Silicon-rich soil amendments impact microbial community composition and the composition of \textit{arsM} bearing microbes

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Abstract

Purpose Arsenic (As) cycling in flooded rice paddies is driven by soil microbes which among other transformations can cause conversion between inorganic and organic As species. Silicon (Si)-rich soil amendments cause increased methylated As species, particularly DMA, in grain likely because they influence the microbial community responsible for As methylation, but the mechanism remains unclear.

Methods To investigate how Si-rich amendments influenced the microbial community, we sequenced the 16S rRNA and \textit{arsM} genes from rhizosphere soil collected at grain ripening from unamended rice paddy mesocosms or those amended with Si-rich rice husk, charred husk, or calcium silicate, and paired these data with geochemistry and As speciation in grain.

Results We found that Si amendments influenced the 16S rRNA and \textit{arsM} community composition. Increased C storage from calcium silicate amendment drove differences in the 16S rRNA community, whereas low soil redox potential drove differences in the \textit{arsM} community. Differences in grain As were observed independent of Si-rich amendments, and did not correspond to differences in either the 16S rRNA or \textit{arsM} community. Instead, methane flux and soil redox potential correlated with differences in grain DMA.

Conclusions Si-rich amendments drove changes in the microbial community composition and the subset of \textit{arsM}-bearing organisms, but higher grain DMA levels were not directly caused by Si-rich amendments. Our findings imply that microbes active at lower soil redox potentials where As is mobilized are likely involved in DMA production, and future work should focus on linking the active community with DMA production.

Keywords Arsenic · Methylation · Rice · Microbiome · Silicon

Introduction

Arsenic (As) cycling in flooded rice paddies impacts human health and is driven by soil microbes. Microbes are responsible for the release of As (iron-reducing bacteria), transformation of As between different oxidation states (As-reducing and oxidizing bacteria), and transformation of As between inorganic and organic forms (As-methylating and demethylating microbes) (Von Endt et al. 1968; Sanders 1979;
Qin et al. 2006; Lomax et al. 2012; Yan et al. 2015). However, As-methylating microbes (AsMM) are poorly understood, and yet have a critical influence on grain As speciation.

Rice grain tends to contain higher concentrations of organic As species, mainly DMA, upon addition of silicon (Si)-rich soil amendments to paddy soil (Liu et al. 2014; Ma et al. 2014; Seyfferth et al. 2016; Teasley et al. 2017; Limmer et al. 2018; Yang et al. 2018). This implies that As methylation is promoted by Si-rich amendments but for reasons that are unresolved. A meta-analysis of Si-rich amendments found that increased grain organic As concentration was positively correlated with increased rice straw Si concentration (Seyfferth et al. 2018). For example, rice husk, charred husk, and calcium silicate all increase grain DMA compared to control, but likely for different reasons (Limmer et al. 2018; Limmer and Seyfferth 2020). Si-rich amendments can release inorganic As (Seyfferth and Fendorf 2012), increase organic matter, and/or decrease soil redox potential (Penido et al. 2016; Yang et al. 2018) depending on the type of amendment. While Si-rich amendments promote As methylation and cause an increase in grain DMA concentration, they also can decrease grain inorganic As concentrations, which is more acutely toxic than DMA (Li et al. 2009; Liu et al. 2014; Ma et al. 2014; Seyfferth et al. 2016, 2018, 2019a; Teasley et al. 2017; Limmer et al. 2018; Yang et al. 2018). Therefore, it is necessary to understand how Si-rich amendments influence the soil microbial community involved in As cycling, especially As methylation.

Silicon-rich amendments may increase available inorganic As (iAs) rather than the less abundant DMA directly and consequently stimulate arsenic methylation (Dykes et al. 2020). Silicon-rich amendments increase the amount of porewater Si, which in the circumneutral pH of flooded rice paddy porewaters is typically found as silicic acid. Silicic acid is a chemical analog of arsenous acid, the dominant As species in flooded rice paddy porewaters (Marin et al. 1993; Takahashi et al. 2004). Therefore, when Si-rich amendments increase porewater Si, increased competition with inorganic As for binding sites causes inorganic As desorption from soil solids (Luxton et al. 2006; Seyfferth and Fendorf 2012). This release of inorganic As increases the substrate for As methylation, and could therefore be responsible for promoting As methylation, resulting in higher levels of methylated As species in porewater and ultimately grain (Zhao et al. 2013b). However, the type of Si amendment affects this process, with some plant-based Si-rich amendments such as rice husk or charred husk differing in Si dissolution rates compared to inorganic Si-rich amendments (Teasley et al. 2017; Seyfferth et al. 2018; Limmer et al. 2018; Linam et al. 2021). This ultimately affects how much Si (and therefore As) is released to the porewater in a given period of time.

Incorporation of plant-based Si-rich amendments such as rice straw and rice husk also lead to an increase in soil organic matter and decrease in soil redox potential, which directly impacts the soil microbial community (Zhao et al. 2013a; Ma et al. 2014; Penido et al. 2016; Wang et al. 2019). Increased organic matter can decrease soil redox potentials and increase As availability by increasing activity of anaerobic organisms that consume organic carbon such as methanogens and Fe and As reducing bacteria (Yang et al. 2018; Wang et al. 2019). Increased activity of Fe-reducers and As-reducers ultimately leads to increased inorganic As(III) in porewater (Yang et al. 2018; Wang et al. 2019) as adsorbed inorganic As is released from soil solids via reductive dissolution of Fe-(oxyhydr)oxides and As(V) is reduced to As(III), which is more mobile. Increasing inorganic As(III) leads to higher levels of porewater and grain DMA, most likely by increasing the amount of substrate (inorganic As) available to be methylated. Low soil redox potentials and high soil organic matter can also enrich for an anaerobic microbial community, which may be important in As methylation (Zhao et al. 2013b). For example, methanogens were recently demonstrated to be important As methylators in low-redox environments (Viacava et al. 2020). Some methanogens have also been suggested as As de-methylators (Chen et al. 2019a). Rice straw incorporation in particular enriched for mcrA/mrtA, (a marker gene for methanogenesis) and increased dissolved CH4 concentrations and As release (Penido et al. 2016). However, straw and husk amendments behave differently as Si-rich amendments (Penido et al. 2016), and the relationship between methanogens, methane flux, and grain organic As is still unclear.

To better elucidate how Si-rich amendments influence the microbial community and the community
of arsM-harboring organisms, we sequenced the 16S rRNA gene and the arsM gene from rhizosphere soil at grain ripening from rice paddy mesocosms that were unamended (Control) or amended with rice husk (Husk), charred husk (Char), or calcium silicate (Silicate) and combined these analyses with extensive geochemical characterization that was previously described from the same field experiment (Limmer and Seyfferth 2020). While AsMM bearing the arsenic methyltransferase arsM gene have been historically thought to be responsible for transforming inorganic As into methylated species (Qin et al. 2006; Lomax et al. 2012), a growing body of literature suggests that in low redox environments, arsM may not be active as an As detoxification strategy (Chen et al. 2019b; Viacava et al. 2020). Instead, methanogens which methylate As through a non-specific side reaction of As may be responsible for As methylation (Thomas et al. 2011; Viacava et al. 2020). We therefore hypothesized that Si-rich amendments would impact soil redox potential and As availability, thereby indirectly impacting the microbial community. This work expands our understanding of how different soil Si-rich amendments impact the microbial community and our understanding of AsMM in rice paddies.

Methods

Field experiment

DNA was extracted from isolated rice rhizospheres in year 2 after Si-rich amendments were added to soil in an outdoor mesocosm facility previously described (Limmer et al. 2018) where geochemical measurements were obtained over the growing season (Limmer and Seyfferth 2020). Briefly, paddy mesocosms (2 m²) were either unamended (Control) or amended with a mixture of silicic acid and calcium silicate (Silicate), rice husk (Husk), or charred husk (Char) in triplicate at a rate of 5 Mg Si/ha prior to the 2015 growing season (Limmer et al. 2018). Husk contained 0.3 mg/kg As, 40% C, and 0.34% N while Char contained 0.63 mg/kg As, 38% C, and 0.59% N; the salts used for Silicate treatment were ACS certified (99.99% pure). The paddy soil was classified as an Ultisol with a silty clay loam texture and contained 3.8 mg/kg As and 2.4% organic matter. The soil pH was 6.1 (1:1 v:v), the cation exchange capacity was 6.1 meq/100 g, and prior to amending in year 1 plant-available Si was 16 mg/kg (16-h 0.01 M CaCl₂ extraction) (Wu et al. 2020). At the start of the 2016 growing season, each mesocosm was fertilized with urea and KCl, but no additional Si-rich amendments were applied. Each paddy was hand planted with 49 rice (Oryza sativa L. c.v. Jefferson) seedlings at the 3–4 leaf stage that had been germinated in field soil. At transplanting, five plants per paddy were contained within 100 µm nylon mesh bags filled with paddy soil to define the rhizosphere for temporal sampling. Bags were 15.2 cm × 31.8 cm for plants and rhizospheres sampled in this study. Paddies were kept under flooded conditions using municipally treated drinking water that had no detectable As.

Microbial sampling

To collect soil samples for microbial analysis, one bag containing one plant was pulled from each paddy at the grain ripening stage (88 days past transplant, (DPT)). Rhizosphere soil was collected by separating roots from the root mass and washing them in 25 mL of 18 MΩ-cm sterile water, twice, by vortexing. Rhizosphere soils were frozen immediately following collection prior to DNA extraction. DNA was extracted from soils using the Qiagen PowerSoil DNA extraction kit, with modifications. From the bead-beating tubes, 200 µL of powerbead solution was replaced with phenol:chloroform:isoamyl 25:24:1 alcohol at neutral pH. The protocol was followed as written by the manufacturer until column binding. Here, equal parts lysate, solution C4, and absolute ethanol were homogenized before being loaded onto the column. Following this the DNA bound column was washed with 100% ethanol (650 µL), then solution C5 (500 µL). DNA was eluted with molecular biology grade water (based on personal communication with Qiagen representative).

16S rRNA gene sequencing and processing

To assess the diversity of the whole microbial community, DNA samples were submitted to the Joint Genome Institute for paired end (2×300) Illumina MiSeq iTag sequencing of the 16S rRNA gene. The
V4-V5 region was amplified using primers 515F-Y and 926R, according to Parada et al. 2015. 16S rRNA sequences were processed with the JGI pipeline. Briefly, sequences were de-multiplexed, quality-filtered, checked for chimeras, and clustered to 97% similarity with the iTagger pipeline (Tremblay et al. 2015). Processed sequences were imported into qiime2 (Bolyen et al. 2019), and filtered to only include sequences that occurred more than 10 times in the data set in at least 2 samples. Taxonomy was assigned to sequences using the sklearn naïve bayes feature classifier, trained with SILVA database version 132 (Quast et al. 2012).

arsM gene sequencing and processing

To assess the diversity of arsM-bearing organisms closest to the grain filling stage of rice growth, arsM genes were amplified and sequenced from rhizosphere samples collected at 88 DPT. DNA was extracted from soil samples for each paddy as described above, then amplified in a reaction with 5 ng template DNA, 0.4 mM barcoded forward and reverse primer, and 1X NEB Taq master mix in a PTC-100 thermocycler (MJ Research Inc.) for 10 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 60 °C for 45 s, 72 °C for 60 s, and a final incubation at 72 °C for 10 min. Barcoded primers were designed from PacBio® barcodes for SMRT® sequencing, based on arsM primers F1 and R2 from Zhao et al. (2013a, b) (Table S1). Successful amplification for each sample was confirmed by gel electrophoresis. Amplicons were purified with MN PCR purification kit, according to manufacturer’s instructions. Amplicon DNA concentration was determined using the Qubit BR DNA assay, according to manufacturer’s instructions and 80 ng of each barcoded amplicon were pooled and submitted for sequencing with PacBio RSII to the University of Delaware Genotyping and Sequencing Center.

arsM amplicons were initially filtered with the PacBio SMRT portal, including only sequences that had at least 5 circular consensus reads. Preliminarily filtered reads were imported into mothur (Schloss et al. 2009), and filtered to only include reads with an average quality score of at least 25, <1 ambiguous bases, <6 homopolymers, minimum length of 200 bp, maximum length of 450 bp, <1 difference in the primer, and <1 difference in the barcode. Sequences were aligned to a custom reference database, developed from the FunGene arsM database (described below) (Fish et al. 2013). Then, sequences were pre-clustered, merging groups within 2 nt of each other, and chimeras were removed. Sequences were then clustered into groups with 99% similarity using the nearest neighbor method. Taxonomy was assigned to representative cluster sequences using BLAST against the NCBI nt database (NCBI Resource Coordinators 2016), taking the top hit with best_hit_overhang parameter set to 0.25, and best_hit_score_edge set to 0.05.

To develop a custom arsM database for reference alignment, all arsM sequences with a score above 50 were downloaded from the FunGene website, resulting in 23,043 sequences (Fish et al. 2013). For the reference nucleotide alignment, sequences were filtered with mothur (Schloss et al. 2009) to only include those with <1 ambiguous bases, <10 homopolymers, minimum length of 200 bp, maximum length of 500 bp, and <1 difference in the primer for downstream analysis (3795 sequences). For the reference alignment, sequences were de-replicated in mothur then aligned with MUSCLE (a widely-used, fast multiple sequence alignment software), then the alignment was refined using the muscle-refine option (Edgar 2004). The alignment was visually assessed using AliView (Larsson 2014).

To assess the range in diversity of our ArsM sequences against other ArsM proteins, we treed our sequences with reference proteins from NCBI. First, filtered unaligned arsM sequences from this study were translated and screened for frameshift sequencing errors using frameshift_polisher (https://github.com/dnasko/frameshift_polisher), implementing the 23,043 reference protein sequences downloaded from FunGene (described above) as a reference. Translated protein sequences were clustered at 90% similarity using CD-HIT (Fu et al. 2012), then aligned with MUSCLE. A separate reference protein sequence database, containing proteins between 200–400 bp from bacteria and archaea matching the search term “arsM” were downloaded from NCBI, along with eleven additional biochemically characterized arsenite methyltransferases, according to Reid et al. (2017). A new reference database was compiled for better comparison to previous literature. The NCBI reference sequences were aligned with MUSCLE, then the two alignments were aligned with each other using MUSCLE. The aligned set of ArsM protein sequences was
treed using RAxML-NG, using a single random starting tree and the JTT substitution model (Kozlov et al. 2019) and visualized using iTol (Moore et al. 2017).

To investigate the diversity and enrichment patterns of \textit{arsM} and 16S rRNA sequences in conjunction with chemical analysis, the \textit{arsM} shared table (generated using mothur) and the 16S rRNA biom table (generated using qiime2), along with associated rooted trees and taxonomy files were imported to R (R Core Team 2019) using qiime2R (Bisanz 2018) and phyloseq (McMurdie and Holmes 2013). To determine OTUs that were indicator species for each treatment, we used the multi-level pattern analysis function with the R indicspecies package, with default parameters (Cáceres and Legendre 2009). 16S rRNA gene sequences are deposited at JGI https://doi.org/10.25585/1488298 and \textit{arsM} sequences are deposited at NCBI accession PRJNA690162.

Chemical analysis

After shoots and cleaned roots were collected, they were separated for chemical analysis of roots, shoots, husk, and grain. Husk, and grain were only analyzed at the last timepoint (98 DPT). After soil was cleaned from the root mass, roots were air dried and the iron plaque was removed using a cold dithionite-citrate-bicarbonate (DCB) extraction (Taylor and Crowder 1983). Rough rice was air-dried to < 10% moisture (dry basis) and dehusked and polished. Other plant parts were oven-dried and finely ground prior to microwave digestion with concentrated nitric acid (Seyfferth et al. 2016). After acid digestion, the samples were centrifuged and the acid fraction was decanted for ICP analysis. The remaining Si-rich precipitate was washed with DI water, centrifuged, and decanted three times prior to dissolving in 2 M NaOH (Kraska and Breitenbeck 2010). The Si-rich fraction was analyzed using the molybdenum blue method. DCB extractions of root plaque and plant tissue acid digestions were subject to elemental analysis using ICP-OES (As, Al, B, Ca, Cu, Fe, K, Mg, Mn, P, Si, and Zn) or ICP-MS (As in grain, husk, straw, and root, Agilent 7500). In addition, grain As speciation was analyzed via HPLC-ICP-MS using the same instrumental setup as for grain As speciation described above. Because samples were preserved with nitric acid and stored under oxic conditions, As(III) and As(V) were summed and are reported as inorganic As. DOC was measured by vario TOC cube (Elementar). Methane flux was also measured on a weekly basis according to Limmer and Seyfferth (2020) using the closed chamber technique with a chamber (1.5 × 1.5 × 1.5 m) able to encompass the entire planted paddy and a portable greenhouse gas analyzer (Los Gatos Research, San Jose, CA). Greenhouse gas fluxes were calculated from the slope of the linear portion of the gas concentration curve over time and units were converted using the ideal gas law. Because porewater between 55–89 DPT was most predictive of mature grain As concentrations (Limmer and Seyfferth 2020), the median porewater or median methane flux value in this range of sampling was used for analysis with microbial data. Following the 2016 growing season, five soil cores from 0–10 cm depth were
collected in diagonal pattern across each rice paddy and composited for analysis of solid-phase As pools by As sequential extraction, as described in detail by (Seyfferth et al. 2019b). The extractants and their defined fractions, described by Wenzel et al (2001), were 0.05 M ammonium sulfate for fraction F1 (non-specifically sorbed As), 0.05 M ammonium phosphate for fraction F2 (specifically sorbed As), 0.2 M acid ammonium oxalate for fraction F3 (As associated with amorphous Fe and Al oxides), 0.2 M ammonium oxalate and ascorbic acid for fraction F4 (As associated with crystalline Fe/Al oxides) and nitric acid and hydrogen peroxide for fraction F5 (residual As).

Statistical analysis

All statistical analyses were performed in R and are noted where described. Here, we compared the median porewater values between 55–89 DPT for each paddy to alpha-diversity parameters and relative abundance of selected OTUs using Spearman’s rank correlation. A Kruskal–Wallis test was used for comparisons between treatment groups and grain DMA groups, with Dunn’s post hoc test. The ADONIS test was used to compared beta-diversity across treatment groups and grain DMA categories. The envfit function included in the R package vegan (Oksanen et al. 2019) was used for comparisons of beta-diversity with continuous variables.

Results

We sequenced the 16S rRNA and arsM genes from the rhizosphere soils collected at grain ripening (88 DPT) of 12 rice paddy mesocosms amended with husk, char, silicate, or unamended control and paired these data with geochemical measurements from the rice paddies that were previously described (Seyfferth et al. 2019b; Limmer and Seyfferth 2020). We obtained 36,829 raw arsM sequences (with at least 5 circular consensus reads) from PacBio sequencing, of which 24,627 (67%) remained after quality filtering. Reads were clustered at 97% similarity into 11,678 OTUs at an average length of 253 base pairs. We obtained a good sequencing depth of 16S rRNA sequences, as determined by the 16S rRNA rarefaction curve (Fig. S2). 16S rRNA gene sequencing effort are summarized by sample in Table S3.

Effect of amendments on porewater and plant chemistry

As described in Limmer and Seyfferth (2020), the Si-rich amendments had contrasting effects on porewater chemistry 2 years after application. The labile C present in Husk was still sufficient to result in significantly increased average methane emissions (2.4x) relative to the Control (p=0.038). However, Husk did not significantly increase average porewater DOC relative to the Control. Husk also decreased average porewater redox potential (12 ± 38 mV [average ± standard deviation]) relative to Control (87 ± 46 mV) and increased average porewater Fe(II) (894 ± 142 μM) relative to Control (286 ± 208 μM). These reduced conditions led to higher porewater As in Husk-treated paddies (187 ± 49 μM) relative to Control (90 ± 49 μM). Most of this As was present as inorganic As (63% on average), with DMA and TMAO comprising the remainder of the As species. Silicate treatment increased average porewater DOC (62 ± 7 mg C/L) relative to Control (34 ± 1 mg C/L) and also increased average porewater Ca (5.1 ± 0.4 mM) relative to Control (3.6 ± 0.3 mM) due to the use of CaSiO₃. Average porewater concentrations of other divalent cations were also elevated by Silicate, including Mg (3.3 ± 0.6 mM) and Mn (630 ± 170 μM), relative to Control (2.2 ± 0.5 mM and 410 ± 19 μM, respectively).

Si-rich amendments significantly affected plant Si, but only affected plant As in vegetative tissues. Si-rich amendments performed similarly, increasing straw and husk Si by 55% and 15%, respectively, relative to Control. Plant As in the vegetative tissues (straw, nodes, flag leaves, rachis) was decreased 49% by Si-rich amendments relative to Control. In contrast, plant As in the reproductive fractions (ripe and unripe grain, bran, and unfilled grain) was minimally affected by Si-rich amendments (6% decrease).
relative to Control. Polished grain As was primarily DMA (69 ± 14% average ± standard deviation, n = 12) and the concentration and speciation was not significantly affected by treatment.

Treatment and 16S rRNA diversity

The 16S rRNA Shannon diversity and Pielou’s evenness in the rhizosphere at ripening was significantly higher in the husk-amended soils than control or silicate-amended soils (Fig. 1a,b, pairwise Kruskal–Wallis, p < 0.05). 16S rRNA alpha-diversity did not significantly correlate with any porewater chemistry factors, methane flux, grain As, or solid phase As pools (Supplemental file Table 1).

The 16S rRNA rhizosphere community composition at ripening differed by treatment, with treatment explaining 54% of the community variation (Fig. 1c, ADONIS, R² = 0.54, p = 0.015). The 16S rRNA community of control and char amended paddies were similar to each other, with husk and silicate amended paddies separate from each other and from control and char-amended soils, which clustered together (Fig. 1c). The 16S rRNA community at grain ripening in the rhizosphere was dominated by Sideroxydans OTU2, Spirochaetaceae OTU3, and Prolixibacteraceae BSV13 OTU8, which were the three most abundant OTUs across all 12 paddies (Fig. 2). To determine OTUs that were indicator species for each treatment, we used the multi-level pattern analysis function with the R indic species package. Several OTUs were identified as indicators of different treatments; 201 were indicators of silicate, 87 were indicators of husk, 35 were indicators of char, and 27 were indicators of control (Fig. S3-S6). Of all the indicator OTUs, most were uncultured, unclassified, or heterotrophs (Fig S7, Supplemental file table 2).

Differences in Si-rich amendments correlated with differences in the soil microbial community composition (Fig. 3, Supplemental file table 3). Silicate-amended paddies led to high porewater DOC, Ca, and Mg concentrations, and we observed that differences in 16S rRNA community composition correlated with porewater DOC (Vegan envfit function, R² = 0.56, p = 0.05) along with Ca (R² = 0.56, p = 0.02) and Mg (R² = 0.52, p = 0.04), which were covariates with DOC (Fig. 3, Supplemental file table 3). In addition, divalent cations in the root (which are a proxy of chemical species available to the root) such as Ca (R² = 0.56, p = 0.03), Mg (R² = 0.53, p = 0.05), and Mn (R² = 0.44, p = 0.07) and shoot Mn (R² = 0.59, p = 0.03) were significantly correlated with differences in 16S rRNA community composition, with a high concentration of divalent cations associated with silicate-amended paddies (Supplemental file table 3). Indicator species associated with silicate amended soils positively correlated with porewater DOC.

Fig. 1 Alpha diversity of 16S rRNA communities by treatment (A) and constrained correspondence analysis of 16S rRNA communities at ripening (88 DPT) in the rhizosphere (B) from rice paddies amended with various Si-rich sources and unamended controls. In alpha diversity plots, data with the same letters above the bars are not significantly different from one another. Samples are represented as points in CCA where treatment constrained the ordination, with treatment arrows overlain.
(Supplemental file table 4) and included organotrophs such as Geobacter and Gemmatimonadaceae.

Because Fe-reducing bacteria (FeRB) and methanogens have previously been implicated as important players in As cycling and DOC consumption in wetland soils, we compared the sum relative abundance of organisms identified as FeRB (OTUs listed in Fig. S8) or methanogens (OTUs listed in Fig. S9) or by taxonomy and their relationship with each other and geochemical factors. The relative abundance of methanogens and FeRB were significantly different across treatments, (Fig. 4a, b Kruskal–Wallis methanogens \( p = 0.10 \), FeRB \( p = 0.08 \)) with methanogens highest in the husk treatment and FeRB lowest in the husk treatment.

Treatment and \( \text{arsM} \) diversity

The ArsM OTUs were widely distributed amongst a set of representative sequences, with some clustering with previously observed ArsM sequences but many clustering independently of previously observed ArsM sequences (Fig. S10). This indicates that our ArsM sequences expand upon the previously observed diversity in ArsM sequences. Of the 165 OTUs that were assigned taxonomy with the
NCBI blast database (3.6% of total OTUs), 55 were designated *Rhodopseudomonas palustris*, the organism from which *arsM* was originally characterized as an As-detoxifying As-methylating protein coding gene (Supplemental file table 5) (Qin et al. 2006). *Rhodopseudomonas palustris* OTUs were found in all samples. Other *arsM* OTUs included *Gemmatirosa kalamazooensis* (10 OTUs), *Planctomycetes* (22 OTUs), *Rubrivivax gelatinosus* (6 OTUs), *Sphaerobacter thermophilus* (9 OTUs), *Stenotrophomonas* sp. NA06056 (8 OTUs), and various *Streptomyces* species (11 OTUs). Four OTUs (0.09% of OTUs) were classified as archaea in the families *Haloarculaceae* and *Halobacteriaceae*.

The Shannon diversity and Pielou’s evenness of *arsM*-bearing organisms in the rhizosphere at ripening generally did not differ between treatments; however, silicate-amended soils had higher Shannon diversity than char-amended soils (Fig. 5a, b, pairwise Kruskal–Wallis, *p* < 0.05). In addition, Shannon diversity was positively correlated with porewater Mg (Spearman’s rank correlation, rho = 0.60, *p* = 0.04) and DOC (rho = 0.57, *p* = 0.06), and negatively correlated with porewater B (rho = −0.70, *p* = 0.02) and fraction F3 As at harvest (associated with amorphous and Fe and aluminum oxides, rho = −0.70, *p* = 0.01, Supplemental file table 1). Pielou’s evenness was positively correlated with methane flux (rho = 0.64, *p* = 0.03), grain inorganic As at harvest (rho = 0.62, *p* = 0.03), and negatively correlated with fraction F2 As at harvest (specifically sorbed As pool, rho = −0.64, *p* = 0.03), fraction F3 As at harvest (rho = −0.63, *p* = 0.03), and B (rho = −0.59, *p* = 0.04, Supplemental file table 1).

Similar to the 16S rRNA community, the distribution of rhizosphere *arsM* differed by treatment at ripening (Fig. 5c, ADONIS of Bray–Curtis dissimilarity *p* < 0.07, homogeneity of dispersion *p* > 0.05). In the CCA, control and char-amended paddies clustered more closely to each other, with
husk- and silicate-amended paddies distinct from other treatments. Treatment explained 61% of the variation in the \textit{arsM} community (ADONIS, $R^2 = 0.61$, $p = 0.034$). Of the thirty most abundant \textit{arsM} OTUs in the rhizosphere at grain ripening, only one was identified beyond the kingdom level as \textit{Rhodopseudomonas palustris} (Fig. 6). Twenty-four OTUs were identified as indicators of husk amended paddies, compared to one indicator of char, six indicators of silicate, and three indicators of control (Fig. S11). Hierarchical clustering of paddies considering just indicator OTUs only grouped husk-amended paddies together (Fig. S12). No indicator OTUs were assigned taxonomy more detailed than the kingdom level.

The \textit{arsM} community composition correlated with treatment-induced differences in geochemical parameters (Fig. 7). Silicate-amended paddies were associated with higher porewater Ca, and the distribution of \textit{arsM} correlated with porewater Ca (envfit, $R^2 = 0.52$, $p = 0.05$, Supplemental file table 6). Other plant nutrients like Cu ($R^2 = 0.58$, $p = 0.03$), Mg

![Fig. 6](image1)

**Fig. 6** Bubble chart of the most abundant \textit{arsM} sequences in the rhizosphere at grain ripening. Size of bubble corresponds to relative abundance, with bubbles colored according to treatment.

![Fig. 7](image2)

**Fig. 7** Constrained correspondence analysis with porewater Ca (µM), methane flux (nmol/m²/s), Mg (µM), pH, inorganic As (µM), and Fe(II) (µM), correlating with differences in \textit{arsM} community composition. Samples are represented as points in CCA and constraining variables are overlain as arrows.

(R² = 0.45, $p = 0.1$) and P (R² = 0.51, $p = 0.06$) were also correlated with the distribution of \textit{arsM} and were associated with silicate-amended paddies. In
contrast, husk-amended paddies were associated with higher \( \text{CH}_4 \) fluxes than other treatments, and \textit{arsM} distribution also correlated with \( \text{CH}_4 \) flux (enfit, \( R^2 = 0.75, p = 0.004 \), Fig. 7). In addition, porewater pH (\( R^2 = 0.56, p = 0.04 \)), Fe(II) (\( R^2 = 0.56, p = 0.03 \)), and inorganic As (\( R^2 = 0.44, p = 0.08 \)) correlated with \textit{arsM} distribution, and were covariates with methane flux. Furthermore, husk indicator OTUs were positively correlated methane flux, Fe(II), porewater inorganic As, and with each other (Fig. S13).

Grain As and microbial communities

Although grain DMA did not differ by treatments, a subset of paddies that were spatially grouped and had low soil redox potentials and had high grain DMA concentrations (Fig. 8a, b); of these, two were husk-amended, one was silicate-amended and one was from the unamended control treatment. To probe this further, we compared the microbial community to grain DMA levels and to porewater geochemistry to better understand biogeochemical factors contributing to high grain DMA. The \textit{arsM} distribution did not correlate with grain As speciation (when treated continuously, enfit, \( p > 0.05 \)) or any soil As fraction (\( p > 0.05 \), enfit, Supplemental file table 6). Furthermore, the distribution of \textit{arsM} did not differ between grain DMA categories (when treated categorically, Fig. 8c, ADONIS, \( R^2 = 0.08, p = 0.7 \)). In addition, Shannon diversity and Pielou’s evenness of \textit{arsM} was not different by grain DMA categories (Kruskal–Wallis, \( p > 0.05 \), Fig. 8e, f). Only Pielou’s evenness was

Fig. 8 Grain DMA varied more by spatial orientation of paddy location than by treatment. A. Diagram of paddy field layout. B. Grain DMA by paddy, colored to represent assigned grain As category. C, D. Correspondence analysis of \textit{arsM} (C) and 16S rRNA (D) microbial community composition colored by grain DMA category. E, F. Boxplot comparing alpha diversity compared across grain DMA categories, with outliers as points for \textit{arsM} (E,F) and 16S rRNA (G,H). No significant differences were observed between grain As categories in E,F,G, and H.

\( \text{arsM} \) distribution also correlated with \( \text{CH}_4 \) flux (enfit, \( R^2 = 0.75, p = 0.004 \), Fig. 7). In addition, porewater pH (\( R^2 = 0.56, p = 0.04 \)), Fe(II) (\( R^2 = 0.56, p = 0.03 \)), and inorganic As (\( R^2 = 0.44, p = 0.08 \)) correlated with \( \text{arsM} \) distribution, and were covariates with methane flux. Furthermore, husk indicator OTUs were positively correlated methane flux, Fe(II), porewater inorganic As, and with each other (Fig. S13).

Grain As and microbial communities

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positively correlated with grain inorganic As (Supple-
mental file table 1, Spearman’s rank correlation,
rho=0.63, p=0.04), however no \textit{arsM} alpha diver-
sity factors were related to grain DMA.

Similar to \textit{arsM}, the distribution of the 16S rRNA
microbial community did not differ between grain
DMA categories (Fig. 8d, ADONIS, R²=0.09, 
p=0.5), and also did not correlate with grain As
(when treated continuously, envfit, p > 0.05, Supple-
mental file table 3). 16S rRNA alpha diversity param-
eters (Shannon diversity, Pielou’s evenness) did not
differ by DMA category or correlate with grain As
(Fig. 8g, h, Supplemental file table 1, Kruskal–Wal-
lis, p > 0.05).

We also specifically examined sum relative abun-
dance of methanogens and FeRB from the 16S rRNA
sequencing dataset, and although FeRB were slightly
higher in paddies in the low grain DMA category,
neither microbial functional group was significantly
different between high and low grain DMA paddies
(Fig. 9a, b, Kruskal–Wallis p > 0.1). However, we did
find that median methane flux was negatively corre-
lated with the relative abundance of FeRB (Fig. 9c,
Spearman’s rank correlation, rho = −0.67, p=0.02)

Fig. 9 Boxplot of summed relative abundance of
methanogens (A) and FeRB (B) in the rhizosphere at
grain ripening compared across grain As categories,
and correlations between median methane flux
between 55–89 DPT and the
summed relative abundance
of FeRB (C), and grain
DMA (D). In A and B no
significant differences were
observed across grain DMA
categories. Also shown
are correlations between
median porewater chemistry
related to grain organic As.
Grain DMA and median
porewater DMA between
55 and 89 DPT (A):
median porewater As and
median porewater redox
potential between 55 and 89
DPT (B); grain DMA and
median porewater redox
potential between 55 and 89
DPT (C); grain DMA and
median porewater As
between 55 and 89 DPT.
In C–H, points are colored
by paddy, blue line shows
linear regression, and gray
shaded region shows 95%
confidence interval.
and median methane flux was positively correlated with grain DMA (Fig. 9d, Spearman’s rank correlation, $\rho = 0.55, p = 0.07$).

Finally, to investigate the geochemical relationships that may have led to high grain DMA in the spatially-grouped paddies (Fig. 8a, b) we compared grain DMA with various porewater factors. We found that grain DMA was positively correlated with median porewater DMA (Fig. 9e, Spearman’s rank correlation, $\rho = 0.65, p = 0.02$) and median porewater As (Fig. 9h, Spearman’s rank correlation, $\rho = 0.74, p = 0.008$). Median porewater As and grain DMA were both negatively correlated with median porewater redox potential (porewater As Fig. 9f, Spearman’s rank correlation, $\rho = −0.74, p = 0.008$; grain DMA Fig. 9g, Spearman’s rank correlation, $\rho = −0.59, p = 0.05$).

**Discussion**

Different types of soil Si-rich amendments have been explored as a strategy to decrease inorganic As concentrations in rice grain (Liu et al. 2014; Ma et al. 2014; Seyfferth et al. 2016; Teasley et al. 2017; Limmer et al. 2018; Yang et al. 2018) but their impact on the soil microbial community has not been fully explored. Si-rich amendments decrease grain inorganic As concentrations and promote soil production and plant uptake of DMA, but they also affect other important geochemistry like plant nutrition and methane emissions (Liu et al. 2014; Ma et al. 2014; Seyfferth et al. 2016; Teasley et al. 2017; Limmer et al. 2018; Yang et al. 2018) but their impact on the soil microbial community has not been fully explored. Si-rich amendments decrease grain inorganic As concentrations and promote soil production and plant uptake of DMA, but they also affect other important geochemistry like plant nutrition and methane emissions (Liu et al. 2014; Ma et al. 2014; Seyfferth et al. 2016; Teasley et al. 2017; Limmer et al. 2018; Yang et al. 2018). Here, we investigated the influence of Si-rich amendments on the 16S rRNA and *arsM* microbial communities, and how these communities influenced grain As speciation by sequencing 16S rRNA and *arsM* genes from the rhizosphere of rice plants at the grain ripening stage. It has previously been suggested that Si-rich amendments would indirectly influence the microbial community, for example by As desorption from increased competition with Si for binding sites on soil solids (Seyfferth and Fendorf 2012; Dykes et al. 2020) and increasing substrate for inorganic As methylation. We hypothesized that Si-rich amendments would indirectly impact the microbial community by impacting redox potential and As availability. We found that Si-rich amendments caused geochemical differences that corresponded with change in both the 16S rRNA and *arsM* community composition.

The study described here provides a microbial analysis of an experiment described by Limmer and Seyfferth (2020) who reported that after two years of growth in soil that was treated with Si-rich amendments at the beginning of year 1, rice plants continued to have higher Si levels in Si-amended paddies compared to non-amended control paddies regardless of the type of Si amendment. That study also found that husk amendment increased methane emissions whereas silicate amendment increased porewater DOC and some divalent cations. Here, we paired these findings with analysis of the microbial community composition, which was hypothesized to be influenced by amendment-driven changes in geochemistry (Limmer and Seyfferth 2020). We found that treatment with calcium silicate led to higher porewater levels of DOC and divalent cations, whereas treatment with Si-rich rice husk led to lower porewater Fe(II) and inorganic As, both of which shaped the microbial community. Regardless of the effect treatments had on shaping the microbial community, grain DMA levels were not controlled by treatment but were driven by over-arching differences in redox-sensitive variables.

**Treatment effects on geochemistry and the microbial community**

Silicate-amended paddies may have impacted the microbial community by influencing C storage in year one growth. In silicate amended paddies, where Ca was elevated due to the addition of Si as calcium silicate, Ca could have facilitated DOC binding to Fe/Mn oxides via Fe(or Mn)-Ca-DOC ternary complexes (Sowers et al. 2018b). In year two, as redox potential continued to decrease from year one (Limmer and Seyfferth 2020), reductive dissolution of Fe and Mn oxides would release Mn, Fe, and As into solution, and in Ca silicate amended paddies reductive dissolution could additionally release stored DOC and Ca. Mg has additionally been hypothesized to facilitate DOC binding to Fe/Mn oxides and therefore, like Ca, could be released with reductive dissolution of Fe/Mn oxides (Sowers et al. 2018b).

Increased DOC, driven by silicate treatment, was correlated with differences in 16S rRNA community composition, and was associated with several
organoheterotrophic microorganisms (Fig. 1 S7, Supplemental file table 2). In addition, most 16S rRNA silicate treatment indicator OTUs were organoheterotrophic. In particular, organisms in the family Gemmatimonadaceae were often identified as indicator species, with one of the most abundant Gemmatimonadaceae OTUs (OTU170) slightly enriched in the silicate treatment. Gemmatimonadaceae have previously been identified as possible AsMM (Jia et al. 2013), but no Gemmatimonadaceae were identified in our arsM sequencing data set (Supplemental file table 5). Members of Gemmatimonadaceae have previously been identified as poly-phosphate accumulating organisms (Zhang et al. 2003) facultative photoheterotrophs (Zeng et al. 2015), and N₂O reducing organisms under oxygen starvation (Park et al. 2017). The versatility of this family combined with its organic carbon consumption (Zhang et al. 2003; Zeng et al. 2015; Park et al. 2017) could be why these organisms were enriched in the silicate treatment. In addition, several Geobacter OTUs were impacted by Si-rich amendments, with silicate treatment enriching for Geobacter OTU6 and OTU66 (Fig. S7). Geobacter are classified within the delta-proteobacteria, which have previously been associated with AsMM (Jia et al. 2013), but likely also influence grain As by increasing porewater As availability through reductive dissolution of Fe/Mn-(oxy)hydroxides (Yang et al. 2018; Wang et al. 2019). Geobacter are chemoorganotrophs known for their role in Fe(III) and Mn(IV) reduction (Lovley et al. 2011). The high abundance of DOC and Mn in silicate amended paddies could explain why several Geobacter OTUs were silicate treatment indicators.

Because DOC drove differences in the microbial community composition, and DOC is a critical substrate for FeRB and methanogens, we chose to focus on how treatments influenced these microbial groups, and additionally how these groups were related to grain As speciation. Both groups are organisms that can influence the availability and speciation of As in paddy porewater. Methanogens were enriched in the husk treatment, whereas FeRB were lowest in the husk treatment, and methane flux and FeRB relative abundance were negatively correlated (Figs. 4a, b and 9c). Previous work has suggested that an active FeRB community can suppress methanogenic activity through competition for organic C (Rodén and Wetzel 1996; Weiss et al. 2007) in accordance with our findings here. In addition, variation in redox potential could cause shifts between FeRB and methanogen communities. Neither methanogens nor FeRB as functional groups were enriched in the silicate-amended paddies, which had the highest DOC; however, in this study we used bacteria-specific 16S rRNA primers (V4-V5 region) which limited our ability to detect archaea (such as methanogens) (Fig. 4a, b). While it is also possible that the quality of DOC or sorbed C influenced the microbial community composition (Docherty et al. 2006), we did not characterize DOC beyond porewater concentration here, but should be further explored. In addition, we found that methane flux was positively correlated with grain DMA at harvest (Fig. 9d). This suggests, in accordance with recent findings (Viacava et al. 2020), that methanogens may be important As-methylaters independent of arsM in low-redox environments.

While increasingly stronger reducing conditions from year one to year two influenced all paddies (Limmer and Seyfferth 2020), the AsMM community in Husk-amended paddies in particular was shaped by porewater parameters associated with low soil redox conditions (Fig. 7). Husk-amended paddies were associated with higher methane flux, inorganic As, and Fe(II); all factors that were correlated with differences in arsM community composition (Fig. 7). This is similar to previous work in which Fe, NO₃⁻, and total As (among other factors) were associated with AsMM community composition (Zhang et al. 2015). Fe, NO₃⁻, and As are redox-sensitive elements and therefore support our interpretation that redox potential impacts the community composition of AsMM. Redox potential may impact the community of AsMM by enriching for anaerobic organisms and/or by increasing As availability (Zhao et al. 2013b).

Redox effects, grain As, and the microbial community

Previous studies have reported the increase of organic As in grain (Ma et al. 2014; Seyfferth et al. 2016; Limmer et al. 2018; Yang et al. 2018) and porewater (Huang et al. 2012; Jia et al. 2012) with soil amendments such as rice straw, rice husk, dried distillers grain, and clover, but here husk treatment did not correspond to significantly higher grain DMA than control (Limmer and Seyfferth 2020). Rather, the paddies with the highest grain DMA were clustered
spatially in field location (Fig. 8) and belonged to control (1 mesocosm), husk (2 mesocosms), and silicate (1 mesocosm) treatments. This may have been because the soil in these 4 paddies had slightly higher total C (2.7 ± 0.8%) than the other paddies (2.2 ± 0.4) mostly likely due to slightly more A horizon incorporation during paddy construction, which may have resulted in higher labile C available for Fe reducing bacteria. The unexpected result of differential grain DMA allowed us to examine the arsM and 16S rRNA microbial communities between high and low grain DMA paddies. Community composition of either arsM or the 16S rRNA community did not correlate with differences in grain DMA (Fig. 8c, d). We also did not observe differences between paddies with different grain DMA levels within paddies of the same treatment amongst the 30 most abundant arsM or 16S rRNA OTUs (by median relative abundance in rhizosphere at grain ripening, Figs. 2 and 6) or amongst FeRB or methanogens (Fig. S8, S9). While we did not observe differences in the microbial community composition between high grain DMA and low grain DMA rhizosphere soils, there could be differences in arsM activity, or even cryptic cycling of methylated As species between AsMM and As de-methylating microbes (Chen et al. 2019a), but neither were able to be measured in this study due to low-quality RNA extracted from Fe-rich rhizosphere soils. In addition, primer bias could have prevented amplification of all arsM sequences, and all amplified sequences may not have been functional. On the other hand, redox potential was negatively correlated with grain DMA (Fig. 9g), which supports that redox potential and As availability were good predictors of grain DMA levels and this suggests that microbes active under these conditions are responsible for DMA production. In addition, methane flux was positively correlated with grain DMA which implies that methanogen activity could relate to grain DMA levels (Fig. 9d). An alternative hypothesis is that perhaps As demethylators were more active in low DMA paddies. Methanogens have previously been suggested to be involved with As methylation and As de-methylation (Chen et al. 2019a; Viacava et al. 2020).

Redox factors, including porewater inorganic As, but not grain DMA, corresponded with differences in the arsM microbial community composition, primarily separating the husk-amended community from other treatments (Fig. 7). This was surprising, given that redox potential was negatively correlated with grain DMA (Fig. 9g). Of the three husk paddies, paddy 7 and 8 had high grain DMA and low soil redox potential, but paddy 5 had low soil redox potential and low grain DMA (Figs. 8a, b and 9). Low redox potential and low grain DMA were also observed in paddy 10 (control). This implies that low redox potential from husk amendment influences the community composition of arsM, but the change in arsM community composition from husk amendment was not responsible for increased grain DMA under the conditions tested here. Recently Viacava et al. (2020) found that anaerobic microbes carrying arsM did not express arsM in response to elevated As(III), but aerobic organisms did. Instead, the only anaerobic organism that demonstrated the capacity for As-methylation was a methanogen, possibly methylating As through side reactions with methyltransferase mtaA (Thomas et al. 2011; Viacava et al. 2020). Anaerobes may not methylate As for detoxification under low redox conditions due to increased toxicity of trivalent methylated As products compared to inorganic As, and instead could use arsM in microbial chemical warfare (Chen et al. 2019b). This is in accordance with our finding that redox potential influenced arsM community composition (Fig. S11). Viacava et al. (2020) also suggested that when the As(III)-efflux pump acr3 was active, little As(III) was able to accumulate in the cell and be methylated. Perhaps under low-redox conditions organisms with active As(III) efflux pumps are selected for and methanogens methylate As independent of arsM, while under higher redox conditions (where less-toxic pentavalent methylated As species persist) As-methylation via arsM is more active. In this scenario, increased porewater As under low redox conditions would also result in higher grain DMA, as it would still increase the substrate for non-specific methylation by methanogens. We also found grain DMA did not correlate with arsM Shannon diversity (Supplemental file table 1). This is consistent with previous work in acid mine drainage sites where arsM was widely distributed, and arsM distribution and richness did not follow the As pollution gradient (Desoeuvre et al. 2016). Together, these findings indicate that redox-induced changes in arsM community composition are not responsible for observed differences in grain DMA. We hypothesize in paddies with low redox potential and low grain DMA (i.e. paddies 5 and 10, Figs. 8 and 9)
methanogens were less active than in paddies with low redox potential and high grain DMA. This is supported by our observation that methane flux was positively correlated with grain DMA.

Conclusions

We found that Si-rich amendments influenced both the whole microbial community and the As-methylating subset of the microbial community in different ways. Increases in Ca from calcium silicate treatments could have promoted C storage in year one, resulting in C release in year two, which may have driven changes in both 16S RNA and arsM gene distribution. In addition, low porewater redox potentials due to husk amendment may have driven changes in arsM community composition. Neither the 16S rRNA community nor the arsM community composition was associated with differences in grain DMA levels; however, methane flux, porewater redox potential, and porewater As were correlated with grain DMA. While these findings imply that the portion of the microbial community that is active at lower redox potentials plays a role in DMA production, future work that can link microbial activity with DMA production and plant-uptake is warranted.

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Data availability

16S rRNA gene sequences are deposited at JGI https://doi.org/10.25585/1488298 and 16S rRNA gene sequences and arsM sequences are deposited at NCBI accession PRJNA690162.

Declarations

Conflict of interest

The authors declare no conflicts of interest.

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