the intermittent-flood treatment. At lower redox conditions, both increased iron-oxide dissolution and the reduction of iAs^{III} could result in higher soluble As concentrations in pore-water (Masscheleyn et al., 1991; Xu et al., 2008). Though the bulk soil contained moderate to high concentrations of iAs^{V}, the pore-water contained no detectable iAs^{V} except in the As-flood plots, in which case < 4% of the total As was present as iAs^{V}.

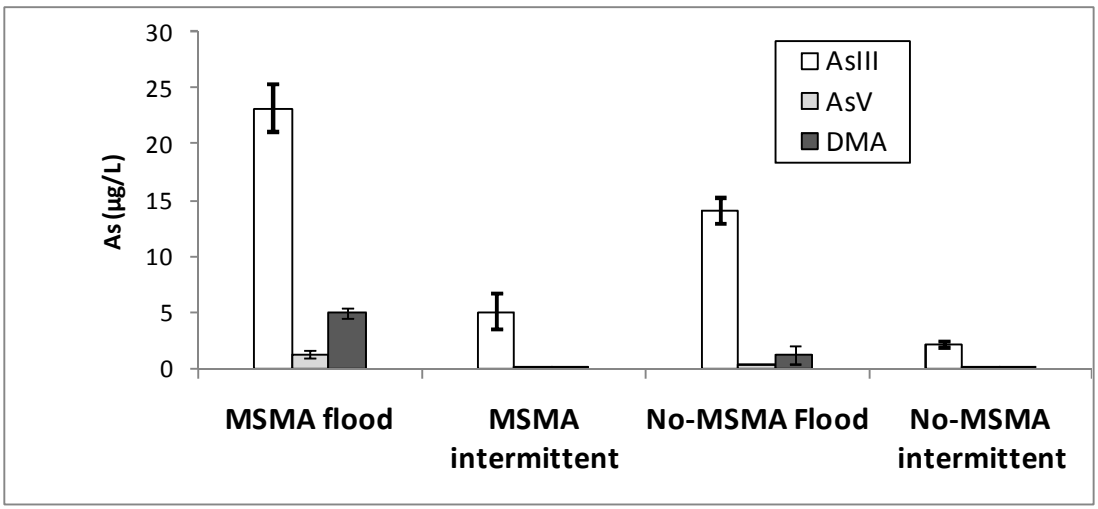

Figure 3.3. Concentrations of various arsenic species in pore-water samples at 120 d samples from yr 2008 under different water management regimes and soil As concentrations. Error bars indicate standard error of mean.

$DMAs^{V}$ was detected in the pore-water of As-flood plots at concentrations of approximately 5 µg L^{-1} in MSMA flood and around 1.2 µg L^{-1} in No-MSMA flood plots (Figure 3.3). $MMAs^{V}$ was not detected in any of the pore-water samples. $DMAs^{V}$ is a considerably more soluble species compared to $MMAs^{V}$ and iAs^{V} and is only negligibly adsorbed by iron oxides at pH > 7 (Lafferty and Loeppert, 2005). Similar concentrations of iAs^{III} and $DMAs^{V}$ have been observed in pore-water samples in other pot-scale studies (Li et al., 2009a; Xu et al., 2008). The presence of $DMAs^{V}$ in No-MSMA plots that did not receive any As-containing products indicate that microbial methylation processes were active in these systems, though there is no direct evidence from this study that methylation had proceeded to the volatile trimethyl-As species. Microbial methylation and volatilization of As is plausible under anoxic conditions (Bright et al., 1994) with studies reporting As loss up to 8.3% of the total As by volatilization from a As contaminated cattle-dip soil over a 5-month incubation (Edvantoro et al., 2004). The higher iAs^{III} concentrations in pore-water samples of the continuously flooded plots might have possibly induced microbial methylation of iAs^{III} to DMA^{V}, since microbial As-methylation is thought to be a detoxification process as DMA^{V} and MMA^{V} are less toxic compared to the iAs^{III} and iAs^{V} (Mukhopadhyay et al., 2002).

3.4.3 As Concentration and Speciation in Root-Plaque Samples

Root-plaque accumulated a higher proportion of As (based on total dry mass of root), with up to 10-times higher concentrations compared to the adjacent bulk soil (Figure 3.4). The total root-plaque As concentrations were significantly higher in the continuously flooded plots than in the intermittently flooded plots, for both the MSMA (287 ± 19 mg kg^{-1} for continuous-flood and 124 ± 23 mg kg^{-1} for intermittent-flood treatments) and No-MSMA soils (142 ± 10 mg kg^{-1} for continuous-flood and 70 ± 7 mg kg^{-1} for intermittent-flood treatments). Interestingly, the

total As concentration of the root-plaque from the No-MSMA-flood treatment (142 ± 10 mg kg^{-1}) was higher than that with the As-intermittent treatment (124 ± 23 mg kg^{-1}), though the bulk-soil As concentration was significantly higher with the latter treatment. This result indicates the substantial impact of water management on As accumulation at the root surface.

Arsenate was the predominant As species in each of the root-plaque samples and accounted for approximately 80% of the total As present on the root-plaques (Figure 3.4). Though iAs^{III} was the predominant As species detected in most pore-water samples (Figure 3.3), it seems that processes at the root surface might favor the oxidation of iAs^{III} to iAs^{V}, since the root surface is relatively more highly oxidized compared to the bulk soil. Arsenate concentrations were significantly higher in the MSMA compared to the No-MSMA samples and in continuously flooded compared to the intermittently flooded samples.

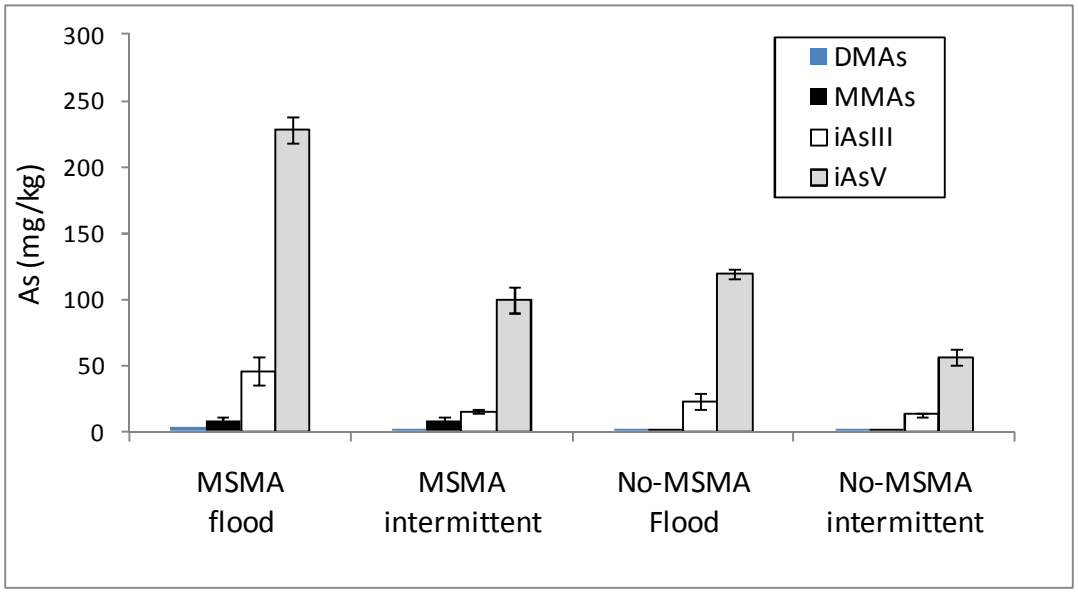

Figure 3.4. Concentrations of various As species in root-plaque samples at 120 d samples from yr 2008 under different water management regimes and soil As concentrations. Error bars indicate standard error of mean.

The major form of iron oxide comprising rice-root-plaque is reportedly poorly crystalline ferrihydrate, but some lepidocrocite and goethite has also been detected in previous studies (Bacha and Hossner, 1977; Taylor et al., 1984). Arsenate strongly bonds with these iron coatings and is not immediately bioavailable (Otte et al., 1991), thus root-plaque can reduce As uptake by plants (Hossain et al., 2009; Liu et al., 2004a).

Arsenite accounted for 9 to 23% of the total As in the root-plaque samples (Figure 3.4). This proportion contrasts with the As concentrations in the pore-water samples that contained mostly iAs^{III}. The root-plaque iAs^{III} concentrations were impacted by water management, with significantly higher iAs^{III} concentrations in the continuously flooded plots compared to the saturated plots. This trend was similar to that of the pore-water iAs^{III} concentrations. The iAs^{III}: iAs^{V} ratios were significantly higher in MSMA flood (0.223± 0.0135) compared to As intermittent (0.145 ±0.0141); whereas there was no significant difference between No-MSMA flood and No-MSMA intermittent plots. $MMAs^{V}$ was detected in low concentrations but only in the MSMA plots; concentrations did not vary appreciably with water treatment. $DMAs^{V}$ was detected in root-plaque from only the As-flood plots. $MMAs^{V}$ and iAs^{III} are each strongly adsorbed by iron oxides at the pH values normally observed under flooded-rice culture, i.e., pH 6-8 (Lafferty and Loeppert, 2005), and cannot be considered as immediately bioavailable for plant uptake. On the contrary, $DMAs^{V}$ has been reported to not be strongly adsorbed by goethite at pH values above 7 and above pH 8 by ferrihydrite (Lafferty and Loeppert, 2005), thus root-plaque might not impact the bioavailability of DMAs. Microorganisms in the rhizosphere could also impact the release and sequestration of As present on root-plaques, since many microorganisms have the potential for reducing or oxidizing As and Fe in the environment

(Kocar et al., 2006; Oremland and Stolz, 2003). Also localized methylation and demethylation processes are likely to impact As bioavailability.

3.4.4 Grain As Species Concentration

The total grain-As concentrations from the MSMA plots were significantly higher than the No-MSMA plots (Table 3.2). For all treatments, $DMAs^{V}$ and iAs^{III} were the only As species detected in rice grain. The total grain As, iAs^{III} and $DMAs^{V}$ concentrations from No-MSMA soils were similar to concentrations reported by Pillai et al. (2010) for rice grown in the same area with similar treatments. As expected, the total grain-As and $DMAs^{V}$ concentrations of rice grown in MSMA plots in the current study were considerably higher than the concentrations from No-MSMA soils from our study and results reported in several market-basket surveys (Williams et al., 2007a; Zavala and Duxbury, 2008); but iAs^{III} concentrations were similar to the concentrations from No-MSMA plots and previously reported values for rice samples originating from the South Central USA (Zavala et al., 2008). The As concentrations from No-MSMA plots were in similar range to the previously reported concentrations in rice grains originating from the South Central USA. $DMAs^{V}$ was the predominant grain-As species from the MSMA plots and accounted for 70 to 80% of TFA-extracted As. In No-MSMA plots, $DMAs^{V}$ and iAs^{III} were present at similar concentrations in the rice grain. Thus inorganic:organic ratios were much lower in MSMA plots (ratios ranging from 0.2 to 0.3) compared to the No-MSMA plots (0.7 to 1.8). Previous studies have reported higher $DMAs^{V}$ concentrations than inorganic-As concentrations in grain samples originating from US rice fields (Zavala et al., 2008), compared to samples originating from Europe and Asia that generally contained higher proportions of inorganic As (Meharg et al., 2009; Williams et al., 2007a). For example Meharg et al., (2009) compared relationships between total grain As and DMA concentrations on a region-specific basis and

reported that slopes of DMA against total As were high for US (0.777) compared to all other countries (0.137 - 0.199). The reason for higher organic:inorganic As ratios in rice grown in the South-Central USA is still unknown, though it might be at least partially attributable to differences in soil methyl-arsenic concentrations attributable to the microbial methylation of soil As. The organic As species are considered relatively less toxic compared to the inorganic As species.

The intermittent-flood treatment compared to the continuous-flood treatment lowered total grain-As concentrations by 25 to 30 % in the MSMA plots and 30 to 45 % in the No-MSMA plots. This decrease was attributable primarily to the lower grain-$DMAs^{V}$ concentrations with the intermittently flooded plots.

Table 3.2. Total As and As species concentrations ($\mu g\ kg^{-1}$) in rice grains grown under different water management regimes and soil As concentrations.

	Wells-2007			Cocodrie-2008		
	Total	As^{III}	$DMAs^{V}$	Total	As^{III}	$DMAs^{V}$
MSMA flood	1065^{a} (± 38.9)	172^{a} (± 11.9)	724^{a} (± 50.5)	950^{a} (± 29.4)	161^{a} (± 8.9)	654^{a} (± 29.1)
MSMA intermittent	801^{b} (± 49.6)	151^{ab} (± 12.0)	488^{b} (± 28.9)	660^{b} (± 30.8)	152^{ab} (± 14.8)	416^{b} (± 17.2)
No-MSMA flood	304^{c} (± 23.1)	138^{b} (± 18.4)	154^{c} (± 21.6)	315^{c} (± 16.8)	131^{b} (± 6.7)	167^{c} (± 15.4)
No-MSMA intermittent	207^{d} (± 20.8)	107^{b} (± 20.6)	118^{d} (±12.3)	173^{d} (± 21.2)	89^{d} (± 9.9)	97^{d} (± 16.3)

Note : Different letters (within a column) indicate means are significantly different. Values in parenthesis are standard error of mean.

The grain-$DMAs^{V}$ concentrations decreased by 30 to 50%, whereas grain iAs^{III} concentrations were decreased by only 5 to 30%. As a result, the inorganic:organic grain-As ratios were higher with the continuous-flood treatments compared to the intermittent-flood treatments. Pot-scale studies have indicated similar trends of decreased total grain-As concentration when rice was grown aerobically compared to continuously flooded conditions (Li et al., 2009a; Xu et al., 2008).

3.4.5 Relationships between Rhizosphere As and Grain As Concentrations, and Implications for Management

The results of the current study indicate that iAs^{III} accounted approximately for 68 to 99 % of the total dissolved As in the pore-water samples and $DMAs^{V}$ was only detected in MSMA-flood at about 14 % of total As and 7% of total As in the No-MSMA flood plots. While iAs^{III} accounted for only 14 to 56 % of the grain-As, $DMAs^{V}$ accumulated at higher concentrations in grain, accounting for approximately 78% of the grain-As in MSMA-amended plots and 52 % of the grain-As in No-MSMA plots. This phenomenon suggests that inorganic iAs^{III} was not as readily translocated to rice grains even if we assume that pore-water iAs^{III} was taken up; whereas it is possible that some of the grain $DMAs^{V}$ was either taken up from the rhizosphere soil and then translocated to the gains or produced as a result of within plant methylation of iAs^{III}. Both iAs^{V} and $MMAs^{V}$ were present at negligible quantities in pore-water samples. This is likely the reason that the rice plants did not accumulate iAs^{V} and $MMAs^{V}$ in the grains. A recent study has shown that rice plants can absorb both $MMAs^{V}$ and $DMAs^{V}$ through the aquaporin channel (Li et al., 2009b), and several studies have reported that DMAs was taken up by rice plants at considerably high concentrations (Huang et al., 2008; Marin et al., 1992). Both $DMAs^{V}$ and DMA^{III} species are more soluble and are not strongly adsorbed to iron oxides, whereas, iAs^{V},

iAs^{III} and $MMAs^{V}$ can strongly bind to iron oxides (Lafferty and Loeppert, 2005). Thus, even if we assume that root-plaque could reduce the immediate bioavailability of iAs^{V}, iAs^{III} and $MMAs^{V}$, it may not impact the bioavailability of $DMAs^{V}$. Arsenic methylation within rice plants cannot be completely ruled out; however, there is no experimental data yet to prove it (Meharg and Hartley-Whitaker, 2002). In any case, it is necessary to minimize soluble As concentrations in the rhizosphere in order to reduce grain As concentrations.

3.5 Conclusions

Intermittent flooding seems to be a viable management option to reduce grain As concentrations when rice is grown on soils with moderate to high As concentrations. The intermittent flooding treatment significantly reduced pore-water As-concentrations by 80 to 90% and grain As concentrations by 25 to 45% compared to the continuously flooded treatments. We did not detect $DMAs^{V}$ in either pore-water or in root-plaque samples from the intermittently flooded plots, in both MSMA and No-MSMA plots. $DMAs^{V}$ concentrations in grains were significantly reduced in intermittently flooded plots compared to the continuously flooded plots. As concentrations were significantly higher in root-plaque compared to the rhizosphere soil, and may be impacting the As availability to the plants.

CHAPTER IV

RHIZOPSHERE MICROBIAL POPULATIONS AND ARSENIC CONCENTRATIONS

4.1 Synopsis

Rice cultivated on As-contaminated soils may accumulate high concentrations of As in grains, mostly as a result of the continuous flooding practices commonly used for rice cultivation. Studies have suggested that the use of intermittent flooding might reduce soluble As concentrations in the rice rhizosphere; however, these practices will also likely alter soil microbial populations that may impact As chemistry through oxidation/reduction and methylation/demethylation processes. A field-scale study was conducted to analyze As concentrations and microbial populations in the rice rhizosphere, in response to continuous and intermittent flooding practices under two levels of soil As. Rhizosphere soil and pore-water As-concentrations were quantified using an ICP-MS, while microbial populations in the rice rhizosphere soil were determined using fatty acid methyl ester analysis (FAME), community qPCR and 16S rRNA gene pyrotag sequencing. Average pore-water As-concentrations ranged from 17 $\mu g\ L^{-1}$ in intermittently flooded plots to 44 $\mu g\ L^{-1}$ in the continuously flooded plots, representing a decrease of approximately 60 to 64% with intermittent flooding. Multivariate FAME analysis indicated that microbial communities changed temporally among the treatments. Community qPCR results demonstrated that the relative abundance of *Bacteria* increased over the course of the growing season, while archaeal and fungal gene abundances decreased. Although qPCR results showed little variation in bacterial relative abundance among the treatments, the 16S rRNA sequence libraries demonstrated that bacterial community structure and membership were significantly different among the treatments. Both qPCR and 16S rRNA

sequencing indicated that relative abundance of iron-reducing bacteria and sulfate-reducing bacteria were significantly higher under the continuous flooding relative to the intermittent flooding treatment, implying active iron reduction and possibly As release from the iron oxides. These results indicate that rhizosphere microbial populations were different in intermittently flooded compared to the continuously flooded plots, which may have impacted decreased concentrations of pore-water arsenic in the intermittently flooded plots.

4.2 Introduction

Arsenic is a naturally occurring metalloid that is toxic to most forms of life. Natural As-contamination occurs throughout the world, resulting in more than 50 million people being exposed to high As concentrations through drinking water (BGS and DPHE, 2001; Duxbury et al., 2003). Additionally, As exposure through the consumption of As-rich rice is reported to be an additional major exposure route for a sizeable population in South-East Asia (Mondal and Polya, 2008). This has caused increasing concern regarding the cultivation of rice on As-contaminated soils with several recent studies reporting high As concentrations in rice grain originating from different parts of the world, including the rice grown in the South-Central USA (Meharg et al., 2009; Zavala et al., 2008).

In 2008, a total of 1.19 million ha of rice was grown in the USA with more than 80% of that total acreage being cultivated in South-Central states of Arkansas (45.5%), Louisiana (16.3%), Mississippi (7.8%), Missouri (6.8%) and Texas (5.4%) (USDA-ERS, 2009). Most of the rice fields in this region were historically used for cotton production and received repeated applications of arsenic-based defoliants and pesticides such as sodium hydroxy-methylarsinate, commonly known as monosodium methyl arsenate (MSMA) (Woolson, 1977). Although As-based pesticides are no longer used in cotton or rice production, soils with a history of being

amended with arsenical-pesticides often contain considerable amounts of residual As (Gilmour and Wells, 1980).

Arsenic can exist as a variety of different chemical species under typical environmental conditions, and transformations between these chemical species are largely mediated by microbial processes. The most commonly encountered inorganic species are arsenate (iAs^{V}) and arsenite (iAs^{III}), and the most commonly encountered organic species are monomethylarsonic acid ($MMAs^{V}$) and dimethylarsinic acid ($DMAs^{V}$), which are primarily the products of microbial methylation (Cullen and Reimer, 1989). The species iAs^{III} and DMA^{V} are more soluble and bioavailable than other species (e.g., (iAs^{V}), and thus, are more commonly found in rice rhizosphere, pore-water and rice grain (Xu et al., 2008). Transformations between iAs^{V} and iAs^{III}, and between organic and inorganic As predominantly occur due to microbially mediated As oxidation, reduction, methylation, and demethylation processes (Oremland and Stolz, 2003). These transformations can result from a detoxification mechanism (Cullen and Reimer, 1989; Jackson et al., 2005), or they can be linked to cellular metabolism and growth (Oremland and Stolz, 2003). Other microorganisms including iron- and sulfate-reducing bacteria can also impact As solubility and adsorption through the reductive dissolution of minerals (e.g., iron oxides) that adsorb As (Horneman et al., 2004).

Rice tends to accumulate higher amounts of As relative to other cereals (Williams et al., 2007b), largely due to the continuous flooding practices commonly used in rice production. Recent studies have reported that growing rice under more aerobic (non-flooded or intermittently flooded) conditions decreases As concentrations in both the rice grain and rhizosphere (Li et al., 2009a; Xu et al., 2008). In one of our other field experiments, a significant decrease in soluble-

As concentrations in the rice rhizosphere and total grain-As concentrations was observed when rice was grown using intermittently flooded as compared to the continuously flooded conditions.

While intermittent flooding appears to have potential for reducing the accumulation of As in rice grain, there has been no research to date specifically investigating the impacts of intermittent flooding on rhizosphere microbial communities in soil with a long-term history of exposure to arsenic-based pesticides. Both As concentrations and redox conditions can impact microbial populations (Edvantoro et al., 2003; Zhou et al., 2002), and, as a result, microbial populations are likely to be very different in intermittently flooded than in continuously flooded soil. Studies are needed to understand the impacts of different water management practices on soil microbial communities in order to ultimately understand the roles of the microorganisms in controlling the As biogeochemistry and bioavailability. In order to address these issues, we conducted a field experiment investigating changes in rice rhizosphere As concentrations and microbial communities, over a growing season, under continuous or intermittent flooding and either amended or not amended with MSMA.

4.3 Material and Methods

4.3.1 Field Experiment

This field experiment was conducted in the year 2007 at Dale Bumpers National Rice Research Center, US Department of Agriculture, Agriculture Research Service, Stuttgart, AR, USA. One of the research plots (approximately 1ha area) has had continuous application of monosodium methane-arsonate (MSMA), in alternate years for more than twenty years in order to screen for rice varieties resistant to straighthead disorder (linked to high soil-As concentrations) (Yan et al., 2005). The MSMA was surface applied to the soil before planting, at the rate of 6.7 kg $ha^{-1}yr^{-1}$ (equivalent to 3.1 kg As $ha^{-1}yr^{-1}$) and these plots will be referred as

'MSMA' hereafter. The adjacent native soil (referred as 'No MSMA' hereafter) had not received any As-containing products for at least the last 20 years. The two water treatments included intermittent and continuous flooding, which were superimposed on MSMA and No-MSMA treatments. Under intermittent flooding, the plots were flooded, allowed to dry until surface cracking initiated, and then were re-flooded. The treatment combinations used in this study were (1) MSMA flood (2) MSMA intermittent (3) No-MSMA flood and (4) No-MSMA intermittent. The treatment plots were distributed using a split-split plot design and four replicates within each treatment were arranged in completely randomized design. The seeds were sown in the middle of April and the first flood was introduced four weeks after sowing, when the plants were about 30 cm tall. All of the other management practices were followed as outlined by Yan et al., (2005).

4.3.2 Sampling

The rhizosphere soil samples were collected at planting (1 d after sowing), 4 weeks after first flooding (60 d after sowing) and 3 months after first flooding (120 d after sowing) from three of the four replicates. At 1 d, six soil cores per plot, from a depth of 6 cm, were collected randomly from the experimental plots and composited into one sample per plot. At 60 d and 120 d time-points three rice plants per plot were collected along with the adhering bulk soil. Plants were shaken to remove loose soil and the remaining non rhizosphere soil was removed manually. This left only a few millimeters of soil around the roots, which was then collected and composited into one rhizosphere sample per plot. The rhizosphere samples were then split into two subsamples, one for chemical analysis and the other for microbial analysis. The samples for chemical analysis were transported from the field to the lab at $4^{O}C$, and the samples for microbial analysis were immediately frozen on dry ice and then stored at $-80^{O}C$ until further analysis. The

samples for chemical analysis were split into two more subsamples, one for As analysis and the other for fatty-acid-methyl ester (FAME) analysis. The subsample for As analysis was air-dried and ground to pass through a 0.2 mm sieve. The total soil As concentrations were determined by inductively-coupled-plasma mass-spectrometry (ICP-MS), model DRC-ELAN II (Perkin Elmer, Waltham, MA, USA) after following a open digestion method with HNO_3/H_2O_2 (US-EPA, 2007).

Rhizosphere pore-water samples were collected at 60 d and 120 d (the plots were not yet submerged at 1 d). Bulk soil samples from 0-6 cm depth were collected from the rhizosphere area (between two adjacent plants) using a 2.5 cm diameter corer with brass insert rings. The insert rings with soil samples were capped with polypropylene end caps and stored on ice during transport to the lab. The core samples were then vacuum filtered at a negative pressure of 138 kPa for 20 min to extract pore-water samples, which were then acidified to pH 3 with 100 mM HNO_3, preserved at 4^oC, and subsequently used for total dissolved As analysis. The As concentrations in soil and pore-water samples were determined using an ICP-MS instrument model DRC-ELAN II (Perkin Elmer, Waltham, MA, USA).

Redox potential was measured in the field at each sampling time using a platinum electrode inserted into the soil and a Ag/AgCl reference electrode placed in the flood water. The methodology for measuring redox potential was followed as outlined by Fiedler et al. (2007).

4.3.3 Microbial FAME Analysis

The subsamples for FAME analysis were air-dried for 3 to 6 hr to decrease water content and then extracted for FAMEs following the procedure outlined by Olexa et al. (2000). Briefly, 3 g of soil from each of the 3 sampling points were extracted using a FAME procedure which involved cell lysis, initial saponifcation and methylation of fatty acids and subsequent extraction.

Extracted samples were sent to the University of Delaware Plant and Soil Sciences Department for analysis using an Agilent model 6890 gas chromatograph with flame ionization detector (Agilent, Wilmington, DE). Two micro liters of each sample were injected into a Hewlett Packard (Agilent) Ultra 2 (Cross linked 5% Phenyl methyl silicone) column 25 m x 0.20 mm x 0.33 μm with a 100:1 split ratio and flow rate of 0.6 ml/min using hydrogen as the carrier gas. The injection temperature was 250°C, and the detection temperature was 300°C. The initial oven temperature was 170°C and ramped at 5°C/min to a final temperature of 300°C, for a total run time of 12.0 min. Peaks were named using Sherlock Eukary program (MIDI, Inc., Newark, DE).

The marker FAMEs included 18:1 ω9c, 18:2 ω6c and 18:3 ω6c for *Fungi;* 15:0 iso, 15:0 anteiso, 17:0 iso, 17:0 anteiso for Gram-positive bacteria; 15:0 2OH, 15:0 3OH, 18:0 2OH and 20:0 3OH for Gram-negative bacteria (Olexa et al., 2000).

4.3.4 DNA Extraction

Microbial community DNA from the rhizosphere soil was extracted using MO BIO PowerMax DNA extraction kits (MO BIO Laboratories Inc., Carlsbad, CA, USA). The manufacturer's protocol was modified to include a lysozyme pre-incubation step in order to enhance Gram-positive bacterial DNA yields. Approximately 10 g of soil was weighed into each tube. A 10-μl aliquot of lysozyme solution (1 mg ml^{-1} final concentration) was then added and the tubes were incubated in a water bath for 1 hr at $37^{O}C$ with occasional shaking. The manufacturer's protocol was continued from this step onward. After the final elution step, the DNA samples were concentrated by ethanol precipitation. The DNA samples were then purified using illustra MicroSpin™ G-25 Columns (GE Healthcare Biosciences, Pittsburgh, PA, USA) and stored at $-20^{O}C$ for further analysis.

4.3.5 Gene Copy Number Quantification Using qPCR Targeting

The quantitative PCR (qPCR) assays targeting total *Bacteria*, *Archaea*, *Fungi*, iron-reducing bacteria (FeRB) and sulfate-reducing bacteria (SRB) were performed using the group specific primer sets and qPCR conditions outlined in Table 4.1. The FeRB were determined by targeting *Geobacteracaea* and *Shewanella* spp. and SRB by targeting the *dsrA* gene. The assays were performed in a 10-µL reaction mix containing 4.5 µl SYBR green real master mix (5Prime, Inc., Gaithersburg, MD, USA), 0.5 µl of each primer (concentration of 10 µM for *Bacteria, Fungi* and *Archaea*, 200 nM for *dsrA* and *mcrA* and 300 nM for *Geobacteracaea* and *Shewanella* spp.), 1 µl template (2.5 ng), 1 µl bovine serum albumin (10 mg ml^{-1}) and 2.5 µl molecular grade water. Each analysis run included a set of standards, controls, blank and samples (all including three analytical replicates) on a 96 well plate. The PCR reactions were conducted at the temperatures listed in Table 4.1 with 40 amplification cycles. Melting curve analysis of the PCR products was performed after each assay to confirm PCR amplification quality. The qPCR was performed using an Eppendorf Mastercycler® ep realplex thermal cycler (Eppendorf, Hamburg, Germany). Standards for qPCR were generated by PCR-amplifying each gene of interest from the genomic DNA of pure cultures using the primers and details listed in Table 4.1. The PCR products were confirmed on an agarose gel, and then cloned into a pGEM®-T Easy vector following the manufacturer's instructions (Promega, Madison, WI, USA). Positive clones were isolated and extracted for plasmid DNA using a Wizard SV Miniprep kit (Promega, Madison, WI, USA). The plasmid DNA concentrations ranging from 5.0×10^{-3} to 5.0×10^{-7} ng μl^{-1} DNA were used to generate the qPCR standard curves. Relative abundance was estimated by calculating ratios of gene copy numbers for each microbial population to the total community gene copy numbers (sum of gene copy numbers for *Bacteria, Archaea* and *Fungi).*

Table 4.1. Conditions and primer sets used for quantitative PCR assays for enumerating the relative abundance of selected microbial populations.

Target group	Primers	Annealing temperature	Standard (source)	Reference
Total *Bacteria*	Eub338- -5'-ACT CCT ACG GGA GGC AGC AG -3' Eub518 --5'-ATT ACC GCG GCT GCT GG-3'	53 °C	*Escherichia coli* DH10B(pUC19) (Carlos Gonzales, Texas A&M Univ.)	(Fierer et al., 2005)
Total *Fungi*	ITS1f- 5'-CTT GGT CAT TTA GAG GAA GTA A-3' 5.8s—5'-CGC TGC GTT CTT CAT CG-3'	61 °C	*Neurospora crassa* (Heather Wilkinson, Texas A&M Univ.)	(Boyle et al., 2008)
Total *Archaea*	Arc85f--5′-ACT GCT CAG TAA CAC GTG GA-3′ Arc313r--5′-ATG TCT CAG AAT CCA TCT CC-3′	53 °C	*Methanosarcina acetivorans* C2A (William Metcalf, Univ. of Illinois)	(Lima and Sleep, 2007)
Geobacteracea spp.	Geo564F -5'-AAG CGT TGT TCG GAW TTA T-3' Geo840R -5'-GGC ACT GCA GG GGT CAA T A-3'	57 °C	*Geobacter metallireducans* (Jizhong Zhou, Univ. of Oklahoma)	(Cummings et al., 2003)
Shewanellae spp.	She 120F – 5'-GCC TAG GGA TCT GCC CAG TCG-3' She 220R – 5'-CTA GGT TCA TCC AAT CGC G-3'	60 °C	*Shewanella oneidensis* MR-1 (Jizhong Zhou, Univ. of Oklahoma)	(Himmelheber et al., 2009)
dsrA gene	dsr-1F--5-ACS CAC TGG AAG CAC G-3 dsr-500r--5-CGG TGM AGY TCR TCC TG-3	58 °C	*Desulfovibrio vulgaris* Hildenborough (Jizhong Zhou, Univ. of Oklahoma)	(Wilms et al., 2007)

4.3.6 16S rRNA Gene Sequencing

Equal quantities of community DNA from all 3 field replicates for each treatment at 120 d were composited into one sample per treatment. The composited DNA samples were then adjusted to a concentration of approximately 100 ng μl^{-1} and submitted to the Research and Testing Laboratory (Lubbock, TX, USA) for tag-pyrosequencing. Samples were amplified with modified versions of primers 530F and primer 1100R (Acosta-Martinez et al., 2008), and the amplicons were sequenced using Roche 454 Titanium chemistry.

4.3.7 Statistical and Sequence Analyses

Nonmetric-multidimensional scaling (NMDS) analysis of FAMEs was performed using the PC-ORD software version 5.0 (McCune and Mefford, 2006). SigmaPlot version 11.0 (Systat Software, 2007) was used for calculating Fisher mean difference and standard errors for the experimental data, and also for creating graphs.

The 16S rRNA gene sequence data was analyzed using the pyrosequencing pipeline tools available from the Ribosomal Database Project (RDP) (Cole et al., 2009) and MOTHUR version 1.6.1 (Schloss et al., 2009). The 16S rRNA sequences were first trimmed for primers and chimeras, and then sequences with < 200 bases were removed from the data sets. The total number of sequences in each library ranged from 1200 to 1650 among the treatments. In order to minimize any bias as a result of this divergence in the total number of sequences, we randomly removed sequences from each sequence library to retain a total of 1200 sequences for each treatment. The individual sequence files were combined into one single file using the BioEdit *v7.0.5* software (Hall, 1999) and then were submitted to the RDP aligner tool for multiple sequence alignment. Pairwise distances between the aligned sequences (distance matrix) were

calculated and used to assign sequences to operational taxonomic units (OTUs) (cluster analysis). Both distance matrix and cluster analyses were performed using the dist and cluster analyses tools in MOTHUR using default settings. The OTUs were defined at 97% similarity cutoff for all the analyses. Both α and β-diversity measures were estimated for the data sets using the summary.single and summary.shared tools in MOTHUR. The α-diversity measures included Chao1 richness, Shannon and Simpson measures and the β- diversity measures included the Yue and Clayton theta and Jaccard similarity indices. Jaccard similarity values demonstrate the similarity in community membership, while Yue and Clayton theta describes community similarity as a function both composition and relative abundance.

A dendogram was constructed using the MEGA 4 software (Tamura et al., 2007) after running the Bray-Curtis community structure analysis with MOTHUR. A phylip-formatted distance matrix file was constructed using the dist function in MOTHUR and used to create a neighbor-joining tree with neighbor tool available with the PHYLIP 3.68 package (Felsenstein, 2005). The PHYLIP generated tree was then used as an input file to run the parsimony *p* test (Martin, 2002) using MOTHUR. Parsimony analysis is commonly used to test whether two or more communities harbor greater differences in phylogenetic structure than would be expected by chance; and a *p* value of ≤ 0.001 is considered to be statistically significant (Martin, 2002). Relative abundance of taxonomic phyla and families among the treatment samples were determined with RDP Library Compare.

4.4 Results

4.4.1 Rhizosphere Pore-water and Soil As Concentrations

The total As-concentrations from the rhizosphere soil and pore-water samples from different treatments were analyzed at 3 different time-points during the rice growing season. The soil-As concentrations were significantly higher in MSMA plots with a growing season average (mean) of 21.1 mg kg^{-1} (±2.0) compared to 6.4 mg kg^{-1} (±0.9) in No-MSMA plots (Figure 4.1). There were no apparent differences in soil As-concentrations between two water management systems, nor were differences found among the sampling times. The pore-water As was measured only at 60 d and 120 d as the plots were not yet submerged at 1 d. At 60 d, the pore-water As concentrations were significantly lower in No-MSMA plots with an average of 20.2 µg L^{-1} (±2.7) compared to 28.8 µg L^{-1} (±3.7) in MSMA plots. Additionally, intermittently-flooded plots had lower pore-water As concentrations than continuously flooded plots with average values of 14.1 µg L^{-1} (±2.4) compared to 34.9 µg L^{-1} (±4.0), respectively. Similar trends were observed at 120 d with significantly lower pore-water As-concentrations observed in No-MSMA plots (28.5 ±2.7 µg L^{-1}) than in MSMA plots (44.3 ± 4.4 µg L^{-1}); and also in intermittently flooded (19.2 ± 2.6 µg L^{-1}) plots than in continuously-flooded plots (53.6 ± 4.6 µg L^{-1}). The pore-water As-concentrations also varied temporally with considerably higher concentrations observed at 120 d compared to 60 d in all the treatment plots. It is also interesting to note that pore-water As-concentrations were higher in No-MSMA flood than the MSMA intermittent, even though soil As-concentrations were 4 to 5 folds higher in the MSMA -intermittent plots (Figure 4.1). The rhizosphere of continuously flooded plots was more reduced with a growing season redox-potential average of -216 mv (±16.8), compared to -143 mv (±9.6) in the

intermittently flooded plots; and the plots were more reduced at 120 d compared to the 60 d sampling time.

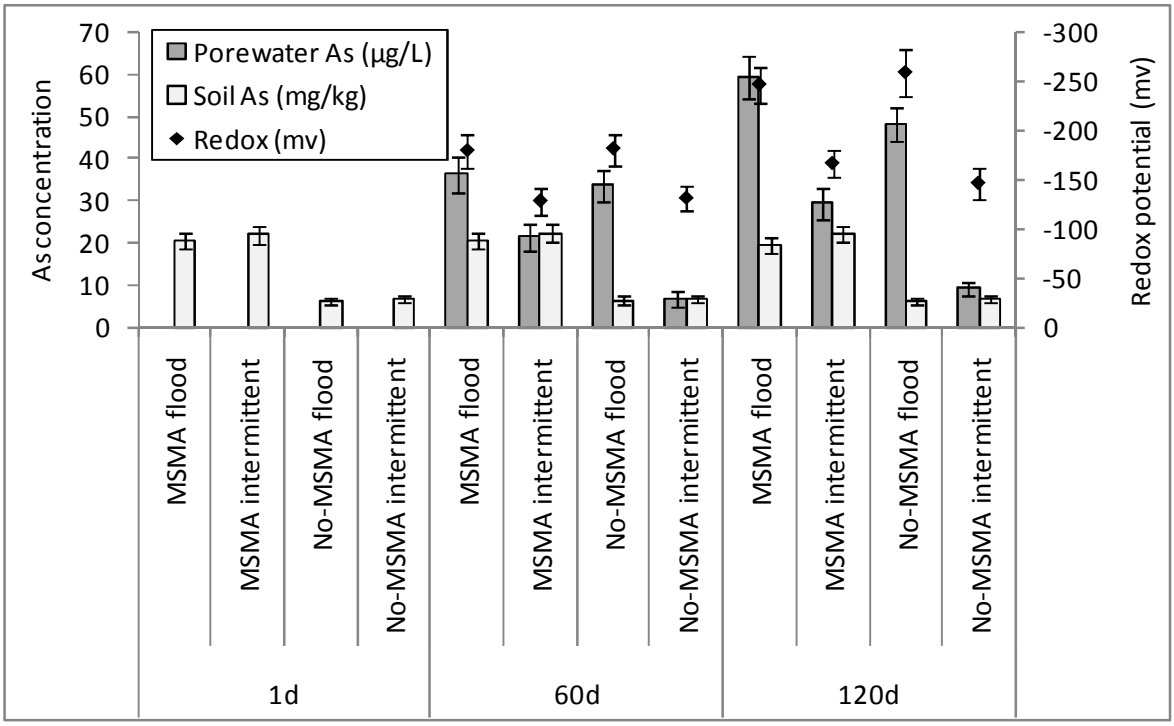

Figure 4.1. Total soil-As and pore-water As concentrations, and redox measurements in rhizosphere soil under different water management and As concentration treatments at 1, 60, and 120 d after sowing. The pore-water samples and redox measurements were taken only at 60 d and 120 d, as the plots were not yet flooded at 1 d. Error bars represent standard error of mean.

4.4.2 Soil FAME Analysis

A nonmetric multidimensional-scaling (NMDS) plot for FAMEs extracted from rhizosphere soil is presented in Figure 4.2. The microbial communities in the experimental plots varied temporally and also among the treatments. At 1 d, the samples grouped by MSMA and No-MSMA, but not by water treatments (water treatments were not yet imposed). At 60 d, the

samples separated both by As and water treatments; similarly at 120 d time-point, the samples separated by water treatments to a greater degree and also to some extent by the As treatments. Further separation of the samples occurred at 120 d among the treatments, compared to 1 d and 60 d time-points.

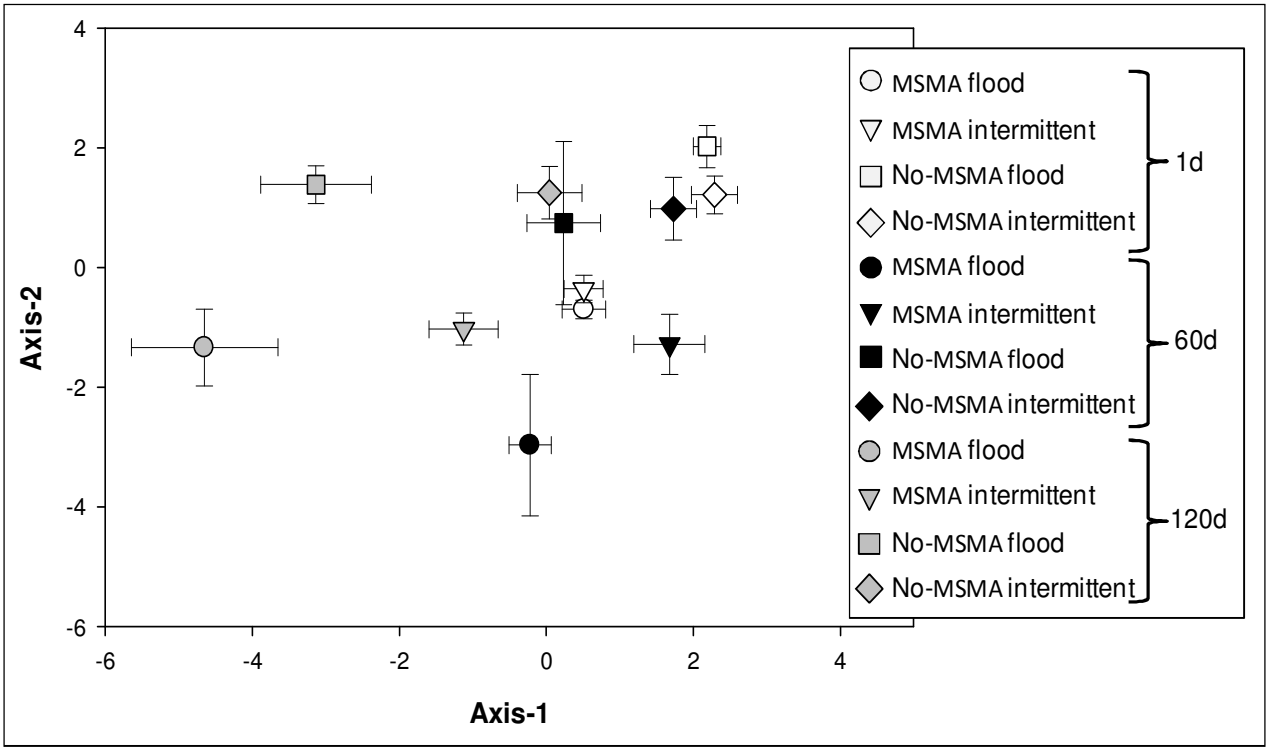

Figure 4.2. Nonmetric multidimensional-scaling of FAMEs from rice rhizosphere under different arsenic and flooding treatments at 1, 60, and 120 d after sowing. Error bars represent standard error of mean.

The relative abundance of *Fungi* and *Bacteria* were calculated by comparing marker FAMEs specific to *Fungi* with those specific to Gram-positive bacteria. No marker FAMEs specific to Gram-negative bacteria were detected. The results indicated that the *Fungi:Bacteria* ratios at 1 d time-point did not vary among the treatments. At the 60 d time-point, however, the ratios were significantly greater in the intermittent plots than the flooded (Figure 4.3). Similarly

at 120 d time-point, the ratios were significantly higher in intermittent than the flooded plots, but they did not differ with As treatment.

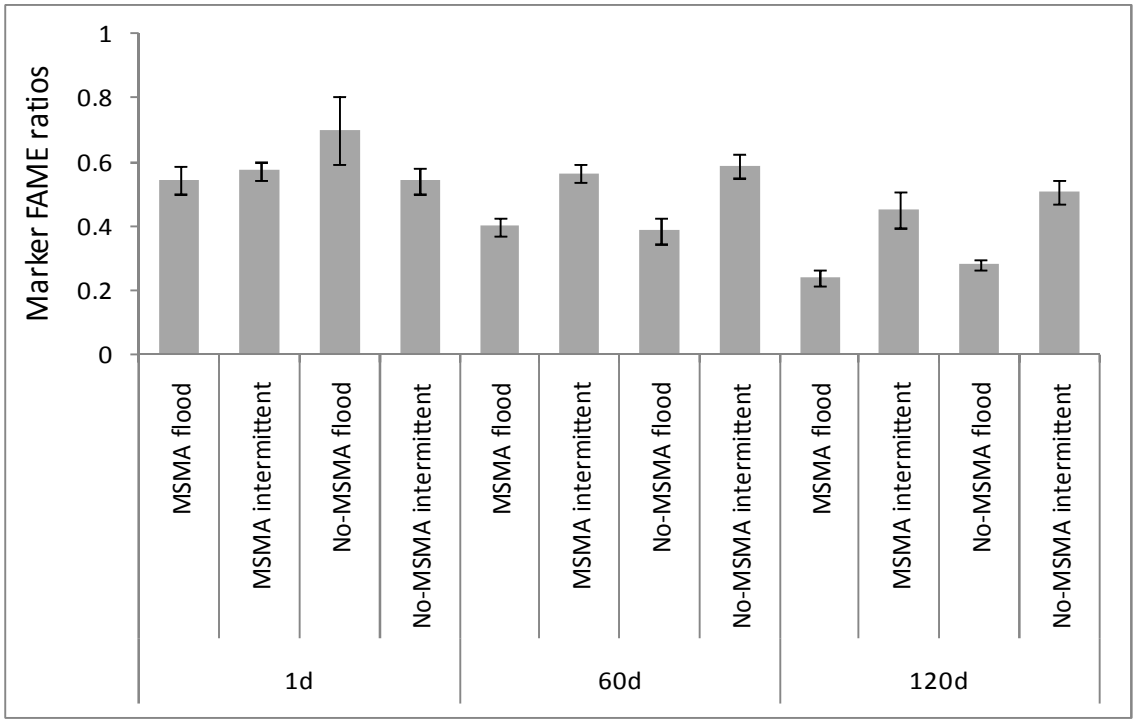

Figure 4.3. Relative abundance of *Fungi* and *Bacteria* in the rhizosphere soil under different water management and As concentration treatments at three time points, as estimated by marker FAME ratios. The values on y-axis represents the marker FAME ratios for *Fungi*:*Bacteria*. Error bars represent ± standard error of mean.

4.4.3 qPCR Assays for Relative Abundance of Microbial Populations

The relative abundances of *Bacteria, Archaea* and *Fungi* to the total microbial-community (sum of gene copy numbers for *Bacteria*, *Archaea* and *Fungi*) are presented in Figure 4.4. At 1 d, the relative abundance of bacterial populations was higher than *Archaea* and *Fungi* in all treatment plots. At 60 d the relative abundance of *Bacteria* decreased slightly

compared to 1 d which correlated with an increased relative abundance of *Archaea* in most treatments. Fungal populations also decreased in most treatments compared to the 1 d time-point. Relative abundance of *Bacteria* and *Archaea* were significantly higher than *Fungi* in most treatments except for the MSMA flood treatment. Archaeal abundance seemed to be slightly higher in flood compared to intermittent with No-MSMA plots, but there was no apparent difference among the water treatments within the MSMA plots or between the MSMA treatments. Fungal abundance was significantly higher in intermittent than the flood with No-MSMA plots and vice versa with MSMA plots. Bacterial abundance did not demonstrate any apparent difference among the treatments. At 120 d, *Bacteria* predominated in all treatments, with relative abundances ranging from 63% in the MSMA-flood plots to 75% in the MSMA-intermittent plots. Archaeal relative abundance was significantly higher in flood compared to intermittent plots with MSMA treatment, but not with No-MSMA treatment; whereas the bacterial and fungal relative abundance demonstrated no significant difference among the treatments.

The abundances of FeRB and SRB, as a proportion of the total microbial community (sum of gene copy number for *Bacteria*, *Fungi* and *Archaea*), are presented in Figure 4.5. At 1 d, both FeRB and SRB were present at relatively low levels with FeRB comprising less than 5% of the total community and SRB less than 2% in most treatments. Relative abundance of FeRB was slightly higher in the No-MSMA plots compared to the MSMA-amended plots, but there was no difference in SRB abundance among the treatments. At 60 d, the relative abundance of FeRB increased to 10 to 20% of the total community, with slight differences among the treatments.

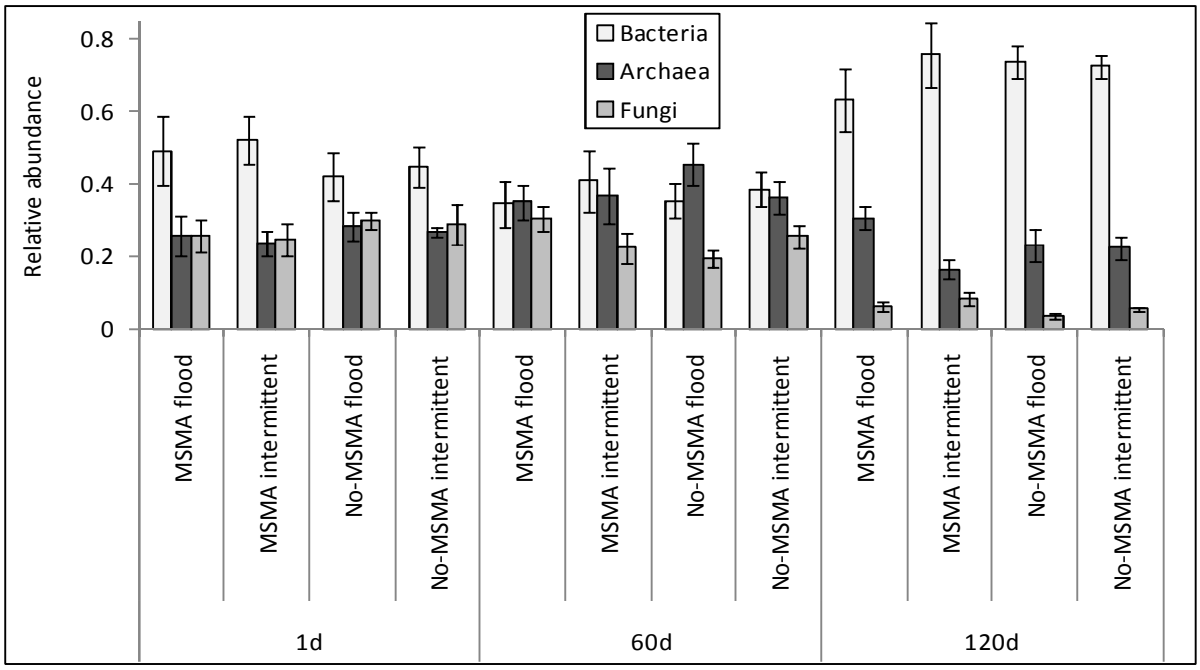

Figure 4.4. Relative abundance of *Archaea, Fungi* and *Bacteria*, determined using qPCR assays, in rice rhizosphere under different arsenic and flooding treatments at 1, 60, and 120 d after sowing. The values on y-axis represent the abundance of *Bacteria* or *Archaea* or *Fungi* to total gene copy numbers of *Bacteria* + *Archaea* + *Fungi*. Error bars represent standard error of mean.

Relative abundance of FeRB was slightly different among the treatments, but there was no consistent difference among the treatments. Relative abundance of SRB also increased slightly at 60 d, relative to the 1 d time-point, comprising approximately 3 to 7% of the total community; but there were no apparent differences among the treatments. At 120 d, the FeRB relative abundance increased to around 16 to 26% in the flooded plots and 5 to 10% in the intermittent plots. The FeRB were found to be significantly higher in continuously flooded plots with an average relative abundance of 21 % compared to about 7 % with the intermittent plots. The relative abundance of FeRB was slightly higher in the MSMA-amended plots compared to the No-MSMA plots. Relative abundance of SRB was also significantly higher in continuously flooded plots compared to intermittent plots.

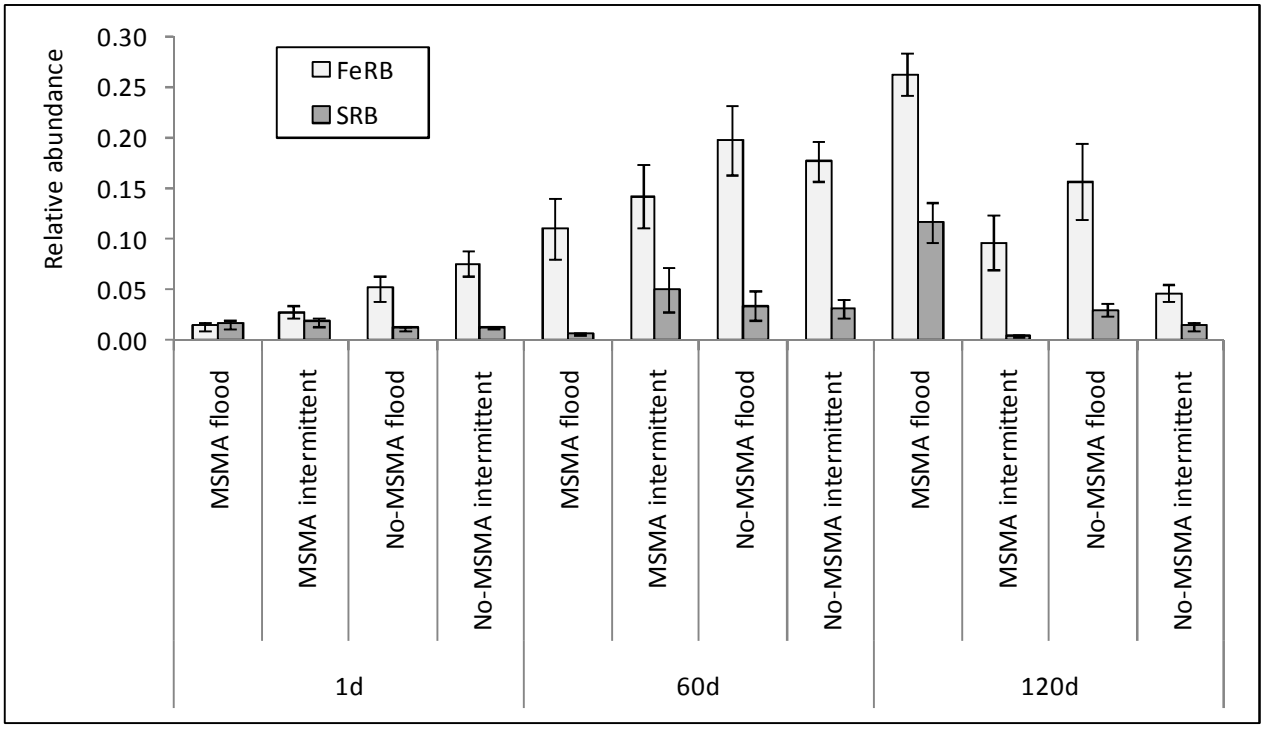

Figure 4.5. Relative abundance of iron-reducing bacteria (FeRB) and sulfate-reducing bacteria (SRB) in rice rhizosphere soil under different arsenic and flooding treatments at 1, 60, and 120 d after sowing. The values on y-axis represent the abundance of FeRB or SRB to total gene copy numbers of *Bacteria* + *Archaea* + *Fungi*. Error bars represent standard error of mean.

4.4.4 16S rRNA Sequence Analyses

The bacterial communities in the rhizosphere soil were evaluated by obtaining over 1200 partial 16S rRNA gene sequences at the 120 d time-point (Table 4.2). There were no discernable differences among the treatments based upon the number of OTUs, Chao1 richness estimates, or Shannon or Simpson's diversity indices. However, parsimony analysis and shared OTU (β–diversity measures) analysis for the pairwise comparisons revealed treatment differences with respect to community structure and membership (Table 4.3). The parsimony analysis indicated that the communities were significantly different from each other with p values < 0.001 observed for each of the comparisons (Table 4.3). The shared OTUs observed for each pair indicated that

MSMA flood and MSMA-intermittent plots shared the greatest amount of OTUs, while MSMA flood and no-MSMA intermittent shared the least amount of OTUs (Table 4.3). A similar trend was also observed among the Jaccard and Yue and Clayton theta indices, with the highest values for MSMA flood and MSMA intermittent (7 to 8% of the total community membership similar among the two treatments) and lowest for the MSMA flood and No-MSMA intermittent pair (<1% of total community membership similar between the two treatments). These results indicate that both MSMA and water management treatments impacted the bacterial communities and that community memberships were significantly different among the treatments. Similarly the dendogram (Figure 4.6a) based on Bray-Curtis values also implied that both redox and As concentrations impacted the bacterial communities. As concentration appeared to be the major determinant of bacterial community composition with the communities grouping primarily by As concentration instead of water treatment. Taxonomic relationships among the treatment communities were estimated by submitting the 16S sequence libraries to RDP lib compare and classifier tools. *Proteobacteria* was the predominant phylum detected in most treatments, ranging from 29% in the MSMA-flood treatment to 24 % in the No-MSMA-intermittent treatment (Figure 4.6b). Other dominant phyla included *Chloroflexi* (20 to 34 %), *Acidobacteria* (5 to 13 %), *Bacteroidetes* (5 to 9 %) and *Firmicutes* (3 to 5 %). With respect to treatment differences, *Proteobacteria* represented a significantly higher proportion of bacteria detected in the MSMA than the No-MSMA plots under both flooding treatments (Figure 4.7a) In contrast, the fraction of *Proteobacteria* was significantly lower in the intermittently flooded treatments than the continuously flooded treatments, both with and without added As. (Figure 4.7b). Other significantly different phyla among the treatments were *Acidobacteria* and *Firmicutes,* both representing lower proportions of sequences in the intermittently flooded than in the

continuously flooded plots. The C*hloroflexi* were higher in the MSMA intermittently flooded than the MSMA-flood plots but were lower in No-MSMA intermittently flooded than the No-MSMA flood plots (Figure 4.7a). The fraction of sequences characterized as *Acidobacteria* was significantly higher in the MSMA amended than the No-MSMA, while *Chloroflexi* sequences were less abundant in MSMA than in the No-MSMA plots (Figure 4.7b). Differences in sequence numbers were analyzed for major taxonomic families among the flood and intermittent, and MSMA and No-MSMA, indicated that the *Geobacteraceae* and *Cystobacteraceae* families, which include many FeRB, were found to be lower in the intermittent than the flood plots, but the differences were only significant for *Geobacteraceae* in MSMA intermittent v. MSMA flood and for *Cystobacteraceae* in MSMA flood v. No-MSMA flood plots (Figures 4.8 and 4.9).

Table 4.2. Diversity and richness estimates for bacterial communities in rice rhizosphere (120 days after planting) under different arsenic and flooding treatments.

	Total No. of Sequences	No. of OTUs*	Chao1 richness	Shannon	Simpson (1/D)
MSMA flood	1200	811	1789 (±257)†	6.51 (±0.05)	811 (±188)
MSMA intermittent	1200	862	2316 (±435)	6.60 (±0.05)	1023 (±302)
No- MSMA flood	1200	819	2026 (± 344)	6.51 (±0.05)	741 (±214)
No- MSMA intermittent	1200	760	1995 (±394)	6.45 (±0.05)	927 (±197)

*Operational taxonomic units (OTUs) were defined at ≥ 97% sequence identity.
†Values in parenthesis are 95% confidence intervals.

Table 4.3. Pairwise shared operational taxonomic unit (OTU)[†], and Jaccard and Yue and Clayton theta community similarity indices for bacterial communities in rice rhizosphere (120 days after sowing) under different arsenic and flooding treatments.

Treatments	MSMA flood	MSMA intermittent	No-MSMA flood
Shared OTUs[†]			
MSMA intermittent	108*		
No-MSMA flood	34*	83*	
No-MSMA intermittent	15*	66*	49*
Jaccard			
MSMA intermittent	0.068		
No-MSMA flood	0.021	0.051	
No-MSMA intermittent	0.010	0.042	0.032
Yue and Clayton			
MSMA intermittent	0.079		
No-MSMA flood	0.015	0.033	
No-MSMA intermittent	0.006	0.027	0.019

[†]OTUs were defined at ≥ 97% sequence identity.

*Indicates parsimony *p* test significantly different between the two treatment communities with *p* value < 0.001.

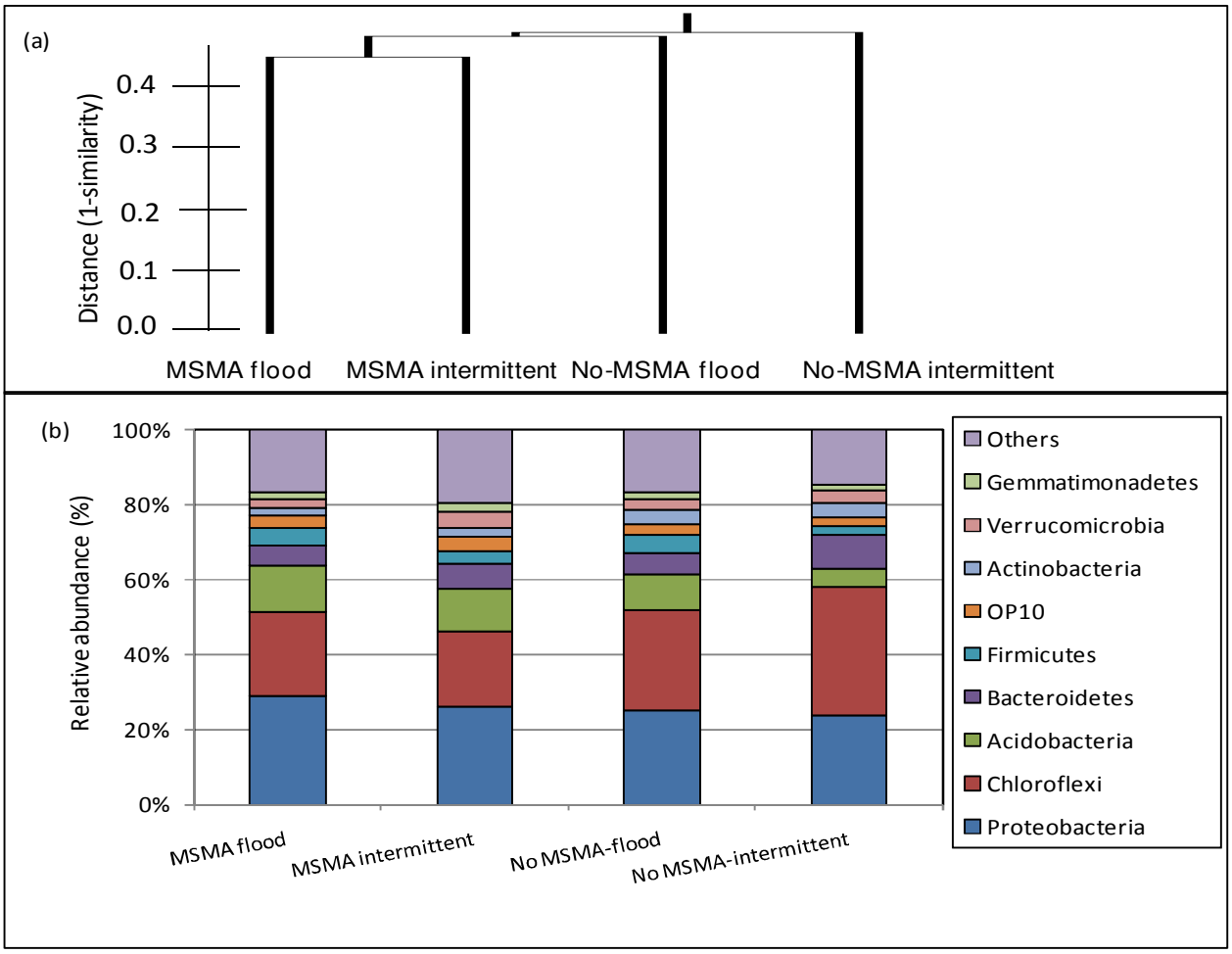

Figure 4.6. Bacterial community composition in rice rhizosphere under different arsenic and flooding treatments at 120 days after sowing. (a) Dendogram represents community structure dissimilarity (1-similarity) among the treatments based upon operational taxonomic unit (OTU) composition. OTUs were defined at ≥ 97% sequence identity. (b) Relative abundance of major bacterial and archaeal phyla as determined using RDP classifier.

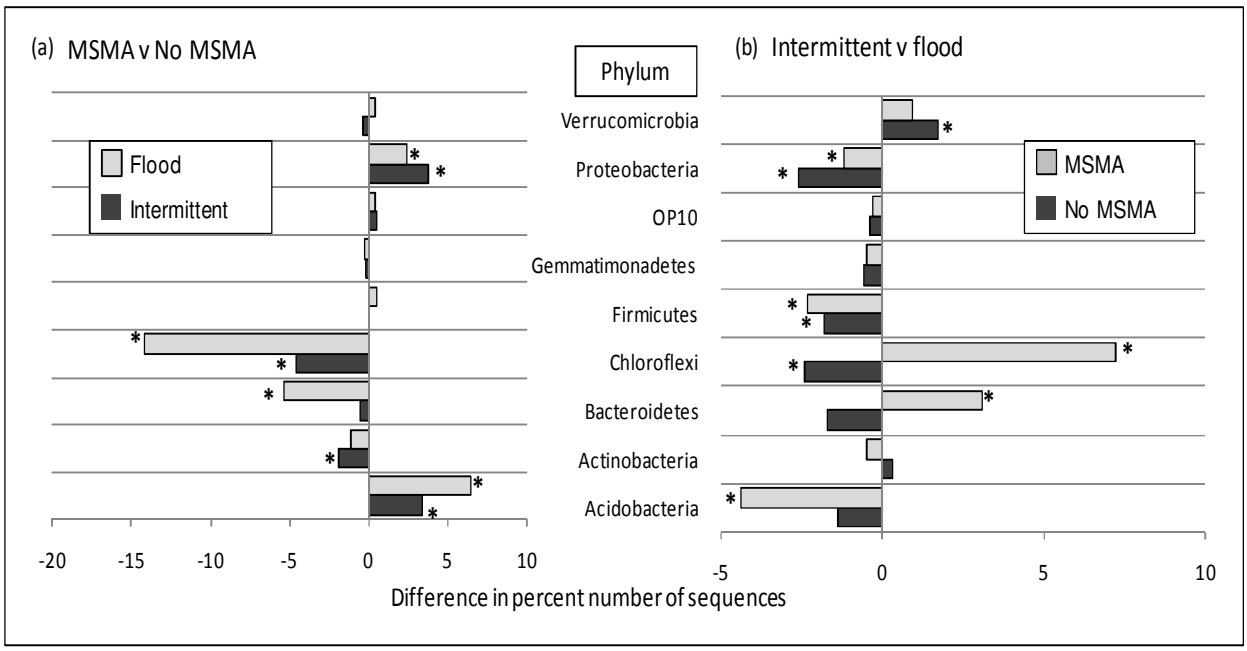

Figure 4.7. Impacts of arsenic and flooding treatments on relative abundance of bacterial phyla in the rice rhizosphere at 120 d. (a) Differences in the relative abundance of sequences in each phylum in the MSMA-amended plots relative to the No-MSMA plots. (b) Differences in the relative abundance of sequences in each phylum in the intermittently flooded plots relative to the continuously flooded plots.

* Significant difference for the phylum between the treatments at 0.05 level using RDP Library Compare.

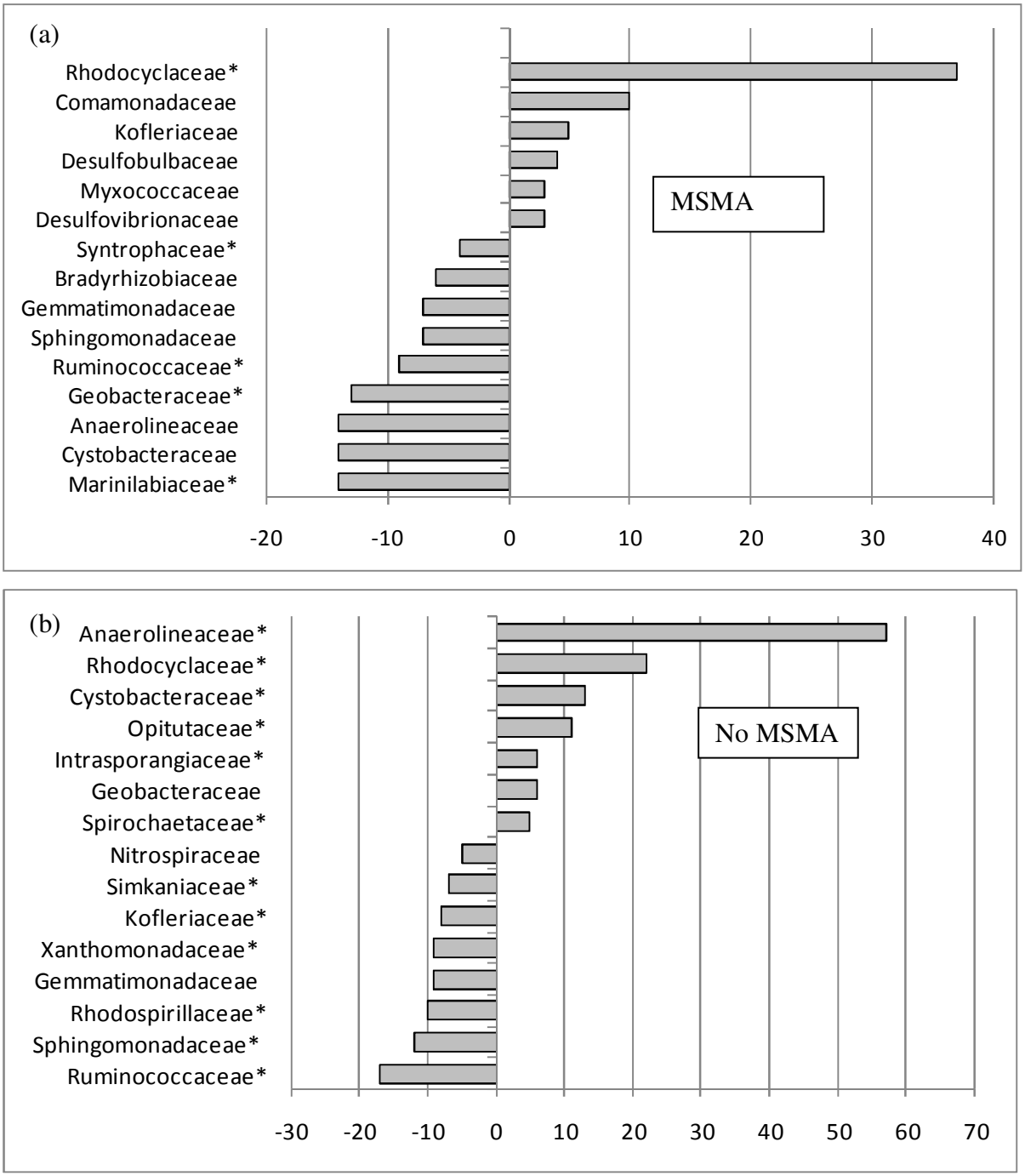

Figure 4.8. Number of sequences observed in intermittently flooded plots relative to the flooded treatment for some of the dominant bacterial families (a) in MSMA-amended and (b) no-MSMA-amended treatments. Stars next to the title mean that the difference in number of sequences was significantly different at 0.05 level according to RDP's library comparison tool.

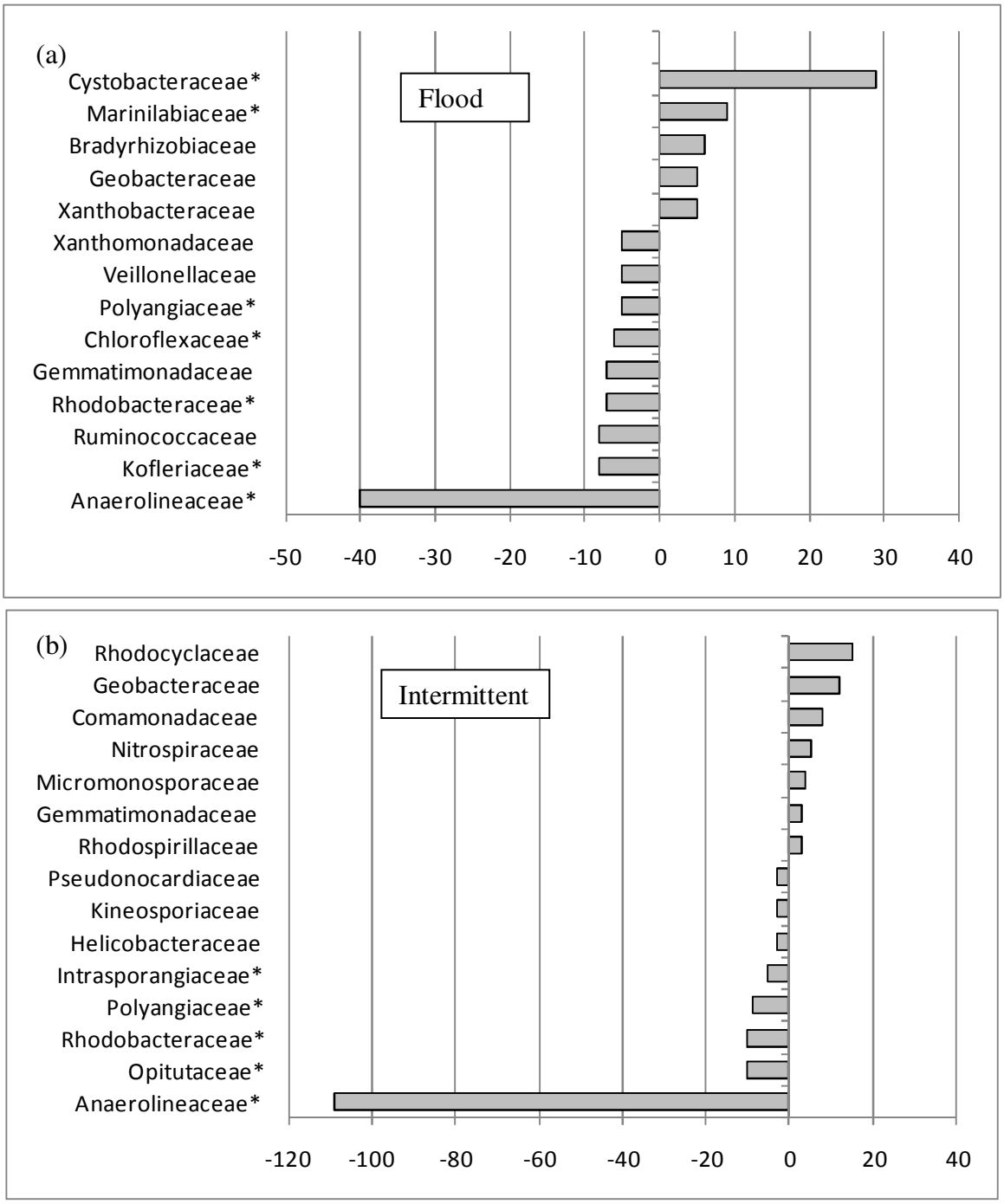

Figure 4.9. Number of sequences observed for some of the dominant bacterial families in MSMA-amended plots sequences relative to the no-MSMA plots in (a) flood and (b) intermittent treatments. Stars next to the title indicate that the difference in number of sequences was statistically significant at 0.05 level, as estimated by RDP's library comparison tool.

4.5 Discussion

4.5.1 Impact of As and Water Treatments on Rhizosphere Soil As Concentrations

The total soil-As concentrations did not significantly change over time indicating that little As was lost from the systems; however, pore-water As concentrations were significantly higher at 120 d compared to 60 d. It was also evident that water management treatments did not impact the total soil-As concentrations, but did significantly changed the pore-water As-concentrations. The pore-water As-concentrations with the intermittent plots decreased by approximately 60% at 60 d and 64% at 120 d time-point relative to the continuous-flood plots. Higher pore-water As-concentrations with the No-MSMA-flood plots than the MSMA-intermittent plots at both the time-points implies that water management had a greater impact than bulk-soil As concentration on the levels of total-dissolved As. The redox measurements taken at 60 d and 120 d sampling time-points indicated that the rice rhizosphere in the continuous-flood treatment was more highly reduced compared to the intermittent-flood treatment, thus indicating that reduced conditions favored As release to pore-water. At lower redox conditions, both increased iron-oxide dissolution and the reduction of iAs^{V} could result in higher soluble As concentrations in pore-water (Masscheleyn et al., 1991; Xu et al., 2008). It has been demonstrated that As mobilization is mostly regulated by reduction and solubilization of iron oxides (Benner et al., 2002; Rowland et al., 2007) and the reduction and dissolution of iron oxides is linked to both biotic and abiotic processes in the rice rhizosphere (Wang et al., 2009); thus implying that either biotic or abiotic processes or both favored more Fe(III) reduction and As release under continuously flooded conditions in these systems.

4.5.2 Response of Rhizosphere Microbial Communities to As and Water Treatments

The NMDS analysis of FAMEs from rice rhizosphere revealed a more distinct separation of treatments at 120 d compared to the 1 d and 60 d time-points (Figure 4.2), which indicates that the microbial communities shifted and diverged over the growing season. The FAME results also indicated that the fungal communities were present at a higher relative abundance in the intermittently-flooded plots but there appeared to be no effect of As treatment on relative abundance of *Fungi* or Gram-positive bacteria. Comparing the fungi:bacteria marker FAME ratios on a temporal scale, there was not much difference between the 1 d and 60 d time points; however the ratios slightly decreased at 120 d indicating that the fungal numbers went down towards the end of the growing season.

The qPCR data also indicated that relative abundance of *Bacteria*, FeRB and SRB proliferated towards the end of the growing season compared to the other groups of microorganisms we evaluated. *Bacteria* were more predominant than either *Archaea* or *Fungi* at 120 d in both flooded and the intermittently flooded plots (Figure 4.3); which was mostly due to increased bacterial gene copy numbers and a decrease in fungal and archaeal gene copy numbers relative to the 60 d time-point. This trend might imply that the *Bacteria* more efficiently used the carbon substrates available towards the end of the growing season. It was reported in a previous study that a few groups of rhizosphere microorganisms can utilize more complex carbon sources like amines and polymers (Chen et al., 2008). As the rice plants matured, it is possible that the more easily degradable carbon sources, such as carbohydrates became limiting, thus favoring specific groups of *Bacteria* capable of metabolizing more recalcitrant carbon compounds.

Higher relative abundances of FeRB and SRB with the continuous-flood compared to the intermittent–flood treatment at 120 d time-point imply that the reduced conditions favored FeRB.

High relative abundances of FeRB of up to 26% in MSMA-flood at 120 d time-point also suggest that iron-reduction reactions dominated these systems. Iron-reducing bacteria are commonly found in rice paddies (Hori et al., 2009; Neubauer et al., 2007b), although they usually only comprise around 10% of the bacterial community in most studies (Weiss et al., 2003). Sulfate-reducing bacteria were also present at slightly higher abundances in the continuously flooded compared to intermittent plots at the 120 d time-point, suggesting active sulfate reduction also occurs in these systems.

Although the qPCR data did not indicate large differences in the relative abundances of *Bacteria* at the 120 d time-point, bacterial community membership was different among the treatments as indicated by parsimony analysis. Jaccard and Yue and Clayton theta analysis of shared OTUs among the treatment pairs also indicated that both As and redox conditions impacted the bacterial communities with distinctly different community structure and membership among the treatments. Interestingly, FAME and 16S rRNA gene analysis indicated that the As treatments had a greater effect than did the flooding treatments on the microbial communities at 120 d. This is likely due to the long-term application of MSMA (>20 years to the MSMA-amended plots. Even at 1 d, FAME indicated differences in the microbial communities in the MSMA and No-MSMA plots. However, the No-MSMA plots also had detectable levels of soil As that were mobilized upon flooding. This actually resulted in pore-water As concentrations that were roughly equal in the continuously flooded MSMA-amended and No-MSMA plots. This implies that there may have been some other factor besides total As, possibly differences in As speciation, that altered the microbial communities either directly and/or indirectly through plant impacts.

In addition to characterizing community diversity, our other objective of 16S rRNA gene sequencing was to identify the taxonomic relationships among the bacterial memberships within the treatments. *Proteobacteria* being the most abundant phylum in MSMA-amended plots suggests that these phyla might comprise high numbers of As-resistant bacteria. Out of curiosity, we explored the NCBI database (accessed Nov, 2009) for different As resistance genes. Search results showed that 49% of all arsenate reductase genes (*arsC*), 76% of all dissimilatory arsenate respiration (*arrA*) and 38% of all arsenic methylation (*arsM*) gene-containing bacteria belonged to phylum *Proteobacteria*; however, it is also true that more proteobacterial sequences have been submitted to NCBI database compared to any other phyla. In a similar study on a metal contaminated site with very high As concentrations, it was observed that 78% of all the bacterial sequences belonged to *β-Proteobacteria* and rest to *γ-Proteobacteria* (Rastogi et al., 2009). Lu and associates also reported that *Proteobacteria* was found to be the dominant phylum in the rice rhizosphere with *α, γ* and *β- Proteobacteria* as the dominant classes (Lu et al., 2007). It is also true that *Proteobacteria* are the dominant phylum observed in most soils as indicated by several reconnaissance studies using different techniques such as DGGE, microarrays and metagenome analysis (Liles et al., 2003; Tringe et al., 2005). It must also be acknowledged that the bias introduced as a result of small length amplification by pyrosequencing techniques might lead to preference of some taxonomic groups such as *Proteobacteria* over others (Elshahed et al., 2008). Several bacterial families that might contain *arrA* and *arsC* genes were identified in most treatments (data not presented); however to make any further comparisons based solely upon the 16S data would be an overstatement.

4.5.3 Relationships between Microbial Populations and As Concentrations

Significantly higher pore-water As-concentrations in the rhizosphere were observed in the continuously flooded plots relative to the intermittent plots, and also at 120 d relative to the 60 d time-point. These trends coincided with the high relative abundance of FeRB in the continuously flooded plots especially towards the end of growing season as indicated by both qPCR results and 16S rRNA gene sequencing. Presence of FeRB does not directly indicate active iron reduction, but increases in their relative abundances (up to 26% of the total microbial community) are suggestive of active iron reduction, and probably As reduction and release. FeRB gain energy by coupling the oxidation of organic compounds with the reduction of Fe(III) oxyhydroxides (Lovley et al., 2004). This could result in the dissolution of solid phase Fe which could subsequently result in solubilization of As sorbed on the surface of iron oxides (Cummings et al., 1999; Rowland et al., 2007), as well as also from the iron oxides precipitated on the rice roots which sequester significant amounts of As (Wang et al., 2009). However, it is still debated whether FeRB would actually result in the release of As, since some of the related studies reported that microbial Fe(III) reduction is also likely to form secondary iron oxide phases which could potentially adsorb to As (Kocar et al., 2006; Tufano et al., 2008). In any case, it is evident that FeRB could potentially impact Fe and As cycling in the rice rhizosphere, which needs to be further investigated in additional studies.

Detection of SRB also suggests that sulfate reduction may be actively occurring in these soils. Sulfides produced as a result of sulfate reduction by SRB, might react with inorganic As compounds to form insoluble thioarsenate compounds (Oremland et al., 2004; Stauder et al., 2005). We observed substantial sulfide-like black coatings in the rice rhizosphere mostly with the MSMA plots; however we do not have direct evidence that sulfides accumulated in the

experimental plots. The pore-water As-concentrations also increased in most treatments towards the end of the growing season, indicating that *Bacteria,* FeRB and SRB could survive the toxic As concentrations more than other group of microorganisms. Several FeRB are reported to be also capable of respiring iAs^V (Islam et al., 2005; Kocar et al., 2006), and thus could proliferate under high As concentrations. It should also be noted that the microbial populations were perhaps resistant to the moderate As-concentrations in our experimental plots relative to high As-concentrations used in other studies (Chopra et al., 2007; Edvantoro et al., 2003; Turpeinen et al., 2004).

4.6 Conclusions

This field-scale study demonstrated that both different water management practices and long-term application of MSMA impacted pore-water As-concentrations and microbial populations in the rhizosphere of rice with the microbial communities for all treatments diverging over the growing season. Results of this study based on FAME, qPCR assays and 16S rRNA sequencing consistently demonstrated that microbial populations do sense and respond to changes in pore-water As-concentrations and redox conditions. Intermittent flooding impacted bacterial community membership and also decreased the relative abundance of FeRB and SRB compared to the flooded plots, which might have favorably contributed towards decreased pore-water As concentrations in the rice rhizosphere. Results of this study demonstrated that intermittent flooding could be a viable management option to reduce soluble As concentrations in the rhizosphere when rice is cultivated on soils with moderate to high As concentrations.

CHAPTER V

INTERMITTENT FLOODING ALTERED RICE ROOT-ASSOCIATED MICROBIAL COMMUNITIES

5.1 Synopsis

Different water management practices could affect microbial populations in the rice rhizosphere and thus potentially impact arsenic (As) chemistry and bioavailability. A field-scale study was conducted to analyze As concentrations and microbial populations in the root-plaque and rhizosphere of rice in response to continuous and intermittent flooding conditions in MSMA (monosodium methyl arsenate)-amended and No-MSMA plots. Rhizosphere, root-plaque, and pore-water As concentrations were quantified, and microbial populations in rhizosphere and root-plaque samples were characterized. Average rhizosphere-As concentrations ranged from 7 mg/kg in No-MSMA to 22 mg/kg in MSMA plots, with no apparent differences due to water treatment. In contrast, average pore-water As concentrations ranged from 4.1 µg/L in intermittently flooded plots to 26.8 µg/L in continuously flooded plots with pore-water As levels being 81 to 86% lower under intermittent flooding. Quantitative PCR indicated that *Bacteria* dominated all samples representing 91 to 94% and 48 to 78% of the total community in root-plaque and rhizosphere, respectively, with smaller proportions of *Archaea* and *Fungi* being detected. The relative abundance of iron-reducing bacteria was lower in rhizosphere under intermittent than continuous flooding. The 16S rRNA gene sequencing indicated that bacterial community composition was significantly different among the treatments and arsenic levels had a greater impact on community composition than did water treatment. *Proteobacteria* was the predominant phylum in root-plaque (51 to 57%) and most rhizosphere samples (23 to 27%).

Chloroflexi (20 to 28%) were also dominant in rhizosphere samples, and their populations increased in response to intermittent flooding and higher As levels. These results indicate that intermittent flooding can alter root-plaque and rhizosphere microbial communities and decrease concentrations of water-soluble As in rice production systems.

5.2 Introduction

Rice is a staple food crop for more than 3 billion people and is cultivated in more than 158 Mha throughout the world (FAO, 2009), mostly under continuous flooding (more than 75% of the total acreage) (Roger et al., 1993). Because of continuous submergence, rice grain tends to accumulate higher concentrations of arsenic (As) than other cereals (Williams et al., 2007b). This is of concern since As is a toxic metal known to cause cancer in humans. Worldwide, more than 50 million people are exposed to higher As levels than is recommended by the World Health Organization (10 µg per day in drinking water) through consumption of As-containing drinking water or food (BGS and DPHE, 2001; Duxbury et al., 2003). Recent reports implicate consumption of As-contaminated rice as one of the major As exposure routes for millions of people in South East-Asia (Mondal and Polya, 2008; Ohno et al., 2007).

Under continuously flooded, anoxic conditions, As is more readily released into soil pore-water by reductive dissolution of iron oxides and increased conversion of iAs^{V} to iAs^{III} (Masscheleyn et al., 1991; Takahashi et al., 2004). iAs^{III}, a neutral species under normal paddy conditions (pH 4 - 8), is more readily bioavailable than iAs^{V}, while iAs^{V} is strongly adsorbed to iron and aluminum oxides and relatively less bioavailable (Lafferty and Loeppert, 2005; Raven et al., 1998), is more prevalent under oxidized conditions. Alternative water management practices might be essential to reducing grain As concentrations, as several studies have reported that aerobic-rice cultivation (Xu et al., 2008) and intermittent flooding with wet/dry cycles (Li et

al., 2009a) result in lower As concentrations in grain than obtained under conditions of continuous flooding. Cultivation of rice with intermittent flooding appears to have significant potential as a management strategy to reduce As accumulation in grain ; however, it is unclear whether intermittent flooding can also affect root-plaque and rhizosphere microbial communities, which may also influence As biogeochemistry.

Under continuous flooding, the rice rhizosphere and bulk soil become anoxic over the growing season; however, the root surface stays relatively oxic because of radial oxygen diffusion from the root aerenchyma structure (Colmer, 2003). An intermediate rhizosphere soil also exists between the anoxic bulk soil and oxic root surface; thus, the rice rhizosphere can be compartmentalized into 3 distinct zones based upon oxygen levels, each with unique biochemical processes. Micro-scale spatial variations of microbial populations exist within the rice rhizosphere (Liesack et al., 2000), suggesting that root-plaque and rhizosphere-soil communities could be distinct, potentially affecting Fe and As chemistry. Dissolved Fe(II) from the anoxic bulk is oxidized to Fe(III) oxyhydroxides that subsequently precipitates on the root surface forming iron-oxide root-plaques. The major form of iron oxide comprising these plaques is reportedly poorly-crystalline ferrihydrate (Bacha and Hossner, 1977; Taylor et al., 1984). These iron oxides provide strong binding sites for many nutrient elements and toxic metals and thus could act as a barrier, reducing bioavailability to the roots (Greipsson, 1994; Otte et al., 1989; Zhang et al., 1998). Several microbial populations can affect rice-root-plaque formation and dissolution, including iron-reducing and iron-oxidizing bacteria that can also impact As adsorption and dissolution (Neubauer et al., 2007a; Wang et al., 2009).

Both As concentrations and redox conditions can affect microbial populations (Edvantoro et al., 2003; Zhou et al., 2002), and microbial populations are thus likely to be different in

intermittently versus continuously flooded soil. The impacts of different water management practices on root-plaque and rhizosphere microbial communities, including the roles of the microorganisms in controlling As biogeochemistry and bioavailability, are not known. Consequently, we conducted a field experiment investigating rice rhizosphere and root-plaque microbial communities and arsenic levels under continuous or intermittent flooding under two levels of soil As.

5.3 Material and Methods

5.3.1 Field Experiment

We conducted our field experiment in 2008 at Dale Bumpers National Rice Research Center (US Department of Agriculture, Agriculture Research Service, Stuttgart, AR). One of the research plots was previously applied with the arsenic-based pesticide monosodium methane-arsonate or MSMA) in alternate years for more than 20 years. The MSMA was applied to the soil surface before rice planting, at the rate of 6.7 kg/ha per year (equivalent to 3.08 kg/ha per year of As). These plots will be referred as “MSMA plots”. The adjacent native soil (referred as “No-MSMA plots”) had not been exposed to any As-containing products for at least the last 20 years. We imposed 2 water treatments: intermittent versus continuous flooding of both the MSMA and No-MSMA plots. Under intermittent flooding, the plots were flooded and allowed to dry until surface cracking initiated before re-flooding. The treatment groups used in this study were (1) MSMA flood (2) MSMA intermittent (3) No-MSMA flood and (4) No-MSMA intermittent. The treatment plots were distributed using a split-split plot design, and 4 replicate plots for each treatment group were arranged in a randomized design (only 3 of these replicates were sampled as part of this study). All other management practices were followed as previously described (Yan et al., 2005).

5.3.2 Sampling

Approximately three months after the first flood and three weeks before the rice was harvested (120 d after planting), we collected rhizosphere, root-plaque, and pore-water samples. We collected 3 rice plants per plot, along with the adhering bulk soil. The plants were shaken to remove loose soil, and the remaining non-rhizosphere soil was removed manually. After we collected the few millimeters of rhizosphere soil left around the roots, we thoroughly washed the roots with deionized water to remove remaining soil. Iron-oxide plaque remained firmly affixed to the roots, and these root-plaque samples, along with the rhizosphere samples, were split into two subsamples (for chemical and microbial analysis). We transported the samples for chemical analysis from the field to the lab at 4°C, and we immediately froze the samples for microbial analysis on dry ice (later storing at -80°C).

For rhizosphere pore-water samples, we collected bulk soil samples from the rhizosphere area (0–6 cm depth, between two adjacent plants) using a 2.5 cm diameter corer with brass insert rings. We capped insert rings containing soil samples with polypropylene end caps and stored them on ice during transport to the lab. We vacuum filtered the core samples at a negative pressure of 138 kPa for 20 min to extract pore-water samples and then acidified to pH 3 with 100 mM HNO_3, preserved at 4°C, and subsequently used for As analysis.

5.3.3 Arsenic Analysis

The total soil As concentrations were determined by inductively-coupled-plasma mass-spectrometry (ICP-MS) model DRC-ELAN II (Perkin Elmer, Waltham, MA, USA) after following a open digestion method with HNO_3/H_2O_2 (US-EPA, 2007).

Ammonium oxalate in the dark (Loeppert and Inskeep, 1996) was used to estimate the reactive root-plaque As concentrations. Approximately 2.5 g of fresh root in a polypropylene

centrifuge tube was covered with aluminum foil to prevent exposure to light, approximately 20 mL of extracting solution was added (0.175 *M* ammonium oxalate + 0.1 *M* oxalic acid at pH 3), and the mixture was agitated for 2 hr on a reciprocating platform shaker. We then centrifuged the suspensions and decanted, diluted, and stored the supernatant solutions for As and Fe analysis. We determined the As concentrations in soil, root-plaque, and pore-water samples using an ICP-MS, model DRC-ELAN II (Perkin Elmer, Waltham, MA, USA). The total iron in ammonium oxalate extraction from the root-plaque samples was analyzed using an AAnalyst 400 atomic absorption spectrophotometer (Perkin Elmer, Waltham, MA, USA).

5.3.4 Gene Copy Number Quantification Using qPCR Targeting

Quantitative PCR (qPCR) assays targeting total *Bacteria*, *Archaea*, *Fungi*, iron-reducing bacteria (FeRB), sulfate-reducing bacteria (SRB), and methanogens were performed using the group specific primer sets and qPCR conditions outlined in Table 5.1. The FeRB were determined by targeting *Geobacteracaea* and *Shewanella* spp. The SRB were determined by targeting the *dsrA* gene. Methanogens were determined by targeting the *mcrA* gene. The assays were performed in a 10-µL reaction mix containing 4.5 µl SYBR green real master mix (5Prime, Inc., Gaithersburg, MD, USA), 0.5 µl of each primer (concentration of 10 µM for *Bacteria, Fungi* and *Archaea*, 200 nM for *dsrA* and *mcrA* and 300 nM for *Geobacteracaea* and *Shewanella* spp.), 1 µl template (2.5 ng), 1 µl bovine serum albumin (10 mg ml^{-1}) and 2.5 µl molecular grade water (DNase free). Each analysis run included a set of standards, controls, blanks and samples (all including three analytical replicates) on a 96-well plate. The PCR reactions were conducted at the temperatures listed in Table 5.1 with 40 amplification cycles. Melting curve analysis of the qPCR products was performed after each assay to confirm qPCR amplification quality. The

Table 5.1. Conditions and primer sets used for qPCR assays for enumerating the relative abundance of microbial populations in rice rhizosphere soil and root-plaque samples under different arsenic and flooding treatments.

Target group	Primer set	Annealing Temp.	Standard (source)	Reference
Total *Bacteria*	Eub338- -5'-ACTCCTACGGGAGGCAGCAG-3' Eub518 --5'-ATTACCGCGGCTGCTGG-3'	53°C	*Escherichia coli* DH10B(pUC19) (obtained from Carlos Gonzales, Texas A&M University)	(Fierer et al., 2005)
Total *Fungi*	ITS1f- 5'-CTTGGTCATTTAGAGGAAGTAA-3' 5.8s—5'-CGC TGC GTT CTT CAT CG-3'	61 °C	*Neurospora crassa* (obtained from Carlos Gonzales, Texas A&M University)	(Boyle et al., 2008)
Total *Archaea*	Arc85f--5′-ACTGCTCAGTAACACGTGGA-3′ Arc313r--5′-ATGTCTCAGAATCCATCTCC-3′	53 °C	*Methanosarcina acetivorans* C2A (obtained from William Metcalf, University of Illinois).	(Lima and Sleep, 2007)
Geobacteracea spp.	Geo564F -5'-AAGCGTTGTTCGGAWTTA T-3' Geo840R -5'-GGCACTGCAGGGGTCAAT A-3'	60 °C	*Geobacter metaloreducans* (genomic DNA obtained from Jizhong Zhou, University of Oklahoma)	(Cummings et al., 2003)
Shewanellae spp.	She 120F – 5'-GCCTAGGGATCTGCCCAGTCG-3' She 220R – 5'-CTAGGTTCATCCAATCGCG-3'	60 °C	*Shewanella oneidensis* MR-1 (genomic DNA obtained from Jizhong Zhou, University of Oklahoma)	(Himmelheber et al., 2009)
dsrA gene	dsr-1F--5-ACSCACTGGAAGCACG-3 dsr-500r--5-CGGTGMAGYTCRTCCTG-3	58 °C	*Desulfovibrio vulgaris* subsp. vulgaris Hildenborough (genomic DNA obtained from Jizhong Zhou, University of Oklahoma)	(Wilms et al., 2007)
mcrA gene	ME1f50-GCMATGCARATHGGWATGTC-30 ME3r50-TGTGTGAASCCKACDCCACC-30	54 °C	*Methanosarcina acetivorans* C2A	(Wilms et al., 2007)

qPCR was performed using an Eppendorf Mastercycler® ep realplex thermal cycler (Eppendorf, Hamburg, Germany).

Standards for qPCR were generated by PCR-amplifying each gene of interest from the genomic DNA of pure cultures using the primers and details listed in Table 5.1. The PCR products were confirmed on an agarose gel, and then cloned into a pGEM®-T Easy vector following the manufacturer's instructions (Promega, Madison, WI, USA). Positive clones were isolated and extracted for plasmid DNA using a Wizard SV Miniprep kit (Promega). Plasmid DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and were used for preparing appropriate dilution standards for the qPCR assays. The plasmid DNA concentrations ranging from 5.0×10^{-3} to 5.0×10^{-7} ng μl^{-1} DNA were used to generate the qPCR standard curves. Relative abundances of specific populations were estimated by calculating ratios of gene copies from respective microbial populations to gene copies for the entire microbial community (sum of *Bacteria, Archaea* and *Fungi).*

5.3.5 Microbial Community Analysis

We extracted microbial community DNA from the rhizosphere and root-plaque samples using MO BIO Power Max DNA extraction kits (MO BIO Laboratories Inc., Carlsbad, CA, USA). We modified the manufacturer's protocol to include a lysozyme preincubation step in order to enhance Gram-positive bacterial DNA yield: Approximately 10 g of rhizosphere or 5 g root-plaque sample were treated with 10 µl of lysozyme solution (15 mg per sample final concentration) and incubated in a water bath for 1 hr at 37.5°C with occasional shaking, after which the manufacturer's protocol was resumed. Resulting DNA samples were concentrated by

ethanol precipitation, purified using Illustra MicroSpin™ G-25 columns (GE Healthcare Biosciences, Pittsburgh, PA, USA), and stored at -20°C.

The root-plaque community DNA samples from all 3-field replicates were sequenced individually; whereas with the rhizosphere soil equal quantities of community DNA from all 3-field replicates for each treatment were composited into one sample per treatment. The DNA samples were submitted to the Research and Testing Laboratory (Lubbock, TX, USA) for tag-pyrosequencing. Samples were amplified with modified versions of primers 530F and 1100R (Acosta-Martinez et al., 2008), and the amplicons were sequenced using Roche 454 Titanium chemistry.

5.3.6 Analysis Pipeline for 16S rRNA Gene Sequences

The 16S rRNA gene sequence data was analyzed using pyrosequencing pipeline tools from the Ribosomal Database Project (RDP) (Cole et al., 2009) and MOTHUR version 1.6.1 (Schloss et al., 2009). The 16S rRNA sequences were first trimmed for primers and chimeras and then sequences with fewer than 350 bases were removed from the data sets. The individual sequence files were combined using the BioEdit *v7.0.5* software (Hall, 1999) and submitted to the RDP aligner tool for multiple alignments. Pairwise distances between the aligned sequences (distance matrix) were calculated and then used to assign sequences to operational taxonomic units (OTUs) (cluster analysis). Both distance matrix and cluster analyses were performed using the dist and cluster analyses tools in MOTHUR (default settings). The OTUs were defined at 97% similarity cutoff for all of the analyses.

Both α- and β-diversity measures were estimated for the data sets using the summary.single and summary.shared tools in MOTHUR. The α-diversity measures consisted of Chao1 richness, Shannon, and Simpson, and the β- diversity measures consisted of Yue and

Clayton theta and Jaccard community structure. After running the Bray-Curtis community structure analysis with MOTHUR, we constructed a dendogram using MEGA 4 software (Tamura et al., 2007). A PHYLIP formatted distance matrix file was constructed using the dist function in MOTHUR and used to create a neighbor-joining tree with neighbor tool available with the PHYLIP 3.68 package (Felsenstein, 2005). The PHYLIP generated tree was then used as an input file to run the parsimony *p* test (Martin, 2002) using MOTHUR. Parsimony analysis is commonly used to test whether two or more communities harbor greater differences in phylogenetic structure than would be expected by chance; and a p value of ≤ 0.001 is considered to be statistically significant (Martin, 2002). Relative abundance of taxonomic phyla and families among the treatment samples were determined with RDP Library Compare.

Analysis of similarity (ANOSIM) was performed on OTU data obtained from root-plaque sequences using PAST software (default settings) (Hammer et al., 2001). A two-way ANOSIM evaluates statistically significant differences between two groups and generates R statics and p values (Clarke, 1993).

5.3.7 Statistical Analyses

We used SigmaPlot version 11.0 (Systat Software, 2007) to calculate mean difference and standard error for the experimental data and to create graphs.

5.4 Results

5.4.1 As Concentrations in the Rhizosphere, Pore-Water, and Root-plaque Samples

The total As-concentrations in the rhizosphere, pore-water, and root-plaque samples are presented in Figure 5.1a. The rhizosphere-As concentrations were significantly lower in No-MSMA plots, with an average (mean) of 6.9 mg/kg, compared to 22.3 mg/kg in MSMA plots. In

contrast, As concentrations were not significantly different in flood plots (13.8 mg/kg) than in intermittent plots (15.4 mg/kg). However, pore-water As was significantly different among the treatments, with concentrations ranging from 3 to 37 µg/L. The pore-water As concentrations were again significantly lower in No-MSMA plots, with an average of 10.0 µg/L, compared with 20.8 µg/L in MSMA plots. In contrast to the soil As results, pore-water As concentrations were also significantly lower in intermittent plots than flood plots, at 4.1 µg/L versus 26.8 µg/L, respectively. The redox potential was also significantly higher in intermittent plots, with an average of -156-mv, compared with -246-mv in flood plots, but there was no difference between the MSMA and No-MSMA plots.

Root-plaque accumulated a large proportion of As (based on total dry mass of root), with concentrations up to 10-times higher than the adjacent rhizosphere soil (74 to 295 mg/kg). Figure 5.1b shows that the root-plaque As concentrations were significantly lower in No-MSMA plots (12.6 mg/kg versus 214.4 mg/kg in MSMA plots) and also in intermittent plots (103.8 mg/kg versus 223.2 mg/kg in flood plots). The As:Fe molar ratios were not significantly different in the No-MSMA plots (0.0022) compared to the MSMA plots (0.0026). However, the As:Fe ratios were significantly lower in MSMA–intermittent plots (0.0018) than MSMA–flood plots (0.0031).

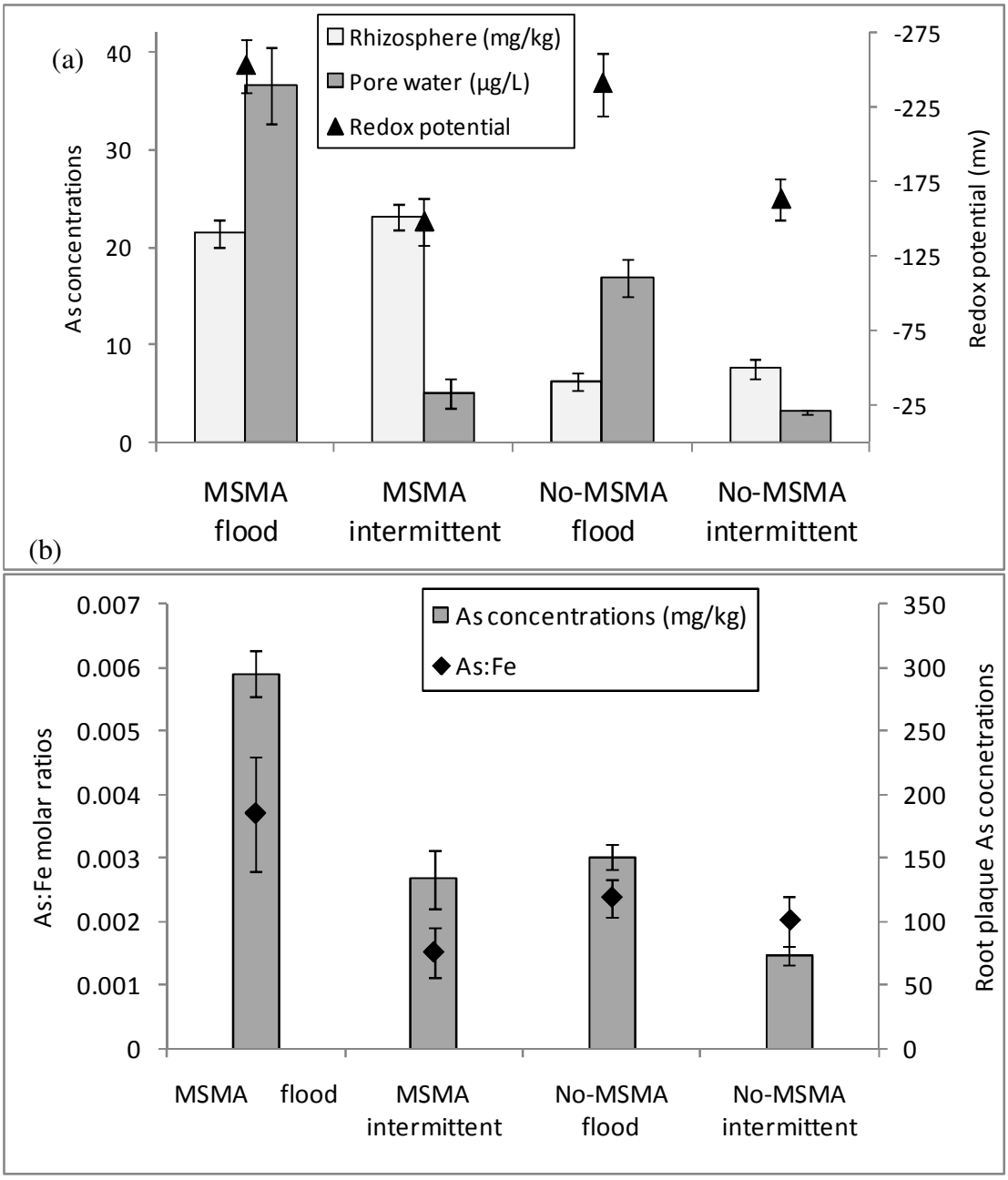

Figure 5.1. (a) Rice rhizosphere and pore-water As concentrations and redox potential, and (b) root-plaque As concentrations and arsenic to iron molar ratios under different arsenic and flooding treatments. Error bars represent standard error of mean.

5.4.2 Relative Abundances of Microbial Populations in Root-plaque and Rhizosphere

The relative abundance of microbial populations to total rhizosphere microbial community (sum of gene copy numbers from *Bacteria*, *Archaea* and *Fungi*) in root-plaque and rhizosphere soil, as represented by the ratio of gene copy numbers for each group are presented in Figure 5.2. *Bacteria* were the most dominant group with relative abundances of approximately 93% of the total community in the root-plaque samples and approximately 60% of the total community in the rhizosphere. There was no significant difference in the relative abundances of *Bacteria* among the treatments in the root-plaque samples, whereas the ratios significantly varied among the treatments with the rhizosphere soil. The relative abundance of *Bacteria* was significantly lower in the flood plots (50%) compared to the intermittent plots (71%).

Archaea represented approximately 37% of the total community in the rhizosphere soil with significant differences among the treatments; whereas with the root-plaque samples the *Archaea* were present at less than 1% of the total community. In the rhizosphere, archaeal populations were significantly higher in the flood plots (47%) compared to the intermittent plots (28%). *Fungi* were present at relatively lower abundance of around 6% of total community in the root-plaque-samples and around 3% in the rhizosphere, without any treatment differences among the treatments.

The relative abundance of FeRB was low in the root-plaque samples representing approximately around 3% of total community with no significant differences between the treatments; whereas in the rhizosphere samples, the FeRB comprised about 20% of the total community. Additionally, the FeRB were significantly more dominant in the flood plots representing around 22% of the total community compared to 17% in the intermittent plots.

The relative abundance of SRB was also low representing approximately 2 to 3% of the total community in the root-plaque and about 2 to 5% in the rhizosphere without any significant differences among the treatments.

Methanogens were almost undetectable in the root-plaque samples with relative abundances of < 0.1%. Their abundance was also low in the rhizosphere at approximately 6% of the total community with significantly higher proportions of around 8% in the flood plots compared to around 3% in the intermittent plots. The relative abundance of microbial populations slightly varied between MSMA and No-MSMA plots; however the qPCR results demonstrated no consistent effect of MSMA treatments on relative abundance of any of the microbial communities.

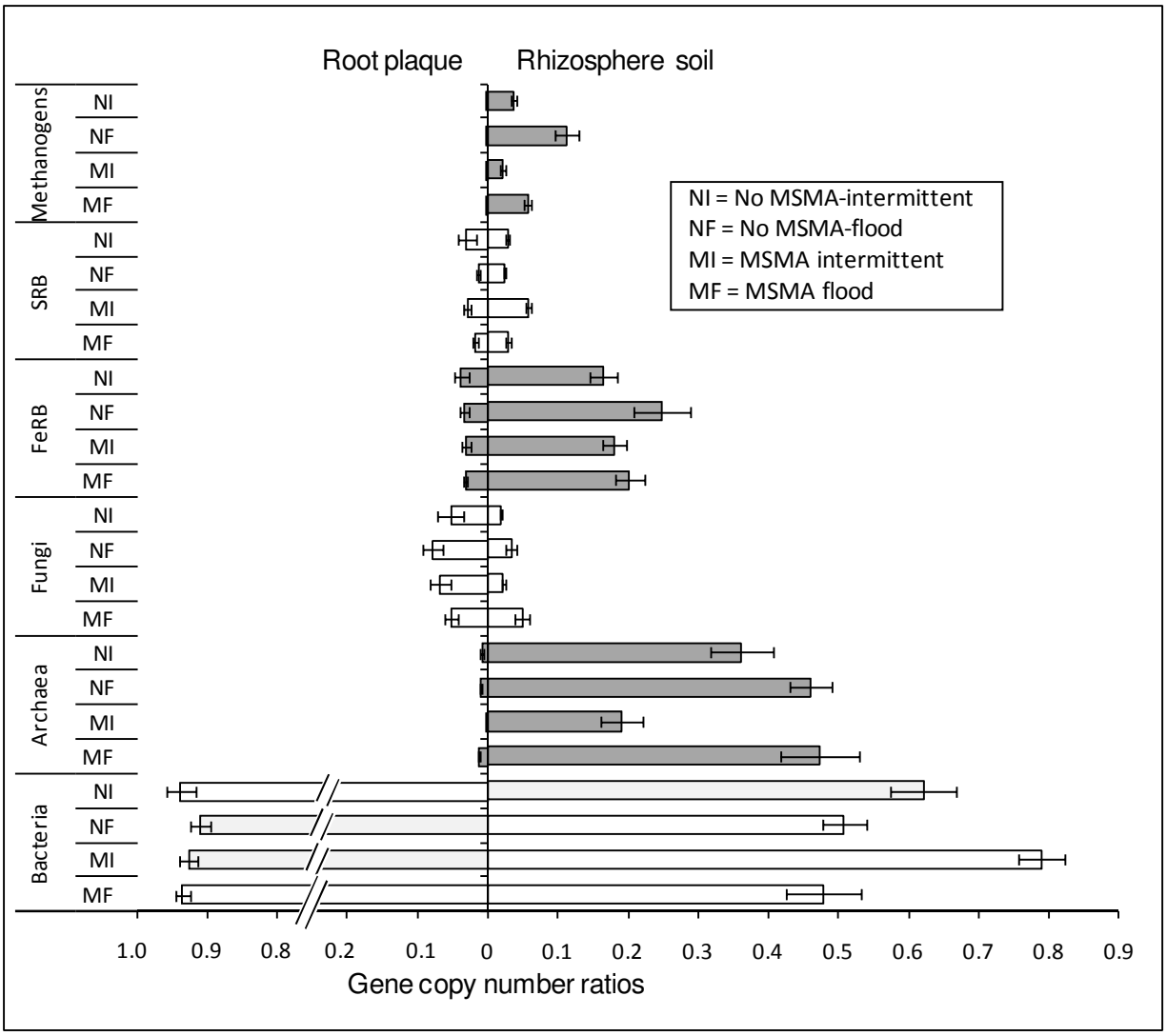

Figure 5.2. Relative abundance of microbial populations in rice root-plaque and rhizosphere under different arsenic and flooding treatments. The ratios were calculated by comparing gene copy numbers for each group, estimated by qPCR targeting with group specific primers. Error bars represent standard error of mean. FeRB = iron reducing bacteria and SRB=sulfate reducing bacteria.

5.4.3 Bacterial Populations in the Root-plaque and Rhizosphere Soil

Approximately 3500 16S rRNA sequences were obtained from each root-plaque field replicate (Table 5.2), with total sequence numbers per treatment type ranging from 8974 to 11724, and number of OTUs from 4313 to 5265 (Table 5.3). Approximately 1103 to 1264 16S rRNA sequences were obtained from the rhizosphere samples with number of OTUs ranging from 730–810 (Table 5.3). The Shannon and Simpson diversity indices varied slightly among the treatments, but were not significantly different both for root-plaque and rhizosphere samples. The parsimony analysis and shared OTUs analysis (β–diversity measures) for the pairwise comparisons revealed some differences in the bacterial community structure and membership (Table 5.4). The parsimony analysis indicated that the communities were significantly different from each other, with $p < 0.001$ for each of the comparisons both for root-plaque and rhizosphere (Table 5.4). With the root-plaque samples, No-MSMA–flood and No-MSMA–intermittent plots shared the most OTUs, while No-MSMA–flood and MSMA–intermittent plots shared the fewest OTUs (Table 5.4). The Jaccard indices were also highest for No-MSMA–flood and No-MSMA–intermittent plots (20% similarity in total community membership) and lowest for the No-MSMA–flood and MSMA–intermittent pair (15% similarity). A similar trend was also observed with Yue and Clayton theta indices. With the rhizosphere soil, MSMA–flood and MSMA–intermittent plots shared the most OTUs, while MSMA–flood and No-MSMA–intermittent shared the fewest OTUs (Table 5.4). The Jaccard values were highest for MSMA–flood and MSMA–intermittent (12% similarity in total community membership) and lowest for the MSMA–flood and No-MSMA–intermittent pair (4% similarity). A similar trend was also observed with Yue and Clayton theta indices. A dendogram based on Bray-Curtis analysis (Figure 5.3a and b) shows root-plaque and rhizosphere samples grouping primarily by As

concentration. The ANOSIM *R* static values were higher than 0.5 for both MSMA and water treatments with significant *p* values confirming that root-plaque bacterial communities were significantly different between MSMA and water treatments (Table 5.5).

Proteobacteria was the predominant phylum detected in root-plaque samples, ranging from 57% in the MSMA–flood plots to 51% in the No-MSMA–intermittent plots (Figure 5.4). Other dominant phyla in root-plaque samples included *Acidobacteria* (8 to 12%), *Firmicutes* (4 to 12%), *Actinobacteria* (6 to7%), and *Bacteroidetes* (3 to 6%). *Proteobacteria* represented a higher proportion of detected rhizosphere bacteria in the intermittent than in the flood plots amended with MSMA (Figure 5.5a and b). The fraction of *Proteobacteria* was significantly higher in the No-MSMA–intermittent than the MSMA–intermittent plots but were lower in No-MSMA–flood than in MSMA–flood plots (Figure 5.5a and b). *Firmicutes* represented significantly lower proportions of sequences in the intermittent than in the flood plots and significantly higher proportions in MSMA–intermittent versus No-MSMA–intermittent plots. *Acidobacteria* and *Actinobacteria* were higher in intermittent plots in MSMA plots and trended higher in No-MMSA plots. *Acidobacteria* proportions were significantly higher in No-MSMA–intermittent versus MSMA–intermittent plots, and *Actinobacteria* proportions were significantly lower in No-MSMA versus MSMA plots in both intermittent and flood plots.

Proteobacteria (23 to 27%) and *Chloroflexi* (20 to 28%) were the predominant phyla detected in most of the rhizosphere samples (Figure 5.4). Other dominant phyla included *Firmicutes* (7 to 22%), *Acidobacteria* (7 to 11%), *Bacteroidetes* (8 to 10%), and *Verrucomicrobia* (3 to 5%). In contrast to root-plaque samples, *Proteobacteria* in the rhizosphere trended toward lower proportions of detected *Bacteria* in intermittent than in flood plots in both MSMA and No-MSMA plots, but these differences were not significant (Figure 5.5a and b). The

fraction of *Proteobacteria* was higher in MSMA than in No-MSMA in both flood and intermittent plots, but was not statistically significant. *Chloroflexi* proportions were significantly higher in the intermittent than in flood plots and significantly lower in MSMA than No–MSMA plots. *Firmicutes* proportions were significantly lower in the MSMA-intermittent than in the MSMA-flood plots and significantly higher in No-MSMA than in MSMA plots. *Acidobacteria* proportions were slightly higher in the MSMA–intermittent than MSMA–flood plots and in No-MSMA–intermittent versus MSMA–intermittent plots (Figure 5.5a and b). Several FeRB groups were also detected at considerable proportions, such as the genus *Anaeromyxobacter* (5 to 10% in root-plaque and 2 to 4% in rhizosphere soil) and family *Geobacteraceae* (around 1% in root-plaque and 2 to 5% in rhizosphere soil (Figure 5.6).

Table 5.2. Root-plaque bacterial communities: number of sequences and OTUs for each replicate under different arsenic and flooding treatments.

	Replicates	Number of Sequences	Number of OTUs
MSMA flood	1	3418	1911
	2	3576	2122
	3	3489	1826
MSMA intermittent	1	3133	1863
	2	3638	2048
	3	3075	1635
No- MSMA flood	1	2589	1533
	2	3246	1880
	3	3139	1926
No-MSMA intermittent	1	3062	1745
	2	4817	2862
	3	3845	2123

Table 5.3. Rice root-plaque and rhizosphere bacterial communities: diversity and richness estimates under different arsenic and flooding treatments.

	Root-plaque				Rhizosphere			
	No. of Sequences	No. of OTUs	Shannon	Simpson (1/D)	No. of Sequences	No. of OTUs	Shannon	Simpson (1/D)
MSMA flood	10483	4530	7.69 (± 0.132)†	863 (± 80)	1264	796	6.45 (±0.126)	694 (±217)
MSMA intermittent	9846	4330	7.78 (± 0.091)	1001 (± 93)	1231	730	6.31 (±0.069)	525 (±107)
No-MSMA flood	8974	4313	7.82 (± 0.108)	896 (± 103)	1103	810	6.51 (±0.057)	772 (±284)
No-MSMA intermittent	11724	5265	7.97 (± 0.146)	1080 (± 98)	1175	774	6.43 (±0.055)	675 (±184)

†Values in parenthesis are 95% confidence intervals.

Table 5.4. Rice root-plaque and rhizosphere soil bacterial communities: pairwise shared OTUs, Jaccard, and Yue and Clayton theta analysis under different arsenic and flooding treatments.

	Rhizosphere			Rhizosphere		
	MSMA flood	MSMA intermittent	No-MSMA flood	MSMA flood	MSMA intermittent	No-MSMA flood
Shared OTUs						
MSMA intermittent	1470*			157*		
No-MSMA flood	1306*	1155*		137*	117*	
No-MSMA intermittent	1485*	1465*	1613*	67*	95*	138*
Jaccard						
MSMA intermittent	0.199			0.124		
No-MSMA flood	0.173	0.154		0.100	0.082	
No-MSMA intermittent	0.179	0.180	0.203	0.044	0.067	0.104
Yue and Clayton						
MSMA intermittent	0.510			0.079		
No-MSMA flood	0.596	0.398		0.015	0.033	
No-MSMA intermittent	0.611	0.497	0.689	0.006	0.027	0.019

*Parsimony *p* test significantly different between the two treatment communities ($p<0.001$).

Table 5.5. ANOSIM statistics for OTUs from the root-plaque samples.

Factor	R value	*P* value
Flood v intermittent	0.630	0.0097
MSMA v No-MSMA	0.963	0.0117

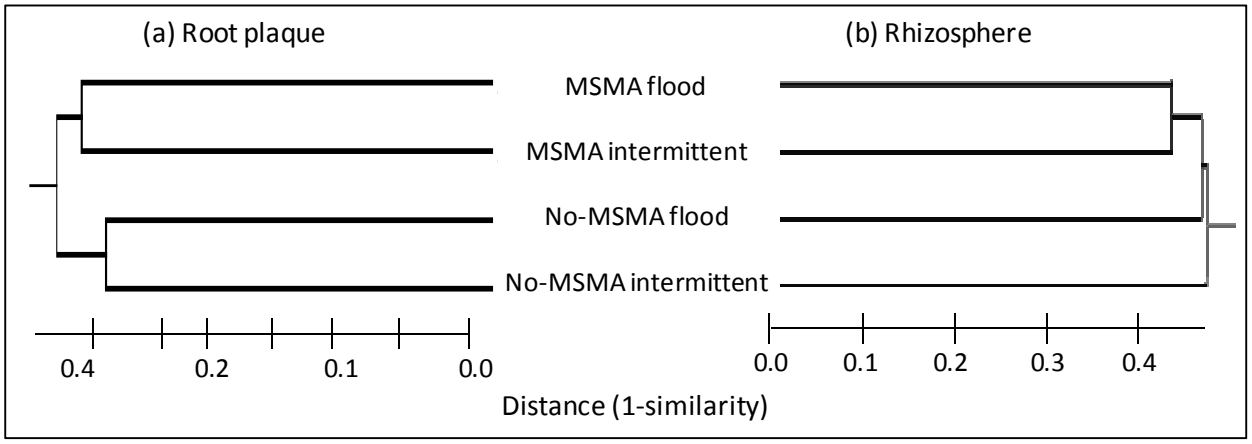

Figure 5.3. The dendogram represents bacterial community structure dissimilarity (1-similarity) among the treatments in rice (a) root-plaque and (b) rhizosphere under different arsenic and flooding treatments, based on Bray-Curtis analysis of 16S rRNA gene sequence data.

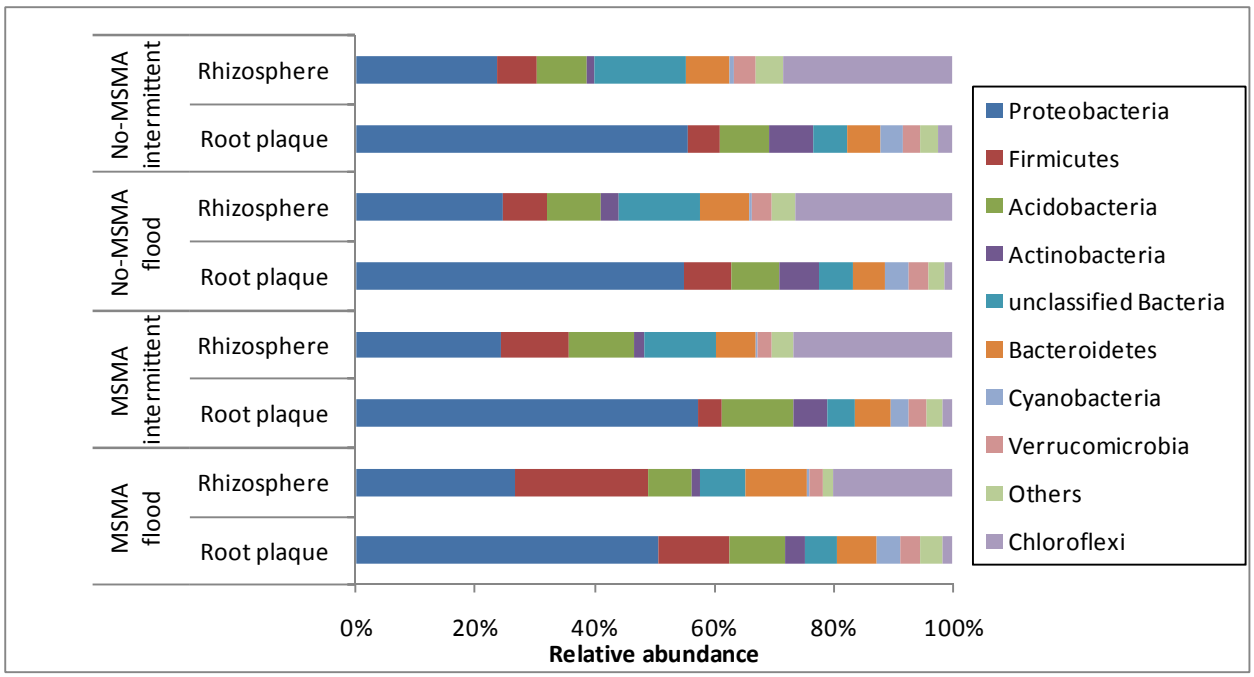

Figure 5.4. Relative abundance of bacterial phyla in rice rhizosphere and root-plaque under different As and flooding treatments, as determined using RDP classifier.

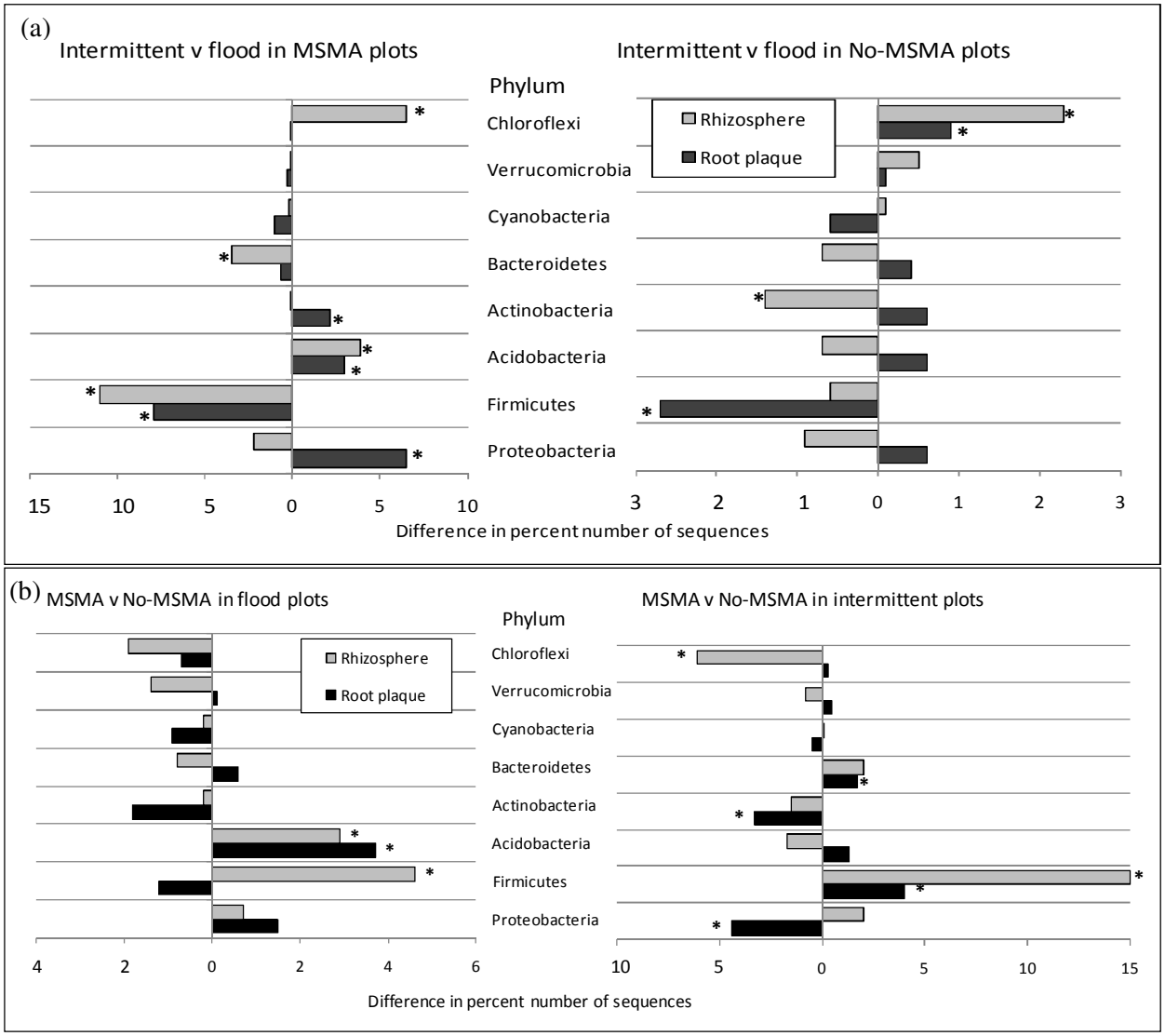

Figure 5.5. Impacts of water and As treatments on relative abundance of bacterial phyla in the rice rhizosphere. (a) Differences in the percentage of sequences in each phylum in the intermittently flooded plots relative to the continuously flooded plots with MSMA and No-MSMA amendment. (b) Differences in the percentage of sequences in each phylum in the MSMA plots relative to the No-MSMA plots with flood and intermittent plots.
* Significant difference at 0.05 level using RDP Library Compare.

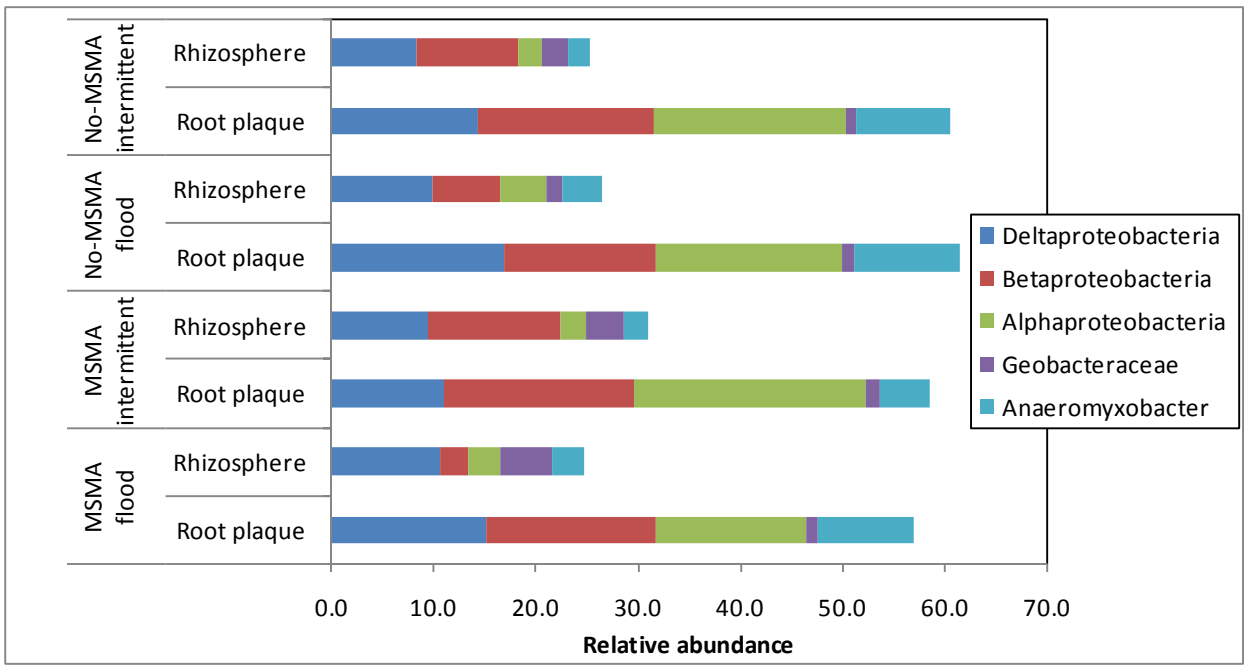

Figure 5.6. Relative abundance of bacterial groups that comprise iron reducing and oxidizing bacteria under different arsenic and flooding treatments, as determined using RDP classifier.

5.5 Discussion

5.5.1 Rhizosphere As Concentrations Varied with Water Management and MSMA Treatments

Soil-As concentrations were higher in MSMA plots due to more than 20 years of MSMA amendment; however, slightly lower total As concentrations in continuously flooded plots might suggest that more As was lost, possibly due to leaching, volatilization, or plant uptake. Conversely, pore-water As concentrations in intermittent plots decreased by approximately 85% relative to flood plots. Coupled with the higher pore-water As concentrations in the No-MSMA–flood plots versus the MSMA–intermittent plots, our results imply that water management had a greater impact than bulk-soil As concentration on the levels of total-dissolved As. Our redox measurements indicated that the rice rhizosphere in the continuous-flood plots was more reduced than in the intermittent-flood plots, thus indicating that reduced conditions might have favored

As release to pore-water. Under anoxic conditions, increased iron-oxide dissolution and reduction of iAs^{V} could result in higher soluble As concentrations in pore-water (Masscheleyn et al., 1991; Xu et al., 2008). Arsenic chemistry in flooded rice soils is primarily controlled by iron oxide minerals (Takahashi et al., 2004) because As mobilization is mostly regulated by reduction and solubilization of iron oxides (Benner et al., 2002; Rowland et al., 2007). The reduction and dissolution of iron oxides is linked to both biotic and abiotic processes in the rice rhizosphere (Wang et al., 2009), thus implying that biotic or abiotic processes (or both) might favor Fe(III) reduction and As release under continuously flooded conditions.

Relative to rhizosphere soil, root-plaque accumulated significantly higher As concentrations, in agreement with previous findings that root-plaque sequesters As (Hossain et al., 2009; Liu et al., 2006). The higher root-plaque As concentrations and As:Fe ratios in flood plots relative to intermittent plots could be due to the combined effects of increased bulk-soil iron-oxide dissolution and resulting As desorption. Increased reduction of iAs^{V} to the more soluble and mobile iAs^{III} species and the subsequent reprecipitation of iron oxide and readsorption of As at the more highly oxidized root surface may also influence As:Fe ratios. Similar ranges of As:Fe ratios have been observed for rice root-plaque under high soil As-concentrations (Hossain et al., 2009). A previous study also reported that continuous submergence results in increased Fe plaque per unit dry weight of root compared to that in lower soil moisture (Chen et al., 2008). Availability of new adsorption sites for As on freshly precipitated iron oxides could decrease the immediate bioavailability of As species that strongly bond to iron oxide minerals, including iAs^{V} and iAs^{III} (Raven et al., 1998). Root-plaque might thus reduce the bioavailability of As to plants, as suggested by previous studies (Hossain et al.,

2009; Liu et al., 2004b); however, whether root-plaque is a sink or a source of As for plants might depend on local conditions, for example localized microbial activity (Wang et al., 2009).

5.5.2 Microbial Community Response to Water Management and Rhizosphere As Concentrations

Microbial populations noticeably varied between the rhizosphere and root surface with the predominance of only *Bacteria* in the root-plaque, but with substantial populations of both *Bacteria* and *Archaea* in the rhizosphere. The relative abundance of rhizosphere *Bacteria* was significantly lower in flood than the intermittent plots, which was mostly due to higher archaeal abundance in the flood plots. These trends agree previous studies by other researchers, which observed that rice rhizosphere could harbor significant numbers of *Archaea* (Conrad et al., 2006; Conrad et al., 2008). *Archaea* and methanogens were likely present at lower abundance in the root-plaque since the surface of an actively metabolizing rice root is more highly oxidized (due to radial O_2 loss from the root) compared to the bulk rhizosphere.

The reduced conditions, especially in the rhizosphere favored FeRB, with relative abundances significantly higher in the continuously flooded plots (up to 26%) compared to intermittent plots. It was somewhat surprising to find such a high relative abundance of FeRB in the soil, which suggests that iron-reducing reactions dominated these systems. Iron-reducing bacteria are commonly found in rice paddies (Hori et al., 2009; Neubauer et al., 2007b), though usually only up to around 10% of total *Bacteria* in most studies (Weiss et al., 2003). The lower relative abundance of SRB compared to FeRB was likely due to competition from the FeRB, which is often the case in rice paddies when soil iron concentrations are several orders higher than sulfate concentrations (Achtnich et al., 1995).

The qPCR data indicated no significant difference in the relative abundance of root-plaque *Bacteria* among the water and MSMA treatments; however the 16S rRNA sequence data

did imply that bacterial community structure was significantly different among the treatments. The ANOSIM *R* static values were higher than 0.5 for both MSMA and water treatments with significant *p* values, thus suggesting that there was no significant difference among the replicated sequence data from same treatment compared to the difference among the treatments. Higher R statistics value in ANOSIM indicated that samples within same group are more similar than the samples from different groups (Clarke, 1993). These trends with the ANOSIM results along with the qPCR results demonstrated a high degree of similarity (low variability) among the DNA samples extracted from the root-plaque field replicates. Although the rhizosphere samples were analyzed by 16S rRNA sequencing as composite samples, the qPCR analysis on the individual replicate samples indicates a high degree of similarity between the replicate rhizosphere samples from each treatment.

The parsimony, Jaccard, and Yue and Clayton theta analysis of OTUs also indicated that bacterial community membership was significantly different among the treatment groups in both root-plaque and rhizosphere. Relative abundance of *Proteobacteria* was higher in root-plaque (50–58%) than in rhizosphere (20–28%), suggesting that these phyla might have more efficiently utilized carbon substrates and better adapted to the high As concentrations in the root-plaque. In a similar study of a metal-contaminated site with very high As concentrations, 78% of all the bacterial sequences belonged to *β-Proteobacteria* and the rest to *γ-Proteobacteria* (Rastogi et al., 2009). Rhizosphere *Proteobacteria* were higher in MSMA than the No-MSMA plots and in flood than the intermittent plots. This included mostly *β-, α-,* and *δ-Proteobacteria* groups (Figure 5.6). Most FeRB classify with *δ-Proteobacteria*, such as genus *Anaeromyxobacter* and family *Geobacteraceae* and most iron-oxidizing *Bacteria* (FeOB) with *β-* and *α-Proteobacteria* (Weber et al., 2006). Several FeRB, including *Anaeromyxobacter* and *Geobacter*, have been

previously detected on the surface of rice roots (Hengstmann et al., 1999; Scheid et al., 2004), and a recent study also showed that *α*- and *β-Proteobacteria* were active in oxic zones of rice paddy soil and *δ-Proteobacteria* in anoxic zones (Shrestha et al., 2009). In our study, *Chloroflexi* were present at significantly higher abundance in rhizosphere soil (20 to 27%) than root-plaque (2 to 5%). *Chloroflexi* comprises both facultative anaerobes and facultative aerobes (Yamada and Sekiguchi, 2009) and has been detected in both oxic and anoxic zones of rice paddies (Shrestha et al., 2009), thus rhizosphere soil may be an ideal niche for *Chloroflexi* spp. Given their high numbers in rhizosphere soil and the finding that their numbers were reduced at higher As levels, additional research should be conducted to determine the role(s) of *Chloroflexi* in these systems.

5.5.3 Relationships between Rhizosphere As Concentrations and Microbial Populations

High concentrations of As in the pore-water of continuously flooded plots correlate with the greater relative abundance of FeRB in the rhizosphere from these treatments. Thus, release of As adsorbed on the iron oxides to pore-water as a result of Fe(III) reduction is a likely mechanism for causing the higher levels of As in pore-water. Presence of FeRB may not directly indicate active iron reduction, but increases in relative abundance of FeRB within the total microbial community (up to 26% in our study) are suggestive of active iron reduction and possibly As reduction and release. FeRB gain energy by coupling the oxidation of organic compounds with the reduction of Fe(III) oxyhydroxides (Lovley et al., 2004). This process could result in the dissolution of solid phase Fe and subsequent solubilization of As from the surface of iron oxides (Cummings et al., 1999; Rowland et al., 2007). Studies have reported that up to 24% of total Fe reduction in rice paddy soils are a result of dissimilatory Fe(III) reduction by *Geobacteraceae* spp., *Anaeromyxobacter* spp., and other related *δ-Proteobacteria* (Hori et al., 2009; Ratering and Schnell, 2001).

Several FeRB and also *Bacteria* related to iron-oxidizing *Bacteria* (FeOB) were detected in root-plaque samples; thus both iron reduction and oxidation could be active on root-plaques, in agreement with previous studies (King and Garey, 1999; Neubauer et al., 2007a; Weiss et al., 2003). The FeRB could proliferate during the reduction of Fe (III) in iron plaque (Neubauer et al., 2005; Scheid et al., 2004) and might result in the release of root-plaque associated As. Our ammonium oxalate iron extractions showed that ferrihydrate was the most predominant phase in the root-plaque (data not presented), which might suggest that the root-plaque area may be an ideal niche for dissimilatory iron reduction, given appropriate redox conditions. The FeRB prefer low crystalline iron phases such as ferrihydrate over high crystalline phases such as goethite (Roden, 2003), and positive correlations between poorly crystalline Fe(III) phases and FeRB in the rhizosphere of wetland plants have been observed (Weiss et al., 2004). It is still debated whether FeRB results in As release, because some studies report that microbial Fe(III) reduction is likely to form secondary iron oxide phases that could adsorb to As (Kocar et al., 2006; Tufano et al., 2008). In any case, these results suggest that FeRB may affect Fe and As cycling in the rice rhizosphere, necessitating further investigation.

5.6 Conclusions

The results of this field-scale study provide additional insight into the impacts of arsenic levels and water management on microbial population dynamics and the levels of water-soluble arsenic. Water management practices and long-term applications of MSMA impacted rhizosphere As concentrations as well as the microbial community composition of rice root-plaque and rhizosphere. Our qPCR and 16S rRNA gene sequencing demonstrated that bacterial populations responded to changes in pore-water As concentrations and redox conditions. Moreover, flooding conditions affected bacterial membership in root-plaque and rhizosphere soil

which might favorably contribute toward decreased pore-water As concentrations in the rice rhizosphere. Additional research is needed to further elucidate the relative importance of biotic versus abiotic mechanisms on arsenic cycling in these systems.

CHAPTER VI

SUMMARY

This field scale study was conducted to evaluate the impact of intermittent and continuous flood practices on rhizosphere As speciation and microbial populations in plots that were either historically amended with MSMA or unamended with any As containing material. A method for As species extraction and analysis using HPLC-ICP-MS has to be standardized to study the As speciation in the treatment plots. Results presented in Chapter II indicated that sequential extraction with 0.4 M H_3PO_4 followed by 0.4 M NaOH provided the highest recovery of As from the soils compared to all of the chemical reagents evaluated. The sequential extraction using the H_3PO_4 and NaOH reagents recovered appreciable quantities of As species from rice paddy soils. The extraction efficiency ranged from 73 to 93% in the order of DMA^V > MMA^V > iAs^V.

The As speciation in different compartments of the rice rhizosphere discussed in Chapter III clearly indicated that intermittent flooding treatment significantly reduced pore-water As-concentrations by 80 to 90 % and grain As concentrations by 25 to 45 % compared to the continuously flooded treatments. Arsenite was predominant in pore-water, whereas iAs^V was predominant in root-plaque and soil. $MMAs^V$ was detected only in MSMA soils and $DMAs^V$ in pore-water and root-plaque samples from the continuously flooded plots. The $DMAs^V$ was not detected in either pore-water or in root-plaque samples from the intermittently flooded plots, in both MSMA and No-MSMA plots. $DMAs^V$ concentrations in grains were significantly reduced in intermittently flooded plots compared to the continuously flooded plots. As concentrations were significantly higher in root-plaque compared to the rhizosphere soil, and may be impacted the As availability to the plants.

The other goal of the field experiments was to study the microbial populations in the rice rhizosphere and root-plaque samples in response to continuous and intermittent flooding practices under two levels of soil. The results presented in Chapters IV and V demonstrated that both different water management practices and long-term application of MSMA impacted microbial populations in the rhizosphere and root-plaque of rice with the microbial communities for all treatments diverging over the growing season. Multivariate FAME analysis indicated that rhizosphere microbial communities changed temporally among the treatments. Community qPCR results demonstrated that the relative abundance of *Bacteria* increased over the course of the growing season, while archaeal and fungal gene abundances decreased in the rhizosphere. Although qPCR results showed little variation in bacterial relative abundance among the treatments, the 16S rRNA sequence libraries demonstrated that bacterial community structure and membership were significantly different in rhizosphere among the treatments. Both qPCR and 16S rRNA sequencing indicated that relative abundance of iron-reducing bacteria and sulfate-reducing bacteria were significantly higher under the continuous flooding relative to the intermittent flooding treatment in rhizosphere samples, implying active iron reduction and possibly As release from the iron oxides.

Quantitative PCR also indicated that *Bacteria* dominated in all samples representing 91 to 94% and 48 to 78% of the total community in root-plaque and rhizosphere, respectively, with smaller proportions of *Archaea* and *Fungi* being detected. *Proteobacteria* was the predominant phylum in root-plaque (51 to 57%) and most rhizosphere samples (23 to 27%). *Chloroflexi* (20 to 28%) were also dominant in rhizosphere samples, and their populations increased in response to intermittent flooding and higher As levels.

The results of FAME, qPCR assays and 16S rRNA sequencing consistently demonstrated that microbial populations do sense and respond to changes in pore-water As-concentrations and redox conditions. Intermittent flooding impacted bacterial community membership and also decreased the relative abundance of FeRB and SRB compared to the flooded plots, which might have favorably contributed towards decreased porewater As concentrations in the rice rhizosphere. Results of this research demonstrated that intermittent flooding could be a potential management option to reduce soluble As concentrations in the rice rhizosphere and grains in rice cultivated on fields with moderate to high As concentrations. Additional research is needed to further elucidate the relative importance of biotic versus abiotic mechanisms on arsenic cycling in these systems.

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