**QUEEN'S
UNIVERSITY
BELFAST**

Inhibition of Microbial Methylation via *arsM* in the Rhizosphere: Arsenic Speciation in the Soil to Plant Continuum

Afroz, H., Su, S., Carey, M., Meharg, A. A., & Meharg, C. (2019). Inhibition of Microbial Methylation via *arsM* in the Rhizosphere: Arsenic Speciation in the Soil to Plant Continuum. *Environmental Science and Technology*, 53(7), 3451. <https://doi.org/10.1021/acs.est.8b07008>

Published in:
Environmental Science and Technology

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

© 2019 American Chemical Society. This work is made available online in accordance with the publisher's policies. Please refer to any applicable terms of use of the publisher.

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Characterization of Natural and Affected Environments

**Inhibition of Microbial Methylation via *arsM* in the Rhizosphere:
Arsenic Speciation in the Soil to Plant Continuum**

Hasina Afroz, Shiming Su, Manus Patrick Carey, Andrew A. Meharg, and Caroline Meharg

Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.8b07008 • Publication Date (Web): 15 Mar 2019Downloaded from <http://pubs.acs.org> on March 19, 2019**Just Accepted**

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

Inhibition of Microbial Methylation via *arsM* in the Rhizosphere: Arsenic Speciation in the Soil to Plant Continuum

Hasina Afroz¹, Shiming Su², Manus Carey¹, Andy A. Meharg^{*,1} and Caroline Meharg^{*,1}

¹*Institute for Global Food Security, Queen's University Belfast, David Keir Building, Malone Road, Belfast, BT9 5BN, UK.*

²*Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences/Key Laboratory of Agro-Environment, Ministry of Agriculture, Beijing, P.R. China.*

***Corresponding authors**

A.A. Meharg, aa.meharg@qub.ac.uk, +44 (0) 28 9097 5413

C. Meharg, caroline.meharg@qub.ac.uk, +44 (0) 28 9097 6880

ABSTRACT

The interplay between rice roots and manuring with respect to arsenic speciation, subsequent assimilation into roots, and translocation to shoots in paddy soil was investigated, alongside bacterial diversity characterization. Planting increased soil Eh and decreased soil solution arsenic species: inorganic arsenic (iAs), monomethylarsonic acid (MMA), trimethylarsenic oxide (TMAO) and dimethylarsinic acid (DMA). Presence of plant roots increased copy number of *Clostridium* and *Tumebacillus* 16S rRNA as well as *Streptomyces* arsenic methylating gene (*arsM*), but decreased *Acidobacteria_GPI* 16S rRNA and *R. palustris* *BisB5 arsM*. Sum of arsenic species decreased under root influence due to the interplay of inorganic arsenic mobilization in bulk soil under anaerobic and immobilization under oxygenated rhizospheric conditions. Manuring increased all soil solution arsenic species (>90%), shoot total arsenic (60%), copy number of *Geobacter* 16S rRNA and *R. palustris* *TIE-1 arsM*, indicative of a shift towards microbes with iron reduction and oxidation as well as arsenic methylation capabilities.

51

52 **INTRODUCTION**

53 Rice grain is *circa.* 10-fold elevated in inorganic arsenic, a class-one, non-threshold
54 carcinogen, compared to virtually all other arable crops.¹ The levels of inorganic arsenic in
55 rice grain is such that they are deemed a threat to human health and have led to legislation to
56 regulate inorganic arsenic's concentrations in rice products by the European Union at 0.2
57 mg/kg As in white rice,² with the WHO setting advisory levels at this concentration.³ Young
58 children are considered to be at particular threat and stricter levels have been set at 0.1 mg/kg
59 of arsenic for rice based baby foods by the European Union² and, at the time of writing, this
60 threshold for rice based baby foods is also under consideration in the USA.⁴ The introduction
61 of food-standards will challenge the food processing industries to produce rice products lower
62 in inorganic arsenic as baby foods currently exceed these standards in circa 50% of samples
63 tested in the EU.⁵

64

65 The reason why rice is problematic with respect to inorganic arsenic is simply due to the soil
66 reduced redox conditions that predominate under paddy rice cultivation.⁶ Arsenic, as
67 arsenate, from sources such as the weathering of rocks, builds up in iron (III) oxyhydroxide
68 (iron plaque) that coats soil particles and sediments under aerobic conditions. Under anoxic
69 conditions insoluble iron (III) is reduced to soluble iron (II) and relatively insoluble arsenate
70 (As(V)) to the more soluble arsenite (As(III)). It is As(V) and As(III) together that are termed
71 "inorganic arsenic", iAs, as they are readily interchangeable depending on prevailing abiotic
72 and biotic conditions. As(V) and As(III) are analogues of the nutrients phosphate and silicic
73 acid, respectively.⁶ Rice has a high demand for both phosphate and is a silicic acid
74 accumulator.⁷ If iAs is available to roots, such as under paddy cultivation, rice will take up

high levels of iAs, to such an extent that rice shoot to soil arsenic concentrations are in the order of 1:1,⁸ remarkable for a non-essential toxicant.

iAs species are not the only forms of arsenic found in rice grain. Dimethylarsinic acid (DMA) can contribute over 50% of the arsenic concentration in grain, with DMA concentrations in rice being highly variable around the globe.¹ DMA is not produced *in planta*⁹ and derives from the methylation of iAs, metabolised by soil bacteria that express *arsM* genes,¹⁰ with subsequent assimilation of DMA by roots.¹¹ Although roots are relatively inefficient at assimilating DMA, as compared to arsenate or arsenite, DMA is much more efficiently translocated to grain than iAs species, primarily as it is not as retarded in its progression through the plants vascular system by phytochelatin complexation that iAs species undergo in planta.¹² Under reducing conditions *arsM* catalyses a stepwise conversion of iAs to monomethylarsonic acid (MMA), DMA, trimethylarsenic oxide (TMAO) and finally tetramethylarsonium (TETRA).¹⁰ All of these species, with the exception of TMAO, can be found in polished rice grain though DMA predominates.¹³

The reason why DMA is variable in rice throughout the globe is not known. Evidence suggests that soil organic matter (SOM), which is a key driver in soil redox has a role with methylated species in soil solution correlating with soluble SOM, and methylation increasing with different organic matter (OM) amendments to soil.^{14,15} The interplay between the rhizosphere and arsenic speciation dynamics also may have a role. Rice aerates its roots through aerenchyma leading to their (partial) coating in iron plaque with subsequent sequestration of arsenic, as both arsenate and arsenite.¹⁶⁻¹⁸ Bulk soil then acts as a source of labile As(III), which is moved due to advective transpiration-driven forces and via molecular diffusion along a concentration gradient to the root.¹⁹⁻²² Microbes expressing *arsM* genes are

100 highly diverse in soil and root rhizosphere, both phylogenetically and ecologically,^{20,23} which
101 could have significant role in biogeochemical cycling of arsenic. However, it is not known
102 how the rhizosphere and amendments with OM interplays with arsenic methylation and
103 subsequent assimilation by the roots, and how this varies with soils of differing origin.
104 Planted and unplanted microcosms, with soils sampled across one of the main EU rice
105 growing regions, Valencia, Spain, were monitored through time to investigate methylation of
106 arsenic to further understand the role of the rhizosphere in rice assimilation of arsenic. Here
107 we investigated the arsenic speciation in the soil to shoot continuum with concurrent
108 monitoring of soil bacterial genomic *arsM* and 16S rRNA copy number through qPCR.
109 Following this, we chose 1 soil to investigate the interaction of planting and manuring, using
110 the same methods plus paired amplicon sequencing^{24,25} of *arsM* and 16S rRNA for in depth
111 phylogenetic analysis of arsenic methylating soil microbial communities.

112

MATERIALS AND METHODS

Experimental design

Paddy soils, classified as Entisols, were collected from five different fields across the region of Valencia, Spain, as described and characterized by Signes-Pastor et al.,²⁶ were used in this study. The location and characteristics of soils are shown in Table S1. Field moist soils were sieved to < 2mm before the experiment. Rice seed (IRGA 425) was the plant cultivar used. Before planting, rice seeds were sterilized with H₂O₂ and then germinated at 25°C for 5 days. Two different microcosm experiments were conducted.

Experiment 1 (5 soils, planted/unplanted): For the first microcosm experiment field moist soil (for details on soil 1-5 see Table S1) was added to 50 ml centrifuge tubes to an equivalent of 30 g dry weight. Pre-germinated seedlings were planted into half the microcosms resulting in unplanted and planted treatments. In total 90 microcosms (5 soils, 2 treatments, 9 time points) were set up to enable 9 destructive harvests at weekly spacing, with each soil represented by one planted and unplanted replicate at each harvest. On planting the microcosms had ultra-pure water added to maintain a 2cm layer of standing water above the soil surface. Microcosms were then placed into plant growth chambers (Fitotron) with day/night temperature 28°C /25°C, light period 16h per day. Rice seedlings were harvested weekly, starting at 2 weeks. Rice seedlings were taken out of tubes by shaking the tube and carefully removing the adhering soils. After repeated rinsing with ultra-pure water rice shoot and root were separated and stored at -20°C until subsequent analysis. Once the plant was removed the centrifuge tubes were placed in a centrifuge (Sorvall Legend RT at 4600 rpm) for 20 minutes, soil porewater decanted and both soil and porewater immediately frozen and stored at -20°C until subsequent analyses. Chemical and molecular analysis (qPCR) from soil, plant and pore water samples was conducted (described below).

139

140 Experiment 2 (1 soil, full factorial design, planted/unplanted and manured/non-manured): For
141 the second microcosm experiment soil-5 (see Table S1) was incubated with four treatment
142 combinations and five replicates of each treatment: non-manured unplanted (NMNP), non-
143 manured planted (NMP), manured unplanted (MNP) and manured planted (MP), for
144 molecular and chemical analysis. Bovine farm Yard Manure (FYM) was dried in an oven at
145 70°C to a constant weight and ground to fine powder. Following this, the total element
146 content of FYM was analysed by X-ray fluorescence spectrophotometry (XRF). For manure
147 treated microcosms the ground and dried FYM was applied to the soil @ 10% dry weight
148 basis, and mixed very well before rice seedlings were planted into half of the manured
149 microcosms. Ultra-pure water was added to maintain flooded condition during the incubation.
150 Microcosms were then placed into a plant growth chamber (Fitotron) for 2 weeks using the
151 same growth condition as in microcosm experiment 1. After 2 weeks, samples were collected
152 as described above for experiment 1. Chemical and molecular analysis (qPCR) from soil,
153 plant and pore water samples was conducted as described below. In addition to these,
154 amplicon sequencing of soil DNA to assess the diversity of bacterial *arsM* and 16S rRNA
155 was conducted (described below). The Eh and pH was measured in planted and non-planted
156 microcosms by inserting an Eh/pH meter through the soil surface, with the live plant still in
157 place in the planted microcosm, to a depth of 2 cm and then waiting for the reading to
158 stabilize before recording that reading.

159

160 **Arsenic speciation in soil pore water**

161 Arsenic species in 0.4 micron Millipore membrane filtered soil porewaters (iAs, MMA,
162 DMA, TMAO and TETRA) were determined using a Dionex IC chromatographic system
163 interfaced with ICP-MS (ICS-5000 DC, Thermo Scientific) as outlined in detail in Signes-

Pastor et al.^{5,26} Using an arsenic speciation specific IC-column, IC-ICP-MS, unlike HPLC – anion exchange – ICP-MS, separates TMAO, and we run authentic TMAO samples, and cross validate with cation exchange IC-ICP-MS²⁷. We have added TMAO to the list of analytes detected in soil porewaters. Results were expressed on a per kg soil ($\mu\text{g/kg}$) and statistical analysis performed in Minitab as outlined below.

Plant analysis

Plant shoot and root material was digested using concentrated nitric acid microwave digestion, again following protocols outlined in Signes-Pastor et al. for total arsenic (experiment 2) and arsenic species (experiment 1).^{5,26} Statistical analysis was performed in Minitab as outlined below. For further details see the supporting information 1.

DNA extraction

Soil DNA was extracted from 0.5g soil from each microcosm using the Powerlyzer[®] Powersoil[®] DNA isolation kit (MOBIO Laboratories, Inc.) following the manufacturer's instructions. For further details see the supporting information 1.

Quantitative PCR

Relative bacterial 16S rRNA and *arsM* copy number were measured on the Eppendorf mastercycler (Realplex⁴, Hamburg, Germany) using precisionPLUS MasterMix premixed with SYBR green (PrimerDesign, USA). Primer pairs of 16S_1369F/16S_1492R²¹ and *arsMF1/arsMR2*²⁰ were used to amplify and quantify the relative copy number of bacterial 16S and *arsM* genes, respectively. For further details see the supporting information 1.

189 **Amplicon sequencing and processing of the resultant data**

190 Following quantification, 20 DNA samples extracted from soil of each microcosm of
191 experiment 2, were submitted for 250bp paired end amplicon sequencing of the 16S rRNA
192 (V4 region) and *arsM* gene on the Miseq. Initial processing of the data involved adapter
193 trimming²⁸ and clipping²⁹ followed by quality control analysis using fastqc³⁰ and merging of
194 forward and reverse paired reads with fastq-join³¹. For further details see the supporting
195 information 1.

196

197 **Bioinformatics and statistical analysis of the amplicon sequencing data**

198 Both 16S rRNA and *arsM* merged sequences were processed using QIIME version 1.8³² for
199 generation of absolute and relative OTU count-tables, taxonomic assignment and alpha and
200 beta diversity analysis. Quality filtering of both 16S rRNA and *arsM* OTUs involved removal
201 of OTUs with lowest abundance. The *arsM* OTUs were also filtered to remove any OTUs
202 with <70% identity and query coverage to *arsM* protein sequences (blastx against nr),
203 followed by taxonomic annotation based on blastn against bacterial genomes and *arsM*
204 related sequences downloaded from the fungene database³³. For 16S rRNA, phyla (level 2)
205 and genera (level 6) showing relative abundance >1% were identified, followed by statistical
206 analysis (see section Minitab below). Statistical analysis on the overall *arsM* OTU and genus
207 level 16S rRNA OTU count tables was performed in R with DESeq2 using the False
208 Discovery Rate (FDR) <0.05 and an absolute log2 foldchange (Log2FC) >1 as cutoff for
209 significance. Identified significant changes in copy number were assigned to 4 groups: Group
210 I: OTUs with significant plant effect and increased copy number in unplanted soil, Group II:
211 OTUs with significant plant effect and increased copy number in planted soil, Group III:
212 OTUs with significant manure effect and increased copy number in manured soil, Group IV:
213 OTUs with significant manure effect and increased copy number in non-manured soil. OTUs

within each group were visualised via a heatmap (R version 3.2.2, R package gplots and heatmap2), variance partition analysis (R version 3.2.2, R package variencePartition³⁴), network analysis (Qiime version 1.8³², Cytoskape version 3.7³⁵) and for each *arsM* OTU in Group I-IV the most highly correlated Group I-IV genus level 16S rRNA OTU identified (Spearman's rank correlation, GraphPad Prism version 6.0 for MAC OS). For further details see the supplementary information 1.

General linear modelling

Minitab version 16 (Minitab, PA, USA) was used as the statistical platform for the analysis of chemical data, qPCR data and relative count data of abundant phyla and genera. For plant and soil solution speciation data, qPCR and relative count data of abundant phyla and genera (Level 2 and level 6 16S rRNA amplicon data), General Linear Modelling (GLM) was employed. For Eh and pH measurements the un-paired t-test was used for identification of significant differences. Results were plotted in GraphPad Prism (version 7). For further details see the supporting information.

RESULTS

Microcosm experiment 1 (5 soils, planted/unplanted)

The location and properties of the 5 soils used in experiment 1 are presented in Table S1. Soil of origin was significant for all arsenic species soil solution concentrations with the exception of TMAO ($P>0.05$), and both *arsM* and 16S relative copy number varied significantly between soils (Figure S1, Table S2). The iAs was the most variable between soils ranging, on average, 4-fold between soils (0.2-0.8 $\mu\text{g/kg}$). The soil solution concentrations of all arsenic species were significantly suppressed in the planted microcosms as compared to non-planted microcosms (Figure S1, Table S2). The As species were dominated by iAs and were

239 suppressed by >30% in planted microcosms in all 5 soils (Figure S1). Similar levels of
240 suppression were observed for DMA and TMAO. The greatest suppression of arsenic species
241 in soil solution when comparing unplanted versus planted soil was observed for MMA.
242 Further to that qPCR showed a significant decrease in relative *arsM* copy number in planted
243 compared to unplanted soils (Figure S1, Table S2).

244
245 Considering the interaction terms in the statistical model, for planted/unplanted*soil, there
246 was a significant interaction ($p < 0.05$) for each arsenic species, and for 16S rRNA relative
247 copy number ($P < 0.05$), but not for *arsM* relative copy number (Table S2). This showed that
248 the soils are inherently variable in the way they interact with planting with respect to arsenic
249 speciation, but that the *arsM* DNA copy number is relatively consistent across soils between
250 the two treatments, i.e. that the treatment was the dominant factor in *arsM* copy number as
251 this was highly significant ($P = 0.002$). Treatment*time interaction for iAs and 16S relative
252 copy number were also significant ($P < 0.05$). Soil*time interactions (Table S2) were
253 significant for iAs, MMA and DMA, indicating that the individual soil
254 microbiology/mineralogy varied with respect to temporal patterns of arsenic release.

255
256 Plant biomass production progressed in a linear manner throughout the experiment, positively
257 correlated with time, with root and shoot biomass being similar in quantity (Figure S2). iAs
258 concentrations were over an order of magnitude higher in roots compared to shoots, with
259 roots initially having 100 mg/kg iAs. The iAs content of both root and shoot declined over
260 time, in shoots more so than roots (Figure S2). Similar magnitudes of differences between
261 roots and shoots were also observed for MMA, though at week 1 MMA was quite high in
262 shoot compared to future time points. MMA concentrations were ~2-orders of magnitude
263 lower than iAs concentrations, though were similar to DMA concentrations in magnitude, at

least towards the end of the experiment. DMA in shoots was initially higher at week 1, but declined through the experiment, while root concentrations remained consistent (Figure S2). TMAO concentrations in both root and shoot converged at 0.01 mg/kg, 3-orders of magnitude lower than iAs in shoots (Figure S2). The sum of arsenic species in plant tissue (root and shoot) constituted 0.17 – 1.24 % of arsenic in the microcosms, from week 1 to week 9, respectively. Given that root biomass is intimately associated with soil, plant biomass arsenic is only a small sink relative to soil stores.

Microcosm experiment 2 (1 soil, planted/unplanted and manured/non-manured)

Soil solution arsenic species analysis and qPCR

Soil solution arsenic species concentrations in microcosm experiment 2 were suppressed significantly in planted soil compared to unplanted soil ($P>0.05$) (Figure 1, Table S3). The As species were dominated by iAs, which decreased in planted microcosm and increased in response to manure treatment (Figure 1). MMA, DMA and TMAO also decreased in planted microcosm and increased in response to manure treatment (Figure 1). Planting was shown to significantly increase Eh from -28 mV to -14 mV ($p<0.001$) and decrease pH from 7.5 to 7.25 ($p<0.001$) (Figure S3). As in experiment 1 there was a significant decrease in relative *arsM* copy number in the planted microcosms ($p=0.001$), as well as relative *arsM*/16S ($p=0.002$), while 16S relative copy number remained unchanged (Figure 1, Table S3).

FYM had significant influence on the soil solution As species concentration and 16S relative copy number (Figure 1, Table S3). In manured soil, the highest concentration was observed for iAs (0.90 $\mu\text{g/kg}$, mean of manured planted and unplanted) followed by TMAO (0.90 $\mu\text{g/kg}$, mean of manured planted and unplanted) and DMA (0.54 $\mu\text{g/kg}$, mean of manured planted and unplanted), with FYM significantly increasing all As species by 94% or more

when compared with non-manured soil. Highest percent increase due to FYM was observed in MMA (from 0.02 mean of non-manure planted and non-manure unplanted) to 0.72 $\mu\text{g/kg}$ (mean of manure planted and manure unplanted). Soil *arsM* relative copy number was not significantly increased by FYM ($p=0.143$) (Figure 1, Table S3). FYM significantly increased the total As concentration in roots by 54% (from 39.67 mg/kg to 61.36 mg/kg) and in shoots by 60% (from 0.54 mg/kg to 0.87 mg/kg) (Figure S4). Non-manured plants contained 0.82 % of arsenic in the microcosms, and manured 0.54 % of this figure; again, as for experiment 1, a small portion of the soil store.

The manure*plant interaction effect was only found to be significant for MMA ($p<0.001$), with manure-planted soil showing a significantly higher concentration of MMA compared to manured-unplanted soil ($p < 0.05$, post-hoc multiple comparison test, Minitab) (Figure 1, Table S3). There was no significant manure*plant interaction observed for iAs, DMA, TMAO and relative copy number of *arsM* and 16S rRNA ($p>0.05$), indicating that the treatments individually influenced these parameters (Figure 1, Table S3).

Amplicon sequencing of the *arsM* and 16S rRNA

Amplicon sequencing of the *arsM* and 16S rRNA genes was used to investigate soil bacterial community composition and diversity. After paired end joining of reads, a total of 6,733,356 sequences for the 16S rRNA gene were retained, ranging from 301,26 to 369,822 per sample (Table S4). Rarefaction curves for alpha diversity (diversity within samples) measures (Chao1, Shannon and Simpson) indicated that sequencing depth was sufficient to detect the majority of the 16S rRNA sequence types in all samples (Figure S5A). Significant differences in species richness (Chao1) and diversity (Shannon and Simpson) were observed. Manure addition significantly increased species diversity (Shannon and Simpson, $P<0.001$, Figure

S6A) and species richness (Chao1, $P < 0.01$, Figure S6A). Principle coordinate (PCoA) plots for overall bacterial community diversity between the samples is shown in Figure S7A. The principle component 1 and 2 explained 47.9 % and 15.5 % of the total variation, respectively (Figure S7A) with samples amended with FYM clearly separated from those without FYM and with a less defined level of separation also observed between the unplanted and planted treatments (Figure S7A).

With respect to 16S rRNA analysis in all treatments, Proteobacteria, Acidobacteria, Actinobacteria, Planctomycetes, Chloroflexi, Firmicutes and Bacteroidetes were the dominant phyla constituting >95% of total bacterial community and showing relative abundance >1% in at least 1 sample (Figure S8A). Among these phyla, Proteobacteria was the most abundant phylum in all treatments. Application of manure significantly increased (GLM, $p < 0.05$, Table S5, Figure S8A) the relative abundance of Proteobacteria, Bacteroides and Firmicutes in both planted and unplanted non-manured treatments, while the Acidobacteria and Chloroflexi significantly decreased (GLM, $p < 0.05$, Table S5, Figure S8A). At genus level, there were 17 dominant genera accounting for >1% in relative abundance this including OTUs significantly increased in manured (*Flavisolibacter*, *Sphaerobacter*, *Singulisphaera*, *Geobacter*, *Desulfocapsa*) and in planted (*Nocardiodes*, *Singulisphaera*, *Clostridium*) soil as well as OTUs significantly increased in non-manured (*Caldilinea*, *Gemmatimona*, *DeFluviicoccus*, *Thiobacillus*, *Syntrophobacter*, *Steroidobacter*) and unplanted (*Caldilinea*, *Gemmatimona*, *Thiobacillus*, *Syntrophobacter*, *Steroidibacter*) soil (Table S6, Figure S8B). However, with respect to abundant genera, only for *Clostridium*, *Geobacter* and *Desulfocapsa* were the foldchanges shown to be large enough for these to be deemed as significant changes in copy number in response to manure treatment, and/or for

planting during DESeq2 analysis, as the significance threshold for Deseq2 was $FDR < 0.05$ and $\log_2FC > 1$ (Table1, Figure 2A, Table S6).

For all genus level 16S rRNA OTUs with at least 5 counts in at least 3 samples, the unique and overlapping DESeq2 results for pairwise comparisons are illustrated in the Venn diagram (Figure S9A). Manure effects (130 in total, Group III and Group IV, Figure 2A) showed 115 significant OTUs in planted, 83 significant OTUs in unplanted, with 68 overlapping OTUs. Compared to this, plant effects (42 in total) showed 1 significant OTU in non-manured soil and 41 in manured soil with 1 overlapping OTU. With respect to the 42 significant genus level 16S rRNA OTUs identified for plant effect, 35 of these also showed a significant manure effect (Figure S8A). Hence, only 7 genera level 16S rRNA OTUs responded solely to planting (Group I and Group II, Figure 2A). DESeq2 analysis of genus level 16S rRNA OTUs, furthermore, showed that the manure treatment resulted in significant >2 -fold increase ($FDR < 0.05$) in copy number of 117 OTUs of specific genera within the phylum Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria and Verrumicrobia (Group III in Figure 2A and Table 1); and 2-fold decrease ($FDR < 0.05$) in 14 OTUs of another set of genera within the Phylum Acidobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria (Group IV in Figure 2A and Table1). Of these only *Desulfocapsa*, and *Geobacter* from Group III were also within the list of most abundant ($>1\%$) genera (Table1, Table S6). Planting resulted in significant >2 -fold increase ($FDR < 0.05$) in copy number of 3 OTUs of genera (*Tumebacillus*, *Clostridium*, *Desulfotomaculum*) within the phylum Firmicutes (Group II in Figure 3A and Table1); and a decrease in 4 OTUs of genera (*Acidobacteria_GPI*, *Dehalogenimonas*, *Cryptomonadaceae* and *Alterococcus*) within the phylum Acidobacteria, Chloroflexi, Cyanobacteria, Verrumicrobia (Group 1 in Figure 2A and Table1). Of all these, only *Clostridium* (Group II)

was also within the list of most abundant (>1%) genera (Table 1, Table S6). Hence 16S rRNA copy number changes of less abundant genera appear to contribute significantly to the overall effect of manure treatment and planting in this study.

The diversity of the *arsM* gene was also assessed using amplicon sequencing and after paired end joining of reads, a total of 5,690,027 sequences for the *arsM* rRNA gene were retained, ranging from 210,483 to 609,670 per sample and a median of 274,036 (Table S4). Rarefaction curves for alpha diversity (diversity within samples) measures (Chao1, Shannon and Simpson) indicated that sequencing depth was sufficient to detect the majority of the sequence types in all samples (Figure S5B). Both FYM and planting significantly decreased the *arsM* diversity (Shannon and Simpson) (Figure S6B). A significant manure*plant interaction effect was observed for *arsM* species richness (Chao1) indicating that planting decreased the overall number of *arsM* OTUs, in particular in non-manured soil (Figure S6B). PCoA plots of the overall diversity of *arsM* in different treatments (Figure S6B), shows a clear separation due to manuring and planting, very similar to that observed for 16S rRNA diversity. Annotation (blastn against bacterial genomes) of the 2680 representative *arsM* OTUs that were retained in the filtered OTU table, returned matches for 2104 OTUs against 97 different bacterial genera. Most *arsM* OTUs showed highest sequence similarity to the genus *Rhodopseudomonas* (25%), followed by *Roseiarcus* (6%), *Gemmatirosa* (5%), *Streptomyces* (4%), *Thiobacillus* (3%) and *Rhodoplanes* (2%); with the rest of the genera matching less than 2% of these OTUs (Table 2). The most numerous were matches of *arsM* OTUs to genomes of the species *Rhodopseudomonas palustris* (638 OTUs) with best matches obtained to 9 different strains within this species: *TIE-1* (212 OTUs), *BisB5* (180 OTUs), *YSC3* (94 OTUs), *XCP* (69 OTUs), *HaA2* (41 OTUs), *CGA009* (19 OTUs), *DX-1* (8 OTUs), *BAL398*, (6 OTUs), *RI* (9 OTUs). With regards to the genus *Rhodopseudomonas*, there were

also 33 OTUs with best matches to *R. pentothentatexigens* strain JA575. Of the 165 *arsM* OTUs with best match against *Roseiarcus ferementans*, 124 returned *R. palustris* TIE-1 as second-best match (blastn against bacterial genomes) and 120 as best match when using blastn against the fungene_arsM database³³.

392

For all significant *arsM* OTUs, the unique and overlapping DESeq2 results for pairwise comparisons are illustrated in the Venn diagram (Figure S9B). Manure effects (319 in total) showed 258 significant OTUs in planted soil and 222 significant OTUs in unplanted soil, with 162 overlapping OTUs. Compared to this, plant effects (73 in total) showed 46 significant OTUs in non-manured soil and 34 in manured soil with 7 overlapping OTUs. With respect to the 73 significant OTUs identified for the plant effect, 12 of these also showed a significant manure effect (Figure S9B).

400

Corresponding *arsM* and genus level 16S rRNA OTUs for 4 identified significant copy number patterns were identified. These are visualized in a heatmap in Figure 2A (genus level 16S rRNA) and Figure 2B (*arsM* OTUs). For *arsM* OTUs 29 were assigned to Group I, 32 to Group II, 256 to Group III and 57 to Group IV. For genus level 16S rRNA, OTUs belonging to Group I, II, III and IV are summarized in Table 1. While overall species richness (number) of *arsM* OTUs was decreased in response to planting and manuring, the largest number of *arsM* OTUs with significantly changed copy number were observed in Group III (Figure 2B). With respect to annotation of significant *arsM* OTUs, blastn against bacterial genomes showed the following: Most Group 1 *arsM* OTUs showed closest sequence similarity to *R. palustris* BisB5 (41%) followed by *R. palustris* YSC3 (10%). Group 2 *arsM* OTUs showed closest sequence similarity to *Streptomyces aurantiacus* (12%) followed by *R. palustris* TIE-1 (9%). Group 3 *arsM* OTUs showed closest sequence similarity to *R. palustris* TIE-1 (29%)

and *Roseiarcus fermentans* (46%) (88% of the latter returned as second-best genome match also *R. palustris TIE-1*), followed by *Thioalbus denitrificans* (3%). Group 4 *arsM* OTUs showed closest sequence similarity to *R. palustris TIE-1* (16%) and *Gemmatirosa kalamazoonesis* (11%) followed by *R. palustris BisB5* (7%). Hence with respect to predominant *arsM* copy number changes, manuring resulted mainly in increase of *R. fermentans* (blast against the fungene-*arsM* database³³ returns for 85% of these *R. palustris TIE-1*) and *R. palustris TIE-1*, while planting resulted in decrease of *R. palustris BisB5* and increase in *S. aurantiacus* annotated OTUs. For further details see Table 2 & Supporting information File 2.

Variance partition analysis for Group I and II 16S rRNA OTUs verified that 60% of the variation in *Acidobacteria_GPI* copy number was due to planting, while for the other 6 OTUs in Group I and II this was around 20-35% (Figure 3). For Group I and II *arsM* OTUs, this analysis verified that 45% of the variation in copy number was due to planting (Figure S10). Variance partition analysis for Group III and IV 16S rRNA OTUs showed that 28% and for Group III and IV *arsM* OTUs that 25% of the variation in copy number was due the manure effect (Figure S11 and Figure S12).

With regards to significant correlation (Spearman's rank correlation, $P < 0.05$) of copy number patterns between genus level 16S rRNA and *arsM* OTUs the following results were obtained. Of the 29 *arsM* OTUs in Group I, 100% correlated with at least 1 Group I genus level 16S rRNA OTU. Of these 97% correlated most strongly with *Acidobacteria_GPI* and 3% with *Dehaligenimonas* (Table S7). Of the 32 *arsM* OTUs in Group II, 65% correlated with at least 1 Group II genus level 16S rRNA OTU. Of these 34% correlated most strongly with *Clostridium* and 31% with *Tumebacillus* (Table S8). All Group III *arsM* OTUs correlated

highly significantly with multiple Group III genus level 16S rRNA OTUs. Of these, highest number of *arsM* OTUs (9%) correlated most significantly with *Luteolibacter* (phylum Verrucomicrobia), followed by *Devosia* (7%), and *Herbaspirillum* (6%) (phylum Proteobacteria). The rest of the Group III *arsM* OTUs correlated most significantly to a highly diverse range of genus level 16S rRNA OTUs (Table S9) within the phyla Proteobacteria (25 genera), Firmicutes (21 genera, this including *Pseudomonas*), Bacteroidetes (5 genera), Acidobacteria (*Geothrix*) and Actinobacteria (*Thermobifida*). All Group IV *arsM* OTUs correlated with at least one Group IV genus level 16S rRNA OTU. Of these 23% correlated most significantly with *Acidobacteria_Gp25*, 14% with *Terrimonas*, 12% with *Acidobacteria_GP22*, 11% with *Streptophyta* and 11% with *Tepidibacter* (Table S10).

Network analysis plots for genus level 16S rRNA OTUs that responded to planting (Group I and II) are shown in Figure 4A-G. The network for *Acidobacteria_GPI* (Figure 4A) shows the strongest connection to all unplanted samples (Group I), due to higher copy number in unplanted soil and was also most highly correlated to Group I *arsM* OTUs (Table S7). In a similar manner *Tumebacillus* and *Clostridium* (Figure 4E and 4F), show the strongest connections to all planted samples (Group II), due to higher copy number in planted soil and these were also most highly correlated to Group II *arsM* OTUs (Table S8). Network analysis plots for genus level 16S rRNA OTUs that responded to manuring are shown in Figure S13. Individual networks are shown for those Group IV genus level 16S rRNA OTUs (Figure S13A-E) that showed highest levels of correlation with Group IV *arsM* OTUs (Table S10), with *Acidobacteria_GP25* (Figure S13A), *Terrimonas* (Figure S13B), *Acidobacteria_GP22* (Figure S13C), *Streptophyta* (Figure S13D), *Tepidobacter* (Figure S13E) all showing strongest connections to non-manured samples, due to higher copy number in non-manured

soil, but with this effect clearly most pronounced for *Acidobacteria_GP22* (Figure S13A). *Acidobacteria_GP22* were also shown to be the most highly correlated to Group IV *arsM* OTUs (Table S10). For Group III genus level 16S rRNA OTUs the overall network shows a clear separation of manure and non-manure samples (Figure S13F), the same applies to networks for Group III and Group IV *arsM* OTUs (Figure 14A and 14B). Contrary to that, network analysis plots for *arsM* OTUs that responded to planting (Group I and Group II) show clear separation of planted versus unplanted samples (Figure S15A and S15B). Hence these results provide further evidence of treatment effects and present potential targets for the study of *arsM* and microbial arsenic methylation efficiency in paddy soil.

DISCUSSION

Microbial driven methylation, and potential subsequent volatilization, mediated by *arsM* plays a crucial role in biogeochemical cycling of arsenic.³⁶ Previous studies on *arsM* diversity in natural rice paddy microbial communities have used low throughput cloning and Sanger sequencing,^{20,23,36} or have limited Illumina sequencing of *arsM* amplicons to the study of enrichment cultures from paddy soil³⁷, or metagenomics analysis to characterize arsenic metabolizing genes in 5 soils³⁸. Our study provides a step-change as it employs arsenic speciation characterization combined with qPCR and high throughput Illumina amplicon sequencing (16S rRNA and *arsM* gene) to study arsenic mobilization and transformation in response to planting and manuring, alongside detailed analysis of the diversity of complex natural bacterial arsenic transforming communities in rice paddy soil.

Phylogenetic annotation based on the protein coding functional gene *arsM*, may provide additional information as well as a higher taxonomic resolution compared to the well-established 16S rRNA gene-based analysis, but is limited by the number of curated *arsM*

protein sequences in the database³³. Correlated 16S rRNA and *arsM* gene copy numbers provide complementary information to highlight organisms that may promote arsenic methylation in paddy soil. The *arsM* based amplicon sequencing analysis revealed a great level of *arsM* sequence diversity with highest number of top matches against phototrophic purple non-sulfur bacteria of the genus *Rhodospseudomonas*. These were dominated by strain *R. palustris TIE-1* with smaller numbers of *arsM* OTUs showing matches to *R. palustris* strains *BisB5*, *YSC3*, *XCP*, *HaA2*, *CGA009* and *DX1*. High frequency of *R. palustris arsM* sequences in rice paddy soil has previously been reported³⁸. The *R. palustris arsM* gene has been proven to methylate As(III) to the relatively nontoxic pentavalent species DMA(V) and TMAO and confer arsenic resistance to an arsenic sensitive *Escherichia coli* strain¹⁰. Further to that, members of this species have been proposed, based on their As detoxification mechanisms, for bioremediation of arsenic contaminated areas surrounding mines as have been shown to be able to oxidise As(III) to As (V)³⁹. Members of this species can grow with or without light or oxygen, fix nitrogen and degrade a wide range of organic compounds, with different strains shown to harbour strain specific genes and exhibit distinctive physiological characteristics with respect to anaerobic fermentation, expanded biodegradation, or expanded light-harvesting capabilities^{40,41} leading to the proposal that they should be reclassified into different species⁴². Hence, differences in copy number of these strains due to plant and manure induced changes in redox potential, pH and available carbon sources is of considerable interest in the context of arsenic in rice grain.

FYM increased all present arsenic species including methylated species in paddy soil solution and with respect to *arsM* gene OTUs led to significant increase in copy number of large numbers of *R. fermentans* and *R. palustris TIE-1* annotated OTUs, with *R. fermentans* reported to grow best under micro-oxic conditions by means of fermentation of sugars and

organic acids⁴³ and *R. palustris* TIE-1 shown to be able to grow on a wide variety of carbon sources as well as perform phototrophic Fe(II) oxidation^{44,45,46}. It is important to note in this context that *R. fermentans* annotated *arsM* OTU sequences (genome match), also showed high similarity to *R. palustris* TIE-1. Further to that, 16S rRNA amplicon sequencing showed manure induced increase in *Geobacter*, an anaerobe shown to perform anaerobic oxidation of aromatic hydrocarbons to carbon dioxide via reduction of Fe(III)^{47,48} as well as thought to be capable of nitrate dependent Fe(II) oxidation⁴⁹. Hence, manuring appears to stimulate microbes involved in arsenic methylation as well as in both iron plaque formation (Fe(II) oxidation) and degradation (Fe(III) reduction), leading to binding and release of arsenic from Fe-plaque around roots.^{22,50}

Higher redox potential of rhizosphere due to oxygenation from rice roots led to suppression of all present arsenic species, including methylated species. This corresponded to decreased *arsM* copy number (qPCR), species richness (amplicon sequencing, Chao1) and diversity (amplicon sequencing, Simpson, Shannon). This implies that in our study, the number of *arsM* organisms decreased in planted soil. With respect to *Rhodopseudomonas* annotated *arsM* OTUs, some with highest sequence similarity to *R. palustris* BisB5 were suppressed by planting. *R. palustris* strain BisB5 was shown to contain a gene cluster for anaerobic phenylacetate degradation and was proposed to be best adapted to degradation of plant material in oxygen depleted environments that are exposed to light⁴⁰. In contrast to this *arsM* OTUs with closest match against *S. aurantiacus* were increased in planted soil. *Streptomyces* are generally aerobic bacteria and isolates from rice paddies have been shown to contribute to better growth of rice plants as well as increased levels of arsenic methylation and uptake of DMA into rice shoots⁵¹. Change in redox potential may therefore play a role in the observed copy number changes of these organisms in response to planting. In our study, the fact that

rhizosphere soils have lower methylated arsenic species than bulk soil coupled with decrease and shift in *arsM* organisms could be attributable to oxygenation of the rhizosphere by the root, as radial oxygen loss (ROL), regulates rice rhizosphere Eh,⁵² and subsequent assimilation of arsenic¹⁸. However, total arsenic species, inorganic as well as methylated, decreased in the rhizosphere suggesting that redox, while part of the answer, does not fully explain the reduction in methylated species.

The root itself is a sink for all arsenic species, but rates of MMA and DMA accumulation by rice roots are low with much higher rates of arsenate and arsenite assimilation.¹¹ Thus, if the root was acting as a major sink, to the extent that they affect soil solution concentrations, then it may be expected that iAs species are preferentially depleted. This was not the case here. Rather than the root tissue, *per se*, being the sink for arsenic species that leads to the lowering of these species in porewater, it may be associated iron plaque in the rhizosphere. Iron plaque preferentially binds arsenate to arsenite, but has a high capacity to sorb both species, and methylated species.¹⁷ It is interesting that our *arsM* amplicon data indicates increase in bacteria with arsenic methylation and Fe(II) oxidation and Fe(III) reduction capacity in manured soil, as this could lead to formation of iron plaques and subsequent release of arsenic from iron plaque.^{44-50,18} As well as the root potentially creating new sinks for arsenic species, iron plaque and root tissue, the enhanced oxygenation of the planted microcosms here may have led to lower mobilization of iAs from sediment stores as it is reducing conditions that drive this mobilization.^{19,22} Lower mobilization of iAs would mean lower substrate for methylation and, hence, lower production of methylated arsenic species.

A number of studies have considered arsenic species mobility in the rhizosphere.^{20,21} The current study differs from these in that it compares planted versus non-planted microcosms,

while the other studies sampled a rhizosphere continuum from bulk soil to root surface. The studies of Jia et al.^{20,21} did not show the inhibition of in culture medium solution of arsenic species concentration in the rhizosphere. In essence these studies have a relatively small and constrained rhizosphere, to enable spatial sampling, with the root-soil interface surrounded by a large volume of bulk soil to resupply arsenic species if they become depleted in the rhizosphere. In the field, particularly in densely planted agronomic systems, the rhizosphere can be considered as a continuum, with dense and overlapping roots.⁵³ In our experiments the soil:fresh root biomass ratio was considerable, being 150:1 at week 2 when Eh measurements were made. Even at these low biomass ratios the presence of the root had a profound effect on both Eh and arsenic speciation.

Here, the detailed reporting of arsenic species in both roots and shoots over a time course illustrates, again, that while iAs concentrations *in planta* are higher than methylated species, it is the methylated species that are more readily translocated to the shoot.¹² The root measurements do not distinguish between surface plaque bound and species actually assimilated into plant tissues and this must be born in mind when interpreting the data as plaque in roots can have high arsenic concentrations.⁶ Here, the shoot measurements are free from such qualifications and only represent arsenic species assimilated by root and then translocated to shoot. Hydroponic experiments have shown that although iAs species are more efficiently assimilated by the root,¹¹ their translocation to shoot are retarded as compared to methylated species, with MMA less effectively translocated to the shoot from roots than DMA.¹² Arsenate is reduced to arsenite in plant cells and then phytochelatin (PC) complexed, with arsenite-PC complexes then transported into vacuoles via ABC transporters.⁶ MMA can be thiol complexed, though less efficiently than arsenite, while DMA has lower affinity for thiol complexation,^{12,54} with the affinity to PCs explaining their

588 relative efficiency in translocation to the shoot.¹² This pattern can also be seen here, with the
589 addition of TMAO which shows equivalency between root and shoot in concentration,
590 indicating further that increasing methylation leads to enhanced translocation to the shoot.
591 Although not tested, to date, it is likely that TMAO has poor affinity to plant thiols.

592

593 Most studies use anion exchange HPLC-ICP-MS to speciate arsenic, where TMAO elutes on
594 the solvent front.¹³ As we used the more discriminatory IC-ICP-MS, TMAO was readily
595 detected and quantified. Even though iAs's movement is retarded through the plant, because
596 initial starting concentrations in soil solution and root tissues are much higher, shoot
597 concentrations were *circa.* 100-fold higher than for MMA, DMA and TMAO. However, in
598 rice grain only iAs and DMA are found in significant quantities, with DMA in many regions
599 of the world reaching equivalency with iAs or exceeding in cases.¹ Traces of MMA can be
600 found routinely, and TETRA in very elevated grain samples, but interestingly not TMAO.¹³
601 The differences in grain unloading of DMA and iAs have been studied for rice,⁵⁵ but MMA,
602 TMAO and TETRA not. It is clear that we still have a way to go in understanding the
603 physiology of all arsenic species found in rice with respect to uptake and translocation.

604

605 As in previous studies in paddy soil, ^{20,23,36-38,56} high diversity of bacterial 16S rRNA and
606 *arsM* were observed, and shown to be affected by soil amendments. Recent studies on *arsM*
607 have identified high levels of diversity with Proteobacteria, Gemmatmonadales and
608 Firmicutes³⁶ as the most common bacterial communities in paddy soil, this including high
609 frequency of *R. palustris*³⁸ and many phylogenetically divergent microbes have been shown
610 to harbor the *arsM* gene and to be able to convert aqueous iAs to methylated species³⁷. Pure
611 culture studies have, furthermore, shown that microbes vary with respect to their methylation
612 efficiency with *Methanosarcina acetivorans* C2A⁵⁷ (phylum Euryarchaeota), *Clostridium* sp.

BXM⁵⁸ (phylum Firmicutes) shown to transform 10%, while *Streptomyces* sp.⁵⁹ (phylum Actinomycete), *Pseudomonas alcaligenes*⁶⁰ (phylum Proteobacteria) and *Arsenicibacter rosenii*⁶¹ (phylum Bacteroidetes) shown to transform more than 50% of aqueous iAs to methylated species. In addition our observed correlated *arsM* and 16S rRNA copy numbers are worth further investigation. The copy number of many Group II *arsM* OTUs was for example correlated to 16S rRNA *Clostridium* OTUs, which is interesting as co-culture of *R. palustris* and *Clostridium* has previously been shown to lead to increased hydrogen production and *R. palustris* genes involved in organic compound catabolism and nitrogen fixation⁶². The copy number of Group III *arsM* OTUs on the other hand showed correlation to a very diverse range of genus level 16S rRNA OTUs, with highest matches to *Luteolibacter* and *Devosia*. *Luteolibacter* have previously reported as one of the 5 most abundant genera in arsenic contaminated soil⁶³ and *Devosia* were shown to exhibit high sequence similarity to arsenic tolerant organisms isolated from arsenic rich environments⁶⁴. Therefore, both the genomic matches of *arsM* OTUs and the correlated copy number patterns in Group I, II, III and IV *arsM* and genera level 16S rRNA OTUs present potential targets for identification of *arsM* organisms and the study of arsenic transformation in paddy soil.

The relative *arsM* copy number is known to be variable between soils,^{20,23,36-38,56} and we have shown this again in the current findings, with highly significant differences also observed between planted and unplanted microcosms. Similarly, arsenic speciation was profoundly influenced by soil origin, planting and manuring. Inherent variation in DMA in soil solution has been shown in other studies,¹⁵ and the results shown here illustrate that this chemical is spatially and temporally variable in soils, interacting with environmental factors. Environmental factors such as soil solution soluble OM was found to correlate well with DMA in soil solution.¹⁵ As plants obtain all their DMA from soil solution,⁹ DMA variability

638 in soil solution will be reflected in root uptake, and ultimately in grain DMA concentrations.¹
639 As DMA is less toxic than iAs to humans, altering grain arsenic through paddy soil
640 management can lead to higher DMA to iAs, but some caution is required before promoting
641 soils microbes to convert iAs to DMA. For example, SOM, while promoting arsenic
642 methylation in paddy soils also liberates more arsenic in general leading to enhanced plant
643 uptake to both inorganic and organic species.¹⁴ Also, while less toxic to humans, DMA is
644 strongly implicated in “straight-head” disease of rice where yield is greatly lowered by DMA
645 (and MMA) exposure due to introducing sterility.^{23,65} It is clear if rice is to be managed to
646 lower grain iAs content then caution needs to be heeded to ensure that adverse consequences
647 are negated. Understanding that the rhizosphere environment greatly alters arsenic
648 concentrations and speciation is a step forward in developing strategies for minimizing grain
649 iAs content without impacting yield. Further studies need to be conducted in a wide range of
650 paddy soils to validate the role of rhizosphere interactions, including microbial mediated
651 arsenic methylation as well as Fe(II) oxidation and Fe(III) reduction and its effect on arsenic
652 bound to iron-plaque, and how these can be manipulated to minimize iAs in rice grain.

653

654 **ACKNOWLEDGEMENTS**

655 We thank the Commonwealth Scholarships for funding Hasina Afroz’ Ph.D. studentship.
656 Illumina Sequencing was performed by the Centre Of Genomics Research, Institute of
657 Integrative Biology University of Liverpool, Crown Street Liverpool L69 7ZB, UK.

658

659 **SUPPORTING INFORMATION.**

660 SupportingInformation1.docx (SI1.docx): additional information on materials and methods,
661 additional results in form of 10 tables and 15 figures, relevant references.

662 SupportingInformation2.xlsx (SI2.xlsx): table with information on significant Group1,

663 Group2, Group3 and Group4 *arsM* OTUs including, sequence, blast results and most highly
664 correlating 16S rRNA OTU.

665

666

667

668

669

670

671

672

673

674

675

676

677

678

679

680

681

682

683

684

685

686

687

REFERENCES

- (1) Meharg, A. A.; Williams, P. N.; Adomako, E.; Lawgali, Y. Y.; Deacon, C.; Villada, A.; Cambell, R. C. J.; Sun, G.; Zhu, Y. G.; Feldmann, J.; Raab, A.; Zhao, F. J.; Islam, R.; Hossain, S.; Yanai, J. Geographical variation in total and inorganic arsenic content of polished (white) rice. *Environ. Sci. Technol.* **2009**, *43* (5), 1612–1617.
- (2) EU Commission Regulation 2015/1006 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of inorganic arsenic in foodstuffs. **2015**. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv%3AOJ.L_.2015.161.01.0014.01.ENG.
- (3) WHO, 2014. Codex alimentarius commission. Project document proposal for new work on a maximum level for arsenic in rice. **2014**
<ftp://ftp.fao.org/codex/meetings/CCCF/cccf5/CRDs/CRD%2024%20ARSENIC%20IN%20RICE.pdf>
- (4) FDA, **2016**. FDA proposes limit for inorganic arsenic in infant rice cereal.
<http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm493740.htm>
- (5) Signes-Pastor, A. J.; Carey, M.; Meharg, A. A. Inorganic arsenic in rice-based products for infants and young children. *Food Chem.* **2016a**, *191*, 128–134.
- (6) Meharg, A. A.; Zhao, F. J. *Arsenic & Rice*; Springer, The Netherlands, **2012**.
- (7) Meharg, C.; Meharg, A. A. Silicon, the silver-bullet for mitigating biotic and abiotic stress, and improving grain quality, in rice. *Environ. Exp. Bot.* **2015**, *120*, 8–17.

713

714 8. Williams, P. N.; Villada, A.; Deacon, C.; Raab, A.; Figuerola, J.; Green, A. J.; Feldmann,
715 J.; Meharg, A. A. Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain
716 levels compared to wheat and barley. *Environ. Sci. Technol.* **2007**, *41* (19), 6854–6859.

717

718 (9) Lomax, C.; Liu, W. J.; Wu, L.; Xue, K.; Xiong, J.; Zhou, J.; McGrath, S. P.; Meharg, A.
719 A.; Miller, A. J.; Zhao, F. J. Methylated arsenic species in plants originate from soil
720 microorganisms. *New. Phytol.* **2012**, *193* (3), 665–672.

721

722 (10) Qin, J.; Rosen, B. P.; Zhang, Y.; Wang, G. J.; Franke, S.; Rensing, C. Arsenic
723 detoxification and evolution of trimethylarsine gas by a microbial arsenite S-
724 adenosylmethionine methyltransferase. *Proc. Natl. Acad. Sci., U. S. A.* **2006**, *103* (7),
725 2075–2080.

726

727 (11) Abedin, M. J.; Cresser, M. S.; Meharg, A. A.; Feldmann, J.; Cotter-Howells, J. Arsenic
728 accumulation and metabolism in rice (*Oryza sativa* L.). *Environ. Sci. Technol.* **2002**, *36* (5),
729 962–968.

730

731 (12) Raab, A.; Williams, P. N.; Meharg, A.; Feldmann, J. Uptake and translocation of
732 inorganic and methylated arsenic species by plants. *Environ. Chem.* **2007**, *4* (3), 197–203.

733

734 (13) Hansen, H. R.; Raab, A.; Price, A. H.; Duan, G. L.; Zhu, Y. G.; Norton, G. J.; Feldmann,
735 J.; Meharg, A. A. Identification of tetramethylarsonium in rice grains with elevated arsenic
736 content. *J. Environ. Monit.* **2011**, *13* (1), 32–34.

737

- 738 (14) Norton, G. J.; Adamoko, E.; Deacon, C.; Carey, A. M.; Price, A. H.; Meharg, A. A..
739 Effect of organic matter amendment, arsenic amendment and water management regime on
740 rice grain arsenic species. *Environ. Poll.* **2013**, *177*, 38–47.
- 741
- 742 (15) Williams, P. N.; Zhang, H.; Davison, W.; Meharg, A. A.; Hossain, M.; Norton, G. J.;
743 Brammer, H.; Islam, M. R. Organic Matter-Solid Phase Interactions Are Critical for
744 Predicting Arsenic Release and Plant Uptake in Bangladesh Paddy Soils. *Environ. Sci.*
745 *Technol.* **2011**, *45* (14), 6080–6087.
- 746
- 747 (16) Liu, W. J.; Zhu, Y. G.; Smith, F. A.; Smith, S.E. Do iron plaque and genotypes affect
748 arsenate uptake and translocation by rice seedlings (*Oryza sativa* L.) grown in solution
749 culture? *J. Exp. Bot.* **2004**, *55* (403), 1707–1713.
- 750
- 751 (17) Liu, W. J.; Zhu, Y. G.; Hu, Y.; Williams, P. N.; Gault, A. G.; Meharg, A. A.; Charnock,
752 J. M.; Smith, F. A. Arsenic sequestration in iron plaque, its accumulation and speciation in
753 mature rice plants (*Oryza sativa* L.). *Environ. Sci. Technol.* **2006**, *40* (18), 5730–5736.
- 754
- 755 (18) Mei, X. Q.; Wong, M. H.; Yang, Y.; Dong, J. Y.; Qiu, R. L.; Ye, Z. H. The effects of
756 radial oxygen loss on arsenic tolerance and uptake in rice and on its rhizosphere. *Environ.*
757 *Poll.* **2012**, *165*, 109–117.
- 758
- 759 (19) Bravin, M. N.; Travassac, F.; Le Floch, M.; Hinsinger, P.; Garnier, J.M. Oxygen input
760 controls the spatial and temporal dynamics of arsenic at the surface of a flooded paddy soil
761 and in the rhizosphere of lowland rice (*Oryza sativa* L.): a microcosm study. *Plant Soil* **2008**,
762 *312* (1-2), 207–218.

763

764 (20) Jia, Y.; Huang, H.; Zhong, M.; Wang, F. H.; Zhang, L. M.; Zhu, Y. G. Microbial arsenic
765 methylation in soil and rice rhizosphere. *Environ. Sci. Technol.* **2013**, *47* (7), 3141–3148.

766

767 (21) Jia, Y.; Huang, H.; Chen, Z.; Zhu, Y. G. Arsenic uptake by rice is influenced by
768 microbe-mediated arsenic redox changes in the rhizosphere. *Environ. Sci. Technol.* **2014**, *48*
769 (2), 1001–1007.

770

771 (22) Williams, P. N.; Santner, J.; Larsen, M.; Lehto, N. J.; Oburger, E.; Wenzel, W.; Glud, R.
772 N.; Davison, W.; Zhang, H. Localized Flux Maxima of Arsenic, Lead, and Iron around Root
773 Apices in Flooded Lowland Rice. *Environ. Sci. Technol.* **2014**, *48* (15), 8498–8506.

774

775 (23) Zhao, F. J.; Harris, E.; Yan, J.; Ma, J.; Wu, L.; Liu, W. J.; McGrath, S. P.; Zhou, J. Z.;
776 Zhu, Y. G. Arsenic methylation in soils and its relationship with microbial *arsM* abundance
777 and diversity, and As speciation in rice. *Environ. Sci. Technol.* **2013**, *47* (13), 7147–7154.

778

779 (24) Caporaso, J. G.; Lauber, C. L.; Walters, W. A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.;
780 Owens, S.M.; Betley, J.; Fraser, L.; Bauer, M.; Gormley, N.; Gilbert, J. A.; Smith, G.;
781 Knight, R. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and
782 MiSeq platforms. *Isme Journal* **2012**, *6* (8), 1621–1624.

783

784 (25) Degnan, P. H.; Ochman, H. Illumina-based analysis of microbial community diversity.
785 *Isme Journal* **2012**, *6* (1), 183–194.

786

787 (26) Signes-Pastor, A.J.; Carey, M.; Carbonell-Barrachina, A.A.; Moreno-Jimenez, E.; Green,

- 788 A.J.; Meharg, A.A. Geographical variation in inorganic arsenic in paddy soils and rice from
789 the Iberian Peninsula. *Food Chem.* **2016b**, *202*, 356–363.
- 790
- 791 (27) Savage, L.S. Carey, M. Hossain, M.; Islam, M.R.; de Silva P.MC.S.; Williams, P.N.;
792 Meharg, A.A. Elevated trimethylarsine oxide and inorganic arsenic in northern hemisphere
793 summer monsoonal wet deposition. *Environ. Sci. Technol.* **2017**, *51*, 12210-12218 .
- 794
- 795 (28) Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads.
796 EMBnet. J. **2011**, *17*, 10–12.
- 797
- 798 (29) Joshi, N. A.; Fass, J. N. Sickle: A sliding-window, adaptive, quality-based trimming tool
799 for FastQ files (Version 1.33). **2011**. Available at <https://github.com/najoshi/sickle>.
- 800
- 801 (30) Andrews, S. FastQC: a quality control tool for high throughput sequence data. **2010**.
802 Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
- 803
- 804 (31) Aronesty, E. ea-utils: “Command-line tools for processing biological sequencing data.
805 **2011**. Available online at: <http://code.google.com/p/ea-utils>
- 806
- 807 (32) Caporaso, J. G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.
808 K.; Fierer, N.; Pena, A.G.; Goodrich, J. K.; Gordon, J. I.; Huttley, G. A.; Kelley, S. T.;
809 Knights, D.; Koenig, J. E.; Ley, R. E.; Lozupone, C. A.; McDonald, D.; Muegge, B. D.;
810 Pirrung, M.; Reeder, J.; Sevinsky, J. R.; Tumbaugh, P. J.; Walters, W. A.; Widmann, J.;
811 Yatsunenko, T.; Zaneveld, J.; Knight, R. QIIME allows analysis of high-throughput
812 community sequencing data. *Nature Methods* **2010**, *7* (5), 335–336.

813

814 (33) Fish, J. A.; Chai B.; Wang, Q.; Sun, Y.; Brown, C.T.; Tiedje, J.M.; Cole, J.R. FunGene:
815 the Functional Gene Pipeline and Repository. *Front. Microbiol.* 2013, 4:291.

816

817 (34) Hoffman, GE, Schadt, EE. variancePartition: interpreting drivers of variation in complex
818 gene expression studies. *BMC Bioinformatics.* **2016**; 17(1):483

819

820 (35) Shannon, P, Markiel, A, Ozier, O, Baliga, NS, Wang, JT, Ramage, D, Amin, N,
821 Schwikowski, B, Ideker, T. Cytoscape: a software environment for integrated models of
822 biomolecular interaction networks. *Genome Res.* **2003**, 13(11):2498-504.

823

824 (36) Zhang, S.-Y.; Zhao, F.-J.; Sun, G.-X.; Su, J.-Q.; Yang, X.-R.; Li, H.; Zhu, Y.-G.
825 Diversity and abundance of arsenic biotransformation genes in paddy soils from southern
826 China. *Environ. Sci. Technol.* **2015**, 49 (7), 4138–4146.

827

828 (37) Reid, M. C.; Maillard, J.; Bagnoud, A.; Falquet, L.; Phu Le Vo; Bernier-Latmani, R.
829 Arsenic Methylation Dynamics in a Rice Paddy Soil Anaerobic Enrichment Culture. *Environ.*
830 *Sci. Technol.* **2017**, 51 (18), 10546–10554.

831

832 (38) Xiao, K.-Q.; Li, L.-G.; Ma, L.-P.; Zhang, S.-Y.; Bao, P.; Zhang, T.; Zhu, Y.-G.
833 Metagenomic analysis revealed highly diverse microbial arsenic metabolism genes in paddy
834 soils with low-arsenic contents. *Environ. Pollut.* **2016**, 211, 1–8.

835

- 836 (39) Nookongbut, P.; Kantachote, D.; Megharaj, M. Arsenic contamination in areas
837 surrounding mines and selection of potential As-resistant purple nonsulfur bacteria for use in
838 bioremediation based on their detoxification mechanism. *Ann Microbiol.* **2016**, *66*, 1419.
- 839
- 840 (40) Oda, Y.; Larimer, F.W.; Chain, P.S.; Malfatti, S.; Shin, M.V.; Vergez, L.M.; Hauser, L.;
841 Land, M.L.; Braatsch, S.; Beatty, J.T.; Pelletier, D.A.; Schaefer, A.L.; Harwood, C.S.;
842 Multiple genome sequences reveal adaptations of a phototrophic bacterium to sediment
843 microenvironments. *Proc Natl Acad Sci U S A.* **2008**, *105*(47), 18543-18548.
- 844
- 845 (41) Lo, K.J.; Lin, S.S.; Lu, C.W.; Kuo, C.H.; Liu, C.T. Whole-genome sequencing and
846 comparative analysis of two plant-associated strains of *Rhodopseudomonas palustris* (PS3
847 and YSC3). *Sci Rep.* **2018**, *8*(1), 12769.
- 848
- 849 (42) Rayyan, A.; Meyer, T.; Kyndt, J.; Draft Whole-Genome Sequence of the Purple
850 Photosynthetic Bacterium *Rhodopseudomonas palustris* XCP. *Microbiol Resour Announc.*
851 **2018**, *7*(4). pii: e00855-18.
- 852
- 853 (43) Kulichevskaya, I.S.; Danilova, O.V.; Tereshina, V.M.; Kevbrin, V.V.; Dedysh, S.N.;
854 Descriptions of *Roseiarcus fermentans* gen. nov., sp. nov., a bacteriochlorophyll a-
855 containing fermentative bacterium related phylogenetically to alphaproteobacterial
856 methanotrophs, and of the family Roseiarcaceae fam. nov. *Int J Syst Evol Microbiol.* **2014**,
857 *64*(Pt 8), 2558-2565.
- 858

(44) Jiao, Y.; Kappler, A.; Croal, L.R.; Newman, D.K.; Isolation and characterization of a genetically tractable photoautotrophic Fe(II)-oxidizing bacterium, *Rhodopseudomonas palustris* strain TIE-1. *Appl Environ Microbiol.* **2005.** 71(8), 4487-96.

(45) Jiao, Y.; Newman, D.K.; The pio operon is essential for phototrophic Fe(II) oxidation in *Rhodopseudomonas palustris* TIE-1. *J Bacteriol.* **2007.** 189(5), 1765-1773.

(46) Hedrich, S.; Schlömann, M.; Johnson, D.B.; The iron-oxidizing proteobacteria. *Microbiology.* **2011.** 157(Pt 6), 1551-64.

(47) Lovley, D.R.; Giovannoni, S.J.; White, D.C.; Champine, J.E.; Phillips, E.J.; Gorby, Y.A.; Goodwin, S. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch Microbiol.* **1993.** 159(4):336-444.

(48) Mahadevan, R.; Bond, D.R.; Butler, J.E.; Esteve-Nuñez, A.; Coppi, M.V.; Palsson, B.O.; Schilling, C.H.; Lovley, D.R.; Characterization of metabolism in the Fe(III)-reducing organism *Geobacter sulfurreducens* by constraint-based modeling. *Appl Environ Microbiol.* **2006.** 72(2), 1558-68.

(49) Coby, A.J.; Picardal, F.; Shelobolina, E.; Xu, H.; Roden, E.E. Repeated anaerobic microbial redox cycling of iron. *Appl Environ Microbiol.* **2011.** 77(17), 6036-6042.

882 (50) Emerson, D.; Weiss, J.V.; Megonigal, J.P.; Iron-oxidizing bacteria are associated with
883 ferric hydroxide precipitates (Fe-plaque) on the roots of wetland plants . *Appl Environ*
884 *Microbiol.* **1999**, 65(6), 2758-2761.

885

886 (51) Kuramata, M.; Sakakibara, F.; Kataoka, R.; Abe, T.; Asano, M.; Baba, K.; Takagi, K.;
887 Ishikawa, S. Arsenic biotransformation by *Streptomyces* sp. isolated from rice rhizosphere.
888 *Environ. Microbiol.* **2015**, 17 (6), 1897–1909.

889

890 (52) Kludze, H.; Delaune, R.; Patrick, W. Aerenchyma Formation and Methane and Oxygen-
891 Exchange in Rice. *Soil Sci. Soc. Am. J.* **1993**, 57 (2), 386–391.

892

893 (53) York, L.M.; Carminati, A.; Mooney, S.J.; Ritz, K.; Bennett, M.J. The holistic
894 rhizosphere: integrating zones, processes, and semantics in the soil influenced by roots. *J.*
895 *Exp. Bot.* **2016**, 67 (12), 3629–3643.

896

897 (54) Park, S. G.; Butcher, D. J. Investigation of the primary interactions between arsenic
898 species and thiols via electrospray ionization tandem mass spectrometry. *Microchem. J.* **2010**,
899 95, 57–66.

900

901 (55) Carey, A. M.; Norton, G. J.; Deacon, C.; Scheckel, K. G.; Lombi, E.; Punshon, T.;
902 Guerinot, M. L.; Lanzirrotti, A.; Newville, M.; Choi, Y.; Price, A. H.; Meharg, A. A. Phloem
903 transport of arsenic species from flag leaf to grain during grain filling. *New Phytol.* **2011**,
904 192, 87–98.

905

906 (56) Gu, Y.; Van Nostrand, J. D.; Wu, L.; He, Z.; Qin, Y.; Zhao, F.-J.; Zhou, J. Bacterial

- community and arsenic functional genes diversity in arsenic contaminated soils from different geographic locations. *PLoS One* **2017**, *12* (5), e0176696.
- (57) Wang, P.-P.; Sun, G.-X.; Zhu, Y.-G. Identification and characterization of arsenite methyltransferase from an archaeon, *Methanosarcina acetivorans* C2A. *Environ. Sci. Technol.* **2014**, *48* (21), 12706-12713.
- (58) Wang, P.-P.; Bao, P.; Sun, G.-X. Identification and catalytic residues of the arsenite methyltransferase from a sulfate-reducing bacterium, *Clostridium* sp. BXM. *FEMS Microbiology Letters* **2015**, *362* (1), 1-8.
- (59) Kuramata, M.; Sakakibara, F.; Kataoka, R.; Abe, T.; Asano, M.; Baba, K.; Takagi, K.; Ishikawa, S. Arsenic biotransformation by *Streptomyces* sp. isolated from rice rhizosphere. *Environ Microbiol.* **2015**, *17*(6), 1897-1909.
- (60) Zhang, J.; Cao, T.; Tang, Z.; Shen, Q.; Rosen, B. P.; Zhao, F.-J. Arsenic methylation and volatilization by arsenite S-adenosylmethionine methyltransferase in *Pseudomonas alcaligenes* NBRC14159. *Appl. Environ. Microbiol.* **2015**, *81* (8), 2852–2860.
- (61) Huang, K.; Chen, C.; Zhang, J.; Tang, Z.; Shen, Q.; Rosen, B. P.; Zhao, F.-J. Efficient Arsenic Methylation and Volatilization Mediated by a Novel Bacterium from an Arsenic-Contaminated Paddy Soil. *Environ. Sci. Technol.* **2016**, *50* (12), 6389–6396.
- (62) Lu, H.; Chen, J.; Jia, Y.; Cai, M.; Lee, P.K.H. Transcriptomic Responses of the Interactions between *Clostridium cellulovorans* 743B and *Rhodopseudomonas palustris*

932 *CGA009* in a Cellulose-Grown Coculture for Enhanced Hydrogen Production. *Appl Environ*
933 *Microbiol.* **2016.** 82(15), 4546-4559.

934

935 (63) Guan, X.; Yan, X.; Li, Y.; Jiang, B.; Luo, X.; Chi, X. Diversity and arsenic-tolerance
936 potential of bacterial communities from soil and sediments along a gold tailing contamination
937 gradient. *Can J Microbiol.* **2017.** 63(9), 788-805.

938

939 (64) Mu, Y, Zhou, L, Zeng, XC, Liu, L, Pan, Y, Chen X, Wang J, Li S, Li WJ, Wang Y.

940 *Arsenicitalea aurantiaca* gen. nov., sp. nov., a new member of the family

941 Hyphomicrobiaceae, isolated from high-arsenic sediment. *Int J Syst Evol Microbiol.* **2016**

942 66(12), 5478-5484.

943

944 (65) Limmer, M.A.; Wise, P.; Dykes, G.E.; Seyfferth, A.L. Silicon decreases dimethylarsinic
945 acid concentration in rice grain and mitigates straighthead disorder. *Environmental science &*
946 *technology* **2018.** 52 (8), 4809-4816.

947

948

949

950

951

952

953

954

955

956

Table 1: Corresponding differential copy number patterns (see heatmap Figure 4a & 4b) identified for 16S rRNA OTUs and *arsM* gene OTUs in planted versus unplanted and manured versus non-manured pairwise comparisons. **Group-I** = significant increase in unplanted soil, **Group-II** = significant increase in planted soil, **Group-III** = significant increase in manured soil, **Group-IV** = significant increase in non-manured soil, ** highest, * second highest (if applicable) correlation to *arsM* OTUs. In bold >1% abundant genera, increase= increase in copy number

Group	Bacterial 16S rRNA OTUs		
	Phylum level	Genus level	
I	Acidobacteria	<i>Acidobacteria_GPI sp.</i> **	
	Chloroflexi	<i>Dehalogenimonas sp.</i>	
	Cyanobacteria	<i>Cryptomonadaceae sp.</i>	
	Verrumicrobia	<i>Alterococcus</i>	
II	Firmicutes	<i>Tumebacillus*</i> , <i>Clostridium</i>**³² , <i>Desulfotomaculum</i>	
III	Acidobacteria	<i>Geothrix</i>	
	Actinobacteria	<i>Aeromirobium</i> , <i>Georgenia</i> , <i>Thermobifida</i> , <i>Patulibacter</i> , <i>Cellulomonadaceae sp.</i>	
	Bacteroidetes	<i>Paludibacter</i> , <i>Flavobacterim</i> , <i>Muricauda</i> , <i>Chitonophaga</i> , <i>Parasegetibacter</i> , <i>Algoriphagus</i> , <i>Dyadobacter</i>	
	Cyanobacteria	<i>Cyanobacteria_GpIV sp.</i> <i>Bacillariophyta sp.</i>	
	Firmicutes	<i>Geobacillus</i> , <i>Ammoniphilus</i> , <i>Brevibacillus</i> , <i>Cohnella</i> , <i>Paenibacillus</i> , <i>Thermobacillus</i> , <i>Paenisporsarcina</i> , <i>Sporosarcina</i> , <i>Planifilum</i> , <i>Thermoactinomyces</i> , <i>Dehalobacter</i> , <i>Thermo flavimicrobium</i> , <i>Oxobacter</i> , <i>Alkalibacter</i> , <i>Desulfitobacterium</i> , <i>Papillibacter</i> , <i>Halocella</i> , <i>Lutispora</i> , <i>Anoxybacillus</i> , <i>Ocenobacillus</i> , <i>Ureibacillus</i> , <i>Desmospora</i> , <i>Garciella</i> , <i>Parasporobacterium</i> , <i>Desulfosporosinus</i> , <i>Sporobacter</i> , <i>Dethibacter</i> , <i>Tepianaerobacter</i>	
	Proteobacteria	<i>Aquabacterium</i> , <i>Cellvibrio</i> , <i>Thermomonas</i> , <i>Brevundimonas</i> , <i>Beijerinckia</i> , <i>Bosea</i> , <i>Devosia*</i> , <i>Kaistia</i> , <i>Rhizobium</i> , <i>Xanthobacter</i> , <i>Paracoccus</i> , <i>Azospirillum</i> , <i>Magnetospirillum</i> , <i>Rickettsia</i> , <i>Novosphingobium</i> , <i>Sphingobium</i> , <i>Sphingopyxis</i> , <i>Sphingosinicella</i> , <i>Achromobacter</i> , <i>Ralstonia</i> , <i>Hydrogenophaga</i> , <i>Herbaspirillum</i> , <i>Herminiimonas</i> , <i>Methylophilus</i> , <i>Methylovorus</i> , <i>Aquaspirillum</i> , <i>Azoarcus</i> , <i>Shinella</i> , <i>Desulfocapsa</i> , <i>Geobacter</i> , <i>Rheinheimera</i> , <i>Halomonas</i> , <i>Pseudomonas</i> ³³ , <i>Pseudoxanthomonas</i>	
	Verrumicrobia	<i>Luteolibacter**</i> , <i>Verrucomicrobiaceae sp.</i> , <i>Optutaceae sp</i>	
IV	Acidobacteria	<i>Acidobacteria_Gp25**</i> , <i>Gp22</i> , <i>Gp11</i> , <i>Gp5 sp.</i>	
	Bacteroidetes	<i>Bacteroides</i> , <i>Terrimonas*</i> , <i>Prevotellaceae sp.</i>	
	Firmicutes	<i>Tepidibacter</i>	
	Cyanobacteria	<i>Cyanobacteria_GpXI sp.</i> , <i>Streptophyta sp.</i>	
	Proteobacteria	<i>Phaselicystis</i> , <i>Methylosarcina</i> , <i>Elioraea sp.</i> , <i>Sinobacteraceae sp.</i>	

Table2: Annotation of representative *arsM* OTUs via Blastn against bacterial genomes using 1e-06 as cutoff for significance. The table shows the percentage of all sequences in each group with best match against each genome: 1) all 2680 *arsM* OTUs in the final filtered OTU table, 2) the 347 *arsM* OTU's with identified significant changes in copy number, G1= 29 OTUs increased in unplanted soil, G2= 32 OTUs increased in planted soil, G3= 256 OTUs increased in manured soil, G4= 57 OTUs significant increase in non-manured soil.

blastn, genomes, top hit	All <i>arsM</i>	Sig. <i>arsM</i>	G1	G2	G3	G4
<i>Rhodopseudomonas palustris</i> TIE-1	7.9	23.3	3.4	9.4	28.9	15.8
<i>Rhodopseudomonas palustris</i> BisB5	6.7	5.6	41.4	0.0	2.0	7.0
<i>Roseiarcus fermentans</i> strain DSM 24875*	6.2	31.8	0.0	3.1	45.7	1.8
<i>Gemmatirosa kalamazoonesis</i> strain KBS708	5.0	2.4	3.4	3.1	0.4	10.5
<i>Rhodopseudomonas palustris</i> strain YSC3	3.5	1.6	10.3	0.0	0.4	3.5
<i>Streptomyces zinciresistens</i> K42	3.3	1.1	0.0	3.1	0.8	1.8
<i>Thiobacillus denitrificans</i> DSM 12475	3.2	0.5	0.0	0.0	0.4	1.8
<i>Rhodopseudomonas palustris</i> strain XCP	2.6	1.6	3.4	0.0	1.6	1.8
<i>Blastopirellula marina</i> DSM 3645	1.6	0.8	0.0	6.3	0.4	0.0
<i>Rhodoplanes roseus</i> strain DSM 5909	1.6	1.6	0.0	0.0	1.6	3.5
<i>Rhodopseudomonas palustris</i> HaA2	1.5	1.6	0.0	0.0	1.2	5.3
<i>Rhodopseudomonas pentothentatexigens</i>	1.2	1.1	0.0	3.1	0.0	5.3
<i>Thioalbus denitrificans</i> strain DSM 26407	1.1	1.9	0.0	0.0	2.7	0.0
<i>Intrasporangium chromatireducens</i> Q5-1	1.0	0.5	0.0	3.1	0.4	0.0
<i>Rhodomicrobium udaipurense</i> JA643	0.9	0.8	0.0	0.0	1.2	0.0
<i>Rhodoplanes elegans</i> strain DSM 11907	0.8	0.8	3.4	0.0	0.0	3.5
<i>Streptomyces aurantiacus</i> JA 4570	0.7	1.6	6.9	12.5	0.0	0.0
<i>Rhodopseudomonas palustris</i> CGA009	0.7	0.8	0.0	3.1	0.8	0.0
<i>Nocardioides</i> sp. JS614	0.7	0.5	0.0	0.0	0.0	3.5
<i>Acidobacteriaceae</i> bacterium SbA1	0.6	0.5	0.0	0.0	0.4	1.8
<i>Stackebrandtia nassauensis</i> DSM 44728	0.4	0.5	0.0	0.0	0.0	3.5
<i>Halobacterium</i> sp. DL1	0.4	0.5	0.0	0.0	0.4	1.8
<i>Streptomyces</i> sp. Root1310	0.4	0.5	0.0	0.0	0.4	1.8
<i>Methanoculleus taiwanensis</i> strain CYW4	0.4	0.5	0.0	0.0	0.0	3.5
<i>Rhodopseudomonas palustris</i> DX-1	0.3	0.5	0.0	3.1	0.4	0.0
<i>Thiocapsa</i> sp. KS1	0.3	0.5	6.9	0.0	0.0	0.0
<i>Planctomyces</i> sp. SH-PL62	0.2	0.5	0.0	6.3	0.0	0.0
<i>Microtholunatus phosphovor</i> NM-1	0.2	0.5	6.9	0.0	0.0	0.0
<i>Telmatospirillum siberiense</i> strain 26-4b1	0.1	0.5	0.0	6.3	0.0	0.0
matches to other genomes	25.0	7.5	10.3	21.9	3.5	15.8
no genome match	21.5	7.2	3.4	15.6	6.6	7.0

*most of these also highly similar to *Rhodopseudomonas palustris* TIE-1 (second best genome match)

Legends to figures

Figure 1. Box plot of soil solution arsenic speciation and relative soil *arsM* and 16S rRNA copy number in replicated microcosms (experiment2). The error bars are standard error of means as calculated in the GLM analysis. Paired lines above bars show identified significant manure (2 larger lines) and plant effect (2 smaller lines), with ‘*’, ‘**’, ‘***’ above indicating $p < 0.05$, $p < 0.01$, $***p < 0.001$, respectively and ‘+’ indicating significant interaction effect of manure and plants (GLM, Minitab). The associated probabilities are reported in Table S3.

Figure 2. Heatmap showing normalized counts of OTUs with significant differences in copy number ($p < 0.05$ and absolute $\log_2FC > 1$, experiment 2). **(A)** level 6 16S rRNA; **(B)** *arsM* gene OTUs. Darker blue indicates greater normalized count. **Group-I** = significantly higher copy number in unplanted soil, **Group-II** = significantly higher copy number in planted soil, **Group-III** = significantly higher copy number in manured soil, **Group-IV** = significantly higher copy number in non-manured soil. **A:** Acidobacteria, **Ac:** Actinobacteria, **B:** Bacteroidetes, **Ch:** Chloroflexi, **Cy:** Cyanobacteria, **F:** Firmicutes, **P:** Proteobacteria and **V:** Verrumicrobia.

Figure 3. Variance partition analysis plot for genus level 16S rRNA OTUs with significant differences in copy number in response to planting (heatmap, Group I and II). **A:** Violin plot, showing the median and variance of the contribution of manure treatment and planting across all OTUs, **B:** Corresponding bar chart for each OTU.

Figure 4. Network analysis of genus level 16S rRNA OTUs that show significant differences in copy number in response to planting. A, B, C, D= selected level 6 OTUs (genera) with significantly higher copy number in non-planted soil (heatmap, Group I), A= *AcidobacteriaGP1*, B=*Dehalogenimonas*, C=*Cryptomonadaceae*, D= *Alterococcus*. E, F, G= selected level 6 OTUs with significantly higher copy number in planted soil (heatmap, GroupII), E= *Tumebacillus*, F= *Clostridium*, G= *Desulfomaculum*. Missing samples in the network for *Cryptomonadaceae* (C) are samples with a copy number count of 0.

1022

1023 **Figure1.**

1024

1025

Figure 2.

Figure 3.

Figure 4.

As_i ➤ MMA ➤ DMA ➤ TMAO