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Redox Dependence of Thioarsenate Occurrence in Paddy Soils and the Rice Rhizosphere

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ABSTRACT

In flooded paddy soils, inorganic and methylated thioarsenates contribute substantially to arsenic speciation besides the much-better-investigated oxyarsenic species, and thioarsenate uptake into rice plants has recently been shown. To better understand their fate when soil redox conditions change, i.e. from flooding to drainage to reflooding, batch incubations and unplanted microcosm experiments were conducted with two paddy soils covering redox potentials from E_H -260 to +200 mV. Further, occurrence of thioarsenates in the oxygenated rice rhizosphere was investigated using planted rhizobox experiments. Soil flooding resulted in rapid formation of inorganic thioarsenates with a dominance of trithioarsenate. Maximum thiolation of inorganic oxyarsenic species was 57% at E_H -130 mV and oxidation caused nearly complete dethiolation. Only monothioarsenate formed again upon reflooding and was the major inorganic thioarsenate detected in the rhizosphere. Maximum thiolation of mono- and dimethylated oxyarsenates was about 70% and 100%, respectively, below E_H 0 mV. Dithiolated species dominated over monothiolated species below E_H -100 mV. Among all thioarsenates, dimethylated monothioarsenate showed the least transformation upon prolonged oxidation. It also was the major thiolated arsenic species in the rhizosphere with concentrations comparable to its precursor dimethylated oxyarsenate, which is especially critical since dimethylated monothioarsenate is highly carcinogenic.

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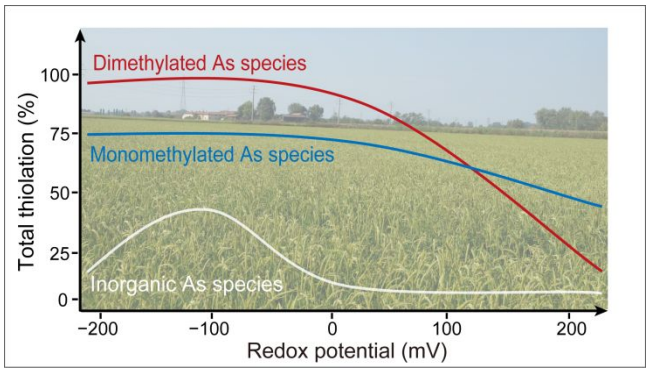

INTRODUCTION

Dietary arsenic (As) exposure from rice is a global problem.[1, 2] Rice grains accumulate approximately 10-fold more As than other cereals[1] and since it is a non-threshold class-1 carcinogen this accumulation poses a potential health risk to over half of the global population, which rely on rice as staple diet.[3]

The reason for the high As accumulation in rice is primarily its growth under flooded conditions.[4] Flood-induced reducing conditions in paddy soils result in the reductive dissolution of Fe(III) (oxyhydr)oxides and release of sorbed inorganic As either as arsenate ($As^{V}O(OH)_3$) or arsenite ($As^{III}(OH)_3$).[5] Arsenate and arsenite are structural analogues to the nutrients phosphate and silicic acid, respectively,[6, 7] therefore inevitably taken up by rice plants[5] and partially translocated to the grain.[6, 7] Microbial methylation of inorganic oxyarsenic species in the paddy soil leads to the formation of methylated oxyarsenates.[8] In the immediate vicinity of rice roots both inhibition[9] and promotion[8] of microbial methylation has been reported. Monomethylated arsenate (MMA) and dimethylated arsenate (DMA) are usually minor species in paddy soils, compared to the predominance of inorganic oxyarsenic species.[8, 10] However, with respect to inorganic oxyarsenic species, plant detoxification strategies are less effective for methylated oxyarsenates, particularly for DMA that can account for 10-90% of total As in rice grains worldwide.[11] Concentration limits imposed for rice and rice-derived products consider only inorganic oxyarsenic due to their higher carcinogenicity, but not methylated oxyarsenates.[12]

Microscale oxic niches within generally reducing paddy soils, e.g. created by root radial oxygen loss, or changes from reducing to oxidizing conditions, e.g. during soil drainage for fertilizer application, have significant impact on pore-water As speciation.[8, 13] Water management practices that involve one or more soil drainage events have long been

suggested to mitigate grain As accumulation.[13] Soil drying and aeration lead to precipitation of newly formed Fe(III) (oxy)hydroxides and oxidation of arsenite, favoring the retention of mobilized inorganic oxyarsenic species onto the solid phase.[14] Methylated oxyarsenates can survive short-term soil oxidation.[15] While MMA sorption to Fe(III) (oxyhydr)oxides is similar to arsenate, DMA sorbs significantly less.[16] Similar to soil drainage, rhizospheric aeration leads to formation of Fe and Mn oxide coatings on rhizospheric soil particles and along O_2-releasing root surfaces with formation of so-called iron plaque,[17] with subsequent retention of inorganic oxyarsenic species, mainly arsenate.[18]

A further group of As species, so-called thioarsenates, have only recently been detected to occur in substantial quantities and under various conditions in flooded paddy soils.[19] Thioarsenates can be divided into two groups: 1) Inorganic thioarsenates which include mono- (MTA; $As^{V}S(OH)_3$), di- (DTA; $As^{V}S_2(OH)_2^-$), and trithioarsenate (TTA; $As^{V}S_3(OH)^{2-}$) and 2) Methylated thioarsenates, which include mono- (MMMTA; $(CH_3)As^{V}S(OH)_2$) and dimethylated monothioarsenate (DMMTA; $(CH_3)_2As^{V}S(OH)$); mono- (MMDTA; $(CH_3)As^{V}S_2(OH)^-$) and dimethylated dithioarsenate (DMDTA; $(CH_3)_2As^{V}S_2^-$). Inorganic thioarsenates form by reaction of arsenite, sulfide, and zero-valent sulfur[20, 21] and are predominantly observed in neutral to alkaline soils.[19] Methylated thioarsenates form by nucleophilic attack of sulfide on the oxy species MMA and DMA[22, 23] and are mostly observed in neutral to acidic soils.[19] Both inorganic and methylated thioarsenates can be taken up by rice plants[24, 25] and DMMTA has already been detected in rice grains before.[26, 27]

In contrast to the broad knowledge about formation and transformation of inorganic and methylated oxyarsenic species under fluctuating redox conditions, there is a lack of studies on the redox-induced formation and transformation of inorganic and methylated

thioarsenates in paddy soils to date, which prevents understanding their environmental fate when redox conditions change, i.e. upon flooding, drainage, and reflooding. Furthermore, it remains unclear which thioarsenate species can survive rhizospheric aeration and thus be available for root uptake. Previous studies on oxidation of thioarsenates in synthetic solutions often showed higher persistence towards oxidation for monothiolated than higher order thiolated species, e.g. for MTA and TTA upon aeration and addition of H_2O_2[28] or for DMMTA and DMDTA in the presence of ferric Fe.[23] Thus, we hypothesized that rhizospheric aeration could act as an oxidative barrier for higher order thiolated species, while monothiolated species could survive and be available for root uptake.

In this study, we first conducted anaerobic soil batch incubations to monitor the temporal formation of thioarsenates under reducing conditions. Subsequently, soil reduction-reoxidation batch incubations were performed to study the oxidative transformation of thioarsenates upon soil reoxidation. We further conducted flood-drain-reflood microcosm incubations to mimic the effect of irrigation-induced redox fluctuations on thioarsenates dynamics under field conditions. Data from all experiments were then combined to evaluate the relevance of thiolation for inorganic and methylated As species over a wide range of redox conditions. Finally, rhizobox rice cultivations were performed to identify thiolated species that occur in the rice rhizosphere.

MATERIALS AND METHODS

Paddy Soil Sampling and Characterization. Soil was collected from the plow layer of two Italian paddy fields: Cascina Veronica (E 8°53′48″, N 45°10′39″; Eutric Gleysol) and Cascina Fornazzo (E 8°57′50″, N 45°13′54″; Umbric Gleysol). These soils were selected

for their high proportion of thiolated As species, based on a previous field survey.[19] Detailed information about soil classification and chemical parameters has been reported previously.[19] Veronica soil contained 5.5 mg kg^{-1} total As, 29 mg kg^{-1} 0.5 M $NaHCO_3$-extractable sulfate, 2.9 g kg^{-1} 0.5 M HCl-extractable Fe, 20 g kg^{-1} organic C, and had a soil pH value of 5.6. Fornazzo soil contained 5.6 mg kg^{-1} total As, 95 mg kg^{-1} 0.5 M $NaHCO_3$-extractable sulfate, 4.0 g kg^{-1} 0.5 M HCl-extractable Fe, 47 g kg^{-1} organic C and had a soil pH value of 5.8. All experimental designs are summarized in Table S1 and described in detail below.

Soil Reduction Batch Incubations. To investigate the temporal formation of thioarsenates under reducing conditions, reduction batch incubations were conducted anaerobically for 20 d. A total of 20 g air-dried Veronica soil was suspended in 40 mL of 10 mM acetate solution without (control) or with (sulfate spike) the addition of 1 mM K_2SO_4. Acetate was selected here to create low E_H rapidly while selectively stimulating sulfate-reducing bacteria [29] to maximize initial thiolation. The acetate solutions were N_2-purged (30 minutes), sterile-filtered (0.2 µm), and added inside a glovebox (COY, N_2/H_2 95/5% (v/v)) to prevent oxygen contamination. Vials were then capped with a butyl rubber septum, covered with aluminum foil, and incubated at room temperature with continuous horizontal shaking (250 rotations min^{-1}). Soil solutions were sampled by sacrificing three bottles each at day 1, 2, 3, 4, 5, 6, 7, 8, 11, 13, 18, and 20. Filtration (0.2 µm cellulose-acetate filters) and all further sampling processing was performed inside the glovebox.

Soil Reduction-Reoxidation Batch Incubations. To study the effect of soil reoxidation on transformation of thioarsenates, reduction-reoxidation batch incubations were conducted with 30 d of soil reduction and 25 d of reoxidation. A spike of 9 mmol L^{-1} sulfate and 100 µg L^{-1} arsenate was added in these incubations to promote formation of thioarsenates. For reduction, 20 g air-dried Veronica soil was suspended in 40 mL

incubation solution containing 9 mmol L^{-1} K_2SO_4, 100 µg L^{-1} arsenate (sodium arsenate dibasic-heptahydrate), and 2.5 mmol L^{-1} D (+)-glucose. Glucose was used here to stimulate anaerobic bacteria in general,[30] not specifically sulfate-reducing bacteria, and induce a slower decrease to low E_H. The suspensions were incubated anaerobically as described above. After 30 d, air was allowed to diffuse into the vials by inserting injection needles (23G, Ø 0.06 × 30 mm) through the rubber septa. Soil solutions were sampled by sacrificing three bottles each at day 5, 15, 30 (reduction period) and at day 35, 44, 55 (reoxidation period). Filtration (0.2 µm cellulose-acetate filters) and all further sampling processing was carried out inside the glovebox.

Flood-drain-reflood Microcosm Incubations. To simulate the irrigation-induced redox fluctuations under field conditions, microcosm experiments were conducted with fresh soils from both rice fields (Fornazzo and Veronica) subjected to a cycle of flood-drain-reflood conditions (Fig S1a). For each microcosm, 400 g fresh soil (2 mm sieved) were premixed with 2.5 g rice straw (cut to pieces of 1 cm in length) and 250 mL tap water was added (tap water properties see https://www.stadtwerke-bayreuth.de/fileadmin/user_upload/wasser/trinkwasseranalyse-eichelberg.pdf). Rice straw was used here as carbon source to mimic natural conditions and likely obtain a slower and less pronounced decrease in E_H compared to easily degradable carbon such as acetate or glucose.

Microcosms (500 mL polyethylene bottles) were equipped with rhizosamplers (Rhizon MOM, pore size 0.15 µm; Rhizosphere Research Products, The Netherlands), each ending in a Teflon shut-off valve. Microcosms were kept flooded for 20 d (without any spike), drained for 4 d, and subsequently reflooded for another 14 d with tap water containing 1 mM $(NH_4)_2SO_4$ to mimic sulfate fertilizer application practice where farmers usually drain fields for a few days before fertilization. Pore-water was extracted by the

rhizosamplers connected to evacuated glass bottles at days 3, 10, 20 (flooded period), days 24 (drainage period), and days 27, 31, and 38 (reflooded period). Microcosm design, experimental setup and pore-water sampling intervals are summarized in Fig S1a.

Rhizobox Rice Cultivations. To further identify thioarsenates that occur in the rice rhizosphere, rhizobox experiments were performed with two rice cultivars (Yangdao 6, YD and Nongken 57, NK), selected on the basis of their different root oxygen loss (0.45 and 1 μmol O_2 per g root and h).[25] For this experiment, Fornazzo soil was used due to its higher potential of thioarsenate formation with respect to Veronica soil. Two rhizoboxes with a volume of 902.5 cm^3 (19.1 cm × 31.5 cm × 1.5 cm) were built (Fig S1b-c). A transparent front panel allowed observing the penetration depth of roots, which was covered with a removable black plate to keep the soil in darkness. On the backside, a rubber panel (0.6 cm thick, fixed by a perforated stainless steel plate to prevent deformation) was equipped with 6 rhizosamplers (MicroRhizon, Rhizosphere Research Products, The Netherlands), which were placed in two rows (three samplers per row) in a distance of 4.5 cm from each other, and at a depth of 9 cm (depth A) and 16.5 cm (depth B) from the soil-water interface. Rhizosamplers had an exposed porous part of 8 mm, with an outer diameter of 1 mm and a mean pore size of 0.15 μm, which enabled sampling of small pore-water volumes (<2 mL) near the root surfaces once the roots spread out in the rhizoboxes. Each rhizobox was filled with 900 gram of air-dried paddy soils, equilibrated with 500 mL tap water and developed a standing water of ~2 cm depth. No rice straw was added to prevent creating further heterogeneities apart from the influence of the rhizosphere in the relatively small rhizobox.

One single rice seedling was transplanted into each rhizobox. After 15 d, rhizoboxes were placed into a climate chamber. Day (15 h, light intensity of 75 μEinstein) and night (9 h, no light) cycles were scheduled with temperatures of 25°C and 20°C, respectively.

Fertilizers used were ammonium sulfate, potassium sulfate, and triple superphosphate. Besides an initial fertilization of 0.2 g N, 0.2 g K_2O, and 0.15 g P_2O_5 per kg soil (added as solution), a second fertilization was applied (one third the amount of the initial fertilization) at day 35. Irrigation was performed daily with tap water to keep flooded conditions. After a total of 100 d (15 d pre-incubation and 75 d in the climate chamber), pore-water was extracted as described above.

Analytical Techniques. During sampling, unstable chemical parameters i.e. pH, E_H, Fe, and sulfide were measured immediately inside a glovebox as described in a previous study.[31] Pore-water samples for total As were acidified with 0.5% H_2O_2 and 0.8% HNO_3 and kept at 4 °C until analysis. Another aliquot was stabilized in 10 mM Diethylenetriamine-pentaacetic acid (DTPA), flash-frozen on dry-ice, and stored at -20 °C for As speciation analysis. Total As was quantified by inductively coupled plasma-mass spectrometry (ICP-MS, XSeries2, Thermo-Fisher) and As was detected as AsO^+ at m/z 91 with an O_2/He mixture (10:90%) serving as reaction gas. Arsenite, arsenate, methylated oxyarsenates, and thioarsenates were quantified by IC (Dionex ICS-3000; AG/AS16 IonPac column, 20−100 mM NaOH at a flow rate of 1.2 mL min^{-1}, using no suppressor) coupled to ICP-MS following the method of Wallschläger and London (2007).[22] Validity of DTPA-based As species preservation and analysis methods has been demonstrated before.[19]

RESULTS

Formation of Thioarsenates in Reduction Batch Incubations. In the sulfate-spiked (1 mmol L^{-1}) incubations of Veronica soil, using acetate as carbon source, E_H dropped continuously from 17 mV at day 1 to -260 mV at day 20, while pH increased from 6.6 to

around 7.0 (Fig 1a). Reducing conditions resulted in a prompt increase of dissolved total Fe up to maximum concentrations of 21 mg L^{-1} at day 2, that subsequently decreased rapidly between day 2 and 4 (Fig 1b). In parallel, thiosulfate (maximum 0.5 mg L^{-1}; Fig S2b) and sulfide (Fig 1b) concentrations increased, along with decreasing sulfate concentrations (Fig S2b). Sulfide peaked at day 4 with a concentration of 8 mg L^{-1}, then decreased to < 2 mg L^{-1} after one week.

Concurrent with Fe and sulfate reduction, a continuous release of total As to soil solution was observed up to day 20 when final concentrations reached 12 µg L^{-1} (Fig 1c). Besides inorganic oxyarsenic species (Fig 1c) and methylated oxyarsenates (Fig 1e-f), seven other As species were detected. These species were the inorganic thioarsenates MTA, DTA, and TTA as well as the methylated thioarsenates MMMTA, MMDTA, DMMTA, and DMDTA. Inorganic thioarsenates formed immediately with the increase of sulfide (Fig 1b, d). Monomethylated thioarsenates increased continuously from day 2 to 20, reaching a final concentration of 0.9 and 2 µg L^{-1} for MMMTA and MMDTA, respectively. Dimethylated thioarsenates increased after a lag phase of 8 d, before reaching concentrations of 0.5 and 1.3 µg L^{-1} for DMMTA and DMDTA, respectively (Fig 1f). Methylated oxyarsenates stayed at relatively low levels (< 1 and < 0.03 µg L^{-1} for MMA and DMA, respectively) compared to their respective thiolated forms (Fig 1e-f).

Control treatments of Veronica soil had lower individual concentrations of thiolated As species in comparison to sulfate-spiked incubations, but revealed a similar temporal pattern of thioarsenate formation (Fig S3).

Transformation of Thioarsenates in Reduction-Reoxidation Batch Incubations. During the reduction-reoxidation batch incubations with Veronica soil, using glucose as carbon source, reducing conditions formed rapidly, but the final E_H was only -162 mV at day 30 (Fig 2a) compared to -260 mV at day 20 with acetate addition as described before.

234 Upon reoxidation, E_H increased up to 97 mV within 5 d, before stabilizing at ~180 mV. The
235 pH increased to neutral conditions during soil reduction, followed by a slight decrease of
236 0.5 units towards the end of the reoxidation period. Total dissolved Fe was 35 mg L^{-1} at
237 day 5, followed by a fast decrease (Fig 2b). Sulfide increased substantially from 3 to 14
238 mg L^{-1} between day 15 and 30. Both total Fe and free sulfide concentrations were close
239 to detection limit after reoxidation.

240 During soil reduction, total As increased sharply, reaching up to 134 µg L^{-1} at day 30. The
241 increase of total As was reflected in a continuous increase of inorganic oxyarsenic species,
242 and methylated oxyarsenates (MMA and DMA), inorganic thioarsenates (MTA, DTA, TTA)
243 and methylated thioarsenates (MMMTA, MMDTA, DMMTA, DMDTA) (Fig 2c-f). TTA was
244 the main inorganic thiolated species (> DTA and MTA). For the methylated thioarsenates,
245 concentrations decreased in the following order MMDTA > MMMTA > DMDTA > DMMTA.
246 Monomethylated thioarsenates dominated over dimethylated thioarsenates (Fig 2e-f) and
247 within each group (mono- or dimethylated) dithioarsenates (blue lines) over
248 monothioarsenates (red lines). Remarkably, concentrations of methylated thioarsenates
249 (up to 41% of total As) were higher than those of the inorganic oxyarsenic species (up to
250 34% of total As) and 2-7 times higher than those of their precursors, the methylated
251 oxyarsenates (Fig S4).

252 Upon reoxidation, total As decreased markedly from day 30 to day 44 before reaching ~70
253 µg L^{-1}. Inorganic oxyarsenic species dropped from 36 µg L^{-1} to 14 µg L^{-1} within 5 d, and
254 remained at that level thereafter (Fig 2c), whereas all inorganic thioarsenates decreased
255 to negligible concentrations after reoxidation. Methylated dithiolated As (MMDTA and
256 DMDTA) dropped steeply after 5 d of reoxidation and were constant at low levels with
257 prolonged oxidation (Fig 2e-f). The decrease of MMDTA was accompanied by the
258 transient rise of MMA and MMMTA. However, both species showed a fast drop to less

than 5 μg L^{-1} at day 44 and thereafter (Fig 2e). The decrease of DMDTA was accompanied by an increase of DMA and DMMTA (day 35). DMA increased slowly afterwards, concurrent with a slight decrease of DMMTA toward the end of reoxidation (Fig 2f). The major As species that survived prolonged reoxidation of 25 d were, in the order DMA, inorganic oxyarsenic species, and DMMTA.

Dynamics of Thioarsenates in Flood-drain-reflood Microcosm Incubations. In the microcosm incubations using rice straw as organic carbon source, E_H values did not decrease as much as during the batch incubations with acetate or glucose but were around 0 mV during the flooded period in both soils (Fornazzo and Veronica). They increased to around 200 mV after drainage for 4 d and dropped again to around 10 mV after reflooding (Fig S5a, c). Flooding induced an increase in total dissolved Fe, reaching 20 mg L^{-1} and 14 mg L^{-1} for Fornazzo and Veronica at day 20, respectively (Fig S5b, d). Total Fe decreased slightly after drainage for 4 d, and was at around 15 mg L^{-1} for both soils after reflooding and addition of 1 mM $(NH_4)_2SO_4$. Sulfide concentrations were always below detection limit (10 μg L^{-1}) during microcosm experiments.

Total As concentrations in pore-water were as high as 81 and 35 μg L^{-1} at day 3 for Fornazzo (Fig 3a) and Veronica, respectively (Fig S6a). Higher concentrations compared to the batch experiments with dry soil suggest that the fresh wet soil used in the microcosm experiments may already have had higher fractions of easily mobilizable As, which was released to pore-water when flooded. Total As (mainly inorganic oxyarsenic species) decreased towards day 10, and remained at low levels thereafter. All As species discussed above were also detected in the microcosm experiments, with higher absolute concentrations and larger proportions of total As of both inorganic and methylated thioarsenates in Fornazzo (Fig 3b-d) than in Veronica soil (Fig S6b-d). During the first flooded period, concentrations of inorganic thioarsenates were generally low (< 1 μg L^{-1})

in both soils. TTA was the dominant species in Fornazzo soil, MTA in Veronica soil. Methylated dithiolated As, i.e. MMDTA and DMDTA, were detected as the main methylated thioarsenates (Fig 3c-d and Fig S6c-d, blue line), which was consistent with observations from the batch incubations.

After drainage and oxidation for 4 d and reflooding, DTA and TTA concentrations were close to detection limit. MTA was the only inorganic thioarsenate species that remained (Fig 3b and Fig S6b). Amongst the methylated thioarsenates, both MMDTA and DMDTA concentrations were substantially lower than before drainage. MMMTA and DMMTA showed an increase in concentrations with reflooding (Fig 3c-d and Fig S6c, red line), in line with the results of the above described reduction-reoxidation batch incubation experiment.

Occurrence of Thioarsenates in Rhizobox Rice Experiments. Total As concentrations in the Rhizobox experiments were between 7.9 and 17.4 μg L^{-1}. At the day of sampling, rice roots had a macroscopically visible length of about 30 cm, so both sampling depths A and B (9 and 16.5 cm below the soil-water interface) were located within the rhizosphere (Fig S1c). Comparing the two different sampling depths, no significant differences neither for total As nor inorganic oxyarsenic species were found for the rice variety YD. For NK, the variety with higher root oxygen loss, both total As and inorganic oxyarsenic species were higher in depth B than A (Fig 4a). Inorganic thioarsenates were all close to detection limit (0.03 μg L^{-1}), with the exception of MTA for NK B (0.7 ± 0.3 μg L^{-1}) (Fig 4b). MMMTA was the second-highest thiolated species (up to 1.5 μg L^{-1}), with concentrations slightly lower than its precursor MMA (Fig 4c). DMMTA was detected as the dominant thioarsenate species (up to 2.2 μg L^{-1}), with concentrations comparable to its precursor DMA (Fig 4d). MMDTA and DMDTA were detected but in much lower concentrations compared to their respective monothiolated As forms (Fig 4c-d).

DISCUSSION

In our previous study, we demonstrated the ubiquitous occurrence and quantitative importance of thioarsenates throughout the rice cropping season and across multiple paddy soils from the major rice cultivation areas in Italy, France, and China.[19] In the present study, we monitored short-term formation and transformation of thioarsenates at different redox conditions to better understand their fate in the paddy soils and the rhizosphere, and thereby estimate their potential availability for root uptake. Combination of all observations from the batch and microcosm experiments based on the prevailing redox potential yielded a pattern of thiolation in relation to E_H ranging from -260 mV (achieved in experiments with acetate addition), via -200 to -100 mV (glucose addition) and -50 to +100 mV (rice straw addition) to + 200 mV (after oxidation) (Fig 5).

Formation and transformation of inorganic thioarsenates. Total thiolation of inorganic oxyarsenic species was much lower than thiolation of methylated oxyarsenic species with a maximum of 57% at E_H -130 mV (Fig 5a-d). This low thiolation is in accordance with previous observations,[19] where low soil pH with a correspondingly low content of soil zero-valent sulfur was found to be a limiting factor for formation of inorganic thioarsenates (oxic soil pH for Veronica and Fornazzo was 5.6 and 5.8, respectively). Especially in controls without sulfate spike, concentrations were very low (<0.5 µg L^{-1}) (Fig S3d). Sulfate spiking of anoxic soil lead to the formation of free sulfide and a rapid formation of inorganic thioarsenates (Fig 1d) with trithioarsenate initially representing the dominant species. When free sulfide concentrations decreased under anoxic conditions over time, likely caused by precipitation of Fe-S minerals[32] and sulfide binding to organic matter,[20] formation of TTA was not favorable anymore due to too little excess sulfide and MTA was the dominant species after 20 d, which is in line with previous observations from

environments with low excess sulfide.[20, 33] Above E_H 0 mV, inorganic As thiolation decreased to only a few %. The lability of TTA towards oxidation has previously been reported,[28] however the rapid disappearance of MTA is rather surprising. During soil drainage, some MTA survived and it was also the only considerable species that was observed after reflooding (microcosm experiment) and in the rhizosphere (rhizobox experiment).

Formation and transformation of methylated thioarsenates. Much higher concentrations of methylated thioarsenates than their oxylated precursors MMA and DMA were observed in soil reduction batch incubations (Fig 1e-f and S3e-f), not only in the sulfate-spiked treatments, but also in those at natural sulfate concentrations. Maximum total thiolation of mono- and dimethylated oxyarsenates was about 70% and 100%, respectively, below E_H 0 mV (Fig 5e, 5h). The high thiolation rate indicates spontaneous thiolation, which is not limited by sulfide supply, but intrinsically by the formation of the oxylated precursors MMA and DMA, which are usually detected only as minor As species in paddy soils.[11] A similar observation was made in our previous large-scale study.[19] Rapid thiolation of methylated oxyarsenates has also previously been reported in kinetic studies of methylated oxyarsenate thiolation at excess sulfide in aqueous solution (abiotic reactions without soil)[23] and in DMA-spiked landfill leachates under sulfidic conditions.[34]

Detectable free sulfide concentrations in our reduction batch incubations were relatively high, ranging from 17 to 66 µM for the control and from 23 to 214 µM for the sulfate-spike treatment (Fig 1b and S3b), thereby greatly exceeding the sum of all As species (< 0.15 µM, Fig 1c and S3c). However, also lower free sulfide concentrations in natural paddy soils, reported to range from 1 to 2 µM for soils high in Fe and manganese and 15 to 25 µM for paddy soils low in Fe but high in organic carbon,[35] are still in an excess of typical

As concentrations (0.1 to 1 μM) by 1 to 2 orders of magnitude. Noticeably, in our microcosm experiments free sulfide concentrations were always below the detection limit (0.3 μM), yet, both mono- and dimethylated thioarsenates increased at the expense of MMA and DMA with prolonged flooding (Fig 3c-d and S6c-d). This observation could be explained by continuous production of below-detection-level concentrations of dissolved sulfide from sulfate as reported before,[36] which can then react immediately in solution to form thiolated species. Alternatively, there could be an additional supply from a pool of reduced sulfur bound to minerals or organic matter which can react with As in solution to form thiolated species, similar to what has been previously suggested for inorganic thioarsenates in peatlands.[20]

A closer look at the individual methylated thioarsenates revealed higher concentrations of dithiolated (MMDTA and DMDTA) compared to monothiolated species (MMMTA and DMMTA) in both batch incubations (Fig 1e-f, 2e-f and S3e-f) and microcosm experiments (Fig 3c-d and S6c-d) when E_H was < 100 mV (Fig 5f, g, I, j). The dithiolated species are the end products of MMA and DMA thiolation at near-neutral to slightly acidic pH in solution.[23, 37] Only at lower pH (≤ 3), monothiolated species would be formed preferentially.[23, 37] Dithiolated species are also expected to be the dominant methylated thioarsenates under reducing conditions in nature because near neutral to slightly acidic pore water pH is typical for paddy soils in general (like in our experiments, see Fig 1a, 2a, S3a, S5a,c). The reason is that pH upon flooding of acidic soils will increase due to proton-consuming reductions of, e.g., Mn(III, IV) and Fe(III) oxyhydroxides, and pH of alkaline soils will decrease due to accumulation of CO_2.[38]

While MMDTA and DMDTA were the dominant methylated species under reducing conditions, fast dethiolation occurred within 4-5 d after re-establishment of oxic conditions in both batch (Fig 2e-f) and microcosm experiments (Fig 3c-d and S6c-d). The driving

processes for dethiolation are likely depletion of sulfide (Fig 2b) by oxidation to thiosulfate and sulfate, formation of strong oxidants like Fe(III) by oxidation of Fe(II) sulfide minerals, or hydroxyl radicals from oxidation of reduced soil humic acids,[39, 40] and the presence of oxygen. The slight decrease in pH observed upon reoxidation (Fig 2a) due to proton-producing oxidation processes, e.g., of Fe(II) oxyhydroxides or inorganic sulfur[41] should have a minor impact on dethiolation of methylated thioarsenates, considering their stability at near-neutral to slightly alkaline pH.[37] The observed lability of MMDTA and DMDTA upon oxidation are in line with the findings of Wallschläger and London (2007), who reported that both species transformed quickly in an unpreserved groundwater sample even when stored without any headspace.[22]

An increase in the share of monothiolated MMA at E_H>-100 to 0 mV (Fig 5f) and DMA at E_H > -150 mV (Fig 5j), confirmed stepwise dethiolation. For DMA, dethiolation continued from DMMTA to DMA which increased in concentration under oxic conditions (Fig 2f) and became dominant at E_H > 100 mV (Fig 5i). For MMA, concentrations started to increase upon oxidation (day35, Fig 2e) which would be consistent with dethiolation of MMDTA and MMMTA, but then decreased upon prolonged oxidation. The share of total MMA thiolation remained around 70% more or less over the whole E_H range with no decreases at oxic conditions (Fig 5e). An explanation could be that dethiolation of MMMTA to MMA takes place but that then MMA is preferentially sorbed to iron (oxy)hydroxides under oxic conditions.[16] Little is known about sorption of thiolated MMA, yet, but first results indicate, compared to MMA, less sorption to goethite and less enrichment in iron plaque.[42] In absolute concentrations, DMMTA concentrations were higher than MMMTA upon prolonged soil reoxidation (Figure 2e-f) which is consistent with results from a recent abiotic air purging experiment in plant nutrient solution.[25] DMMTA, which was already proven to be persistent under oxic conditions,[23, 25] survived for 25 d under oxic soil

409 conditions. In fact, concentrations during the reoxidation period were even higher than
410 those observed during the initial reducing period due to dethiolation of DMDTA (Fig 2f).

411

412 **Occurrence of thiolated species in the rhizosphere.** Previous studies using O_2
413 microsensors showed that the aerenchyma of 0.7 mm thick rice roots contained 32% of
414 atmospheric partial pressure of oxygen and that the oxic zone extended to about 0.3 mm
415 from the roots into the surrounding reducing soil.[43] Since our sampling ports in the rhizobox
416 experiments were all located well within the root-penetrated zone, root oxygen loss was
417 expected to control thioarsenate occurrence in the rhizosphere. Due to the limited amount
418 of sample volume, no E_H was measured in the rhizobox experiment. However, E_H of the
419 same soil (Fornazzo) were measured previously both directly in the planted field (+98 mV)
420 and in mesocosm experiments with rice straw (+132 $\pm$ 45 mV, over a whole growth period,
421 with/without sulfate fertilization, n=28).[19] Redox conditions in the rhizobox of the present
422 study are assumed to be very similar (+100 to +150 mV). Thiolation percentages from the
423 rhizobox experiments are not plotted in Fig 5 due to the lack of a direct E_H measurement,
424 but the data is shown in Table S2. Little thiolation of inorganic As (median 9.2 % total
425 thiolation, 6.1% monothiolation), little dithiolation of methylated oxyarsenates (median
426 16.8% for MMA, 6.1 % for DMA), a high share of monothiolation for MMA (median 32%)
427 and DMA (42%), and a dominance of the methylated oxyarsenates fits well into the general
428 observations for thiolation at +100 to +150 mV as presented in Fig 5.

429 In accordance with previous studies that indicate the potential contribution of DMMTA to
430 rice grain As accumulation,[25, 26] here we provide the first direct evidence that DMMTA
431 occurs in the rhizosphere of paddy soils and is available for root uptake. DMMTA, which
432 derives either from the thiolation of DMA or dethiolation of DMDTA, was identified as the
433 dominant methylated thioarsenate species in the rhizosphere, irrespective of the root

oxygen loss ability of rice cultivars (Fig 4c). In contrast to previous hydroponic studies, which reported significant dethiolation of MMMTA to MMA outside rice roots,[25] our rhizobox experiments identified MMMTA as the second-highest thioarsenate species after DMMTA, with only slightly lower concentrations than MMA (Fig 4d). This discrepancy could be explained by a combination of two possible scenarios: 1) high sulfur turnover rate and enhanced sulfate reduction rate in the rice rhizosphere, as revealed by previous sulfate reduction studies in planted paddy soils,[36] can produce a flow of reduced sulfur species and thus may support the thiolation of MMA and DMA; or 2) continuous diffusion of methylated thioarsenates from the surrounding soil with lower redox potential would further contribute to accumulation of the monothiolated forms MMMTA and DMMTA. Despite the potential availability and resistance to root enzymatic transformation,[25] MMMTA has not been detected in rice grains so far, most possibly due to the sequestration in root cell vacuoles by phytochelatin complexation comparable to what has been reported for MMA before.[44]

Environmental Implications. New insights into As speciation are essential to understand the geochemical behavior of As and to assess the risk associated with As contamination in rice paddies and the potential contribution to grain As concentrations. The substantial contribution of (methylated) thiolated species to total As concentrations shown in the present study raises questions as to why thiolated As species have not been to date reported more widely, and what the lack of their consideration might mean for As risk assessment.

Routinely, samples for species-selective analysis are stabilized with acid, which may result in decrease due to transformation or complete loss of higher order thiolated species (e.g., DTA, TTA, MMDTA, and DMDTA).[22, 45] Additionally, if thioarsenates are not expected, all

sample handling likely is done under oxic conditions, which will lead to transformation to oxyarsenic species. Further, chromatographic separation is commonly done with PRP columns at slightly acidic pH where higher order thiolated arsenates transform and DMMTA does not elute.[26] And, of course, standards are required for species identification by retention time. Filtration and flash-freezing for sample stabilization, sample handling under anoxic conditions, and chromatographic separation on an AS16 column at pH 13, as done in the present study, are recommended for an accurate As speciation information in flooded paddy soils.

Not considering thioarsenates in paddy soil pore-water will lead to an inaccurate estimation of As mobility in soil and neglecting their contribution to As uptake in rice plants will lead to a wrong risk assessment of As exposure from rice grains. The evidence we provide to support the occurrence of DMMTA, formed either from thiolation of DMA or dethiolation of DMDTA, is critical as it is one of most carcinogenic As species.[46, 47] DMMTA has previously been shown to be taken up by rice plants, with both higher root uptake and higher translocation efficiency compared to DMA [25] and it has been detected in rice grains before.[26] However, routine hot acid digestion of rice grains leads to transformation of DMMTA to DMA and therefore underestimation of a highly toxic species which is not regulated by the current As rice grain standards.[25] Based on the observed spontaneous thiolation of methylated oxyarsenates, it is to be expected that agronomic practices that have a potential to increase As methylation, such as the soil incorporation of crop residues [8] and sulfate-based fertilization,[48] may also increase the exposure of rice plants to DMMTA.

Furthermore, results from the present study also provide important insights for As contamination risk assessments in surface or ground waters of analogous systems under periodic redox fluctuations, such as floodplains,[49] wetlands[50] and peatlands.[51] The

formation of highly toxic, oxidation-resistant DMMTA at oxic/anoxic interfaces and its environmental fate needs to be clarified in future studies.

ASSOCIATED CONTENT

Supporting Information

Summary of experimental designs (table and sketches), reduction batch incubations (control: pH, E_H, total Fe, Sulfide, As speciation; control & sulfate spike: sulfate/thiosulfate), reduction-reoxidation batch incubations (As speciation in percentages for Veronica), flood-drain-reflood microcosms (pH, E_H, total Fe for Veronica and Fornazzo; As speciation for Veronica), percentage of total thiolation, mono-, di-, and trithiolation of inorganic oxyAs, MMA, and DMA in the rhizobox experiment.

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Notes

The authors declare no competing financial interest.

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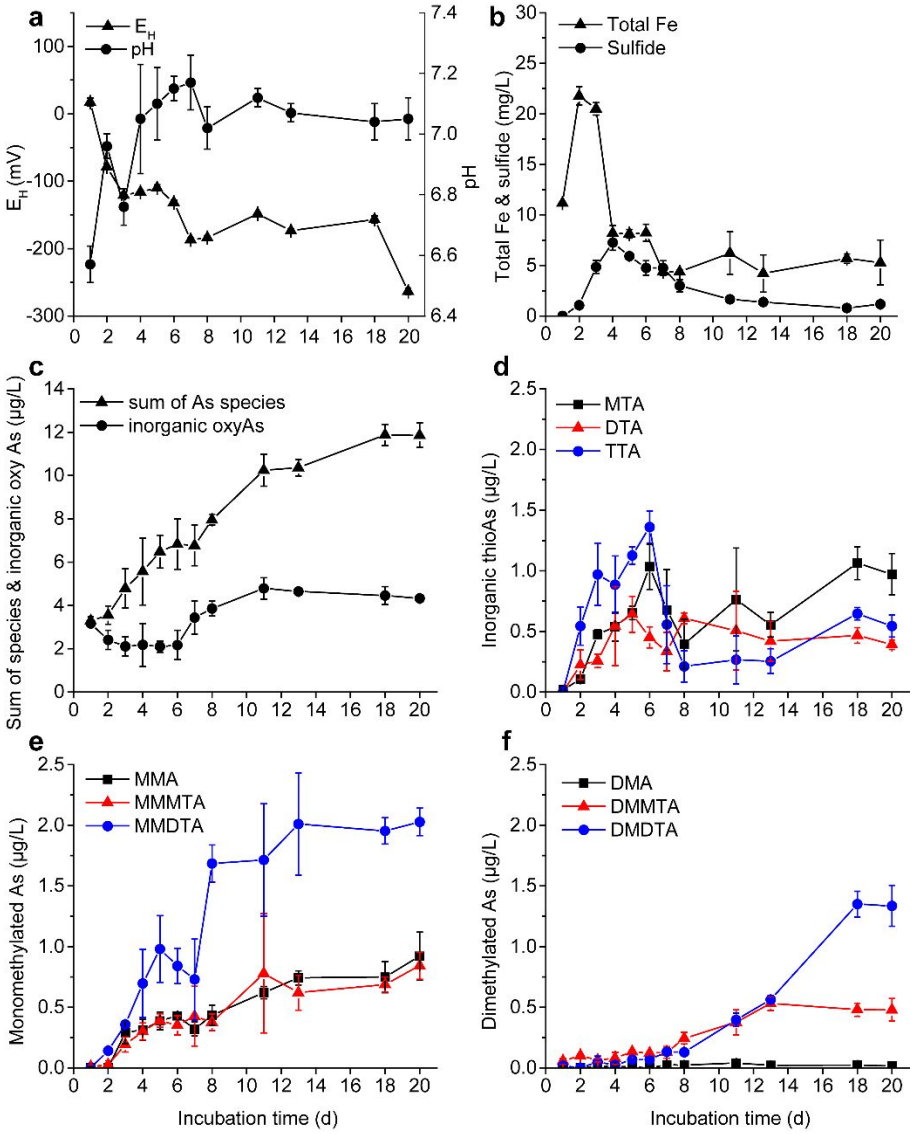

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665 **Figure 1.** Pore-water chemistry (a, b) and As speciation (c, d, e, f) dynamics in reduction batch
666 incubations using Veronica soil (spiked with 1 mmol L^{-1} sulfate). Bars represent standard errors
667 (n = 3). For data of control experiment without sulfate spike see Figure S3.

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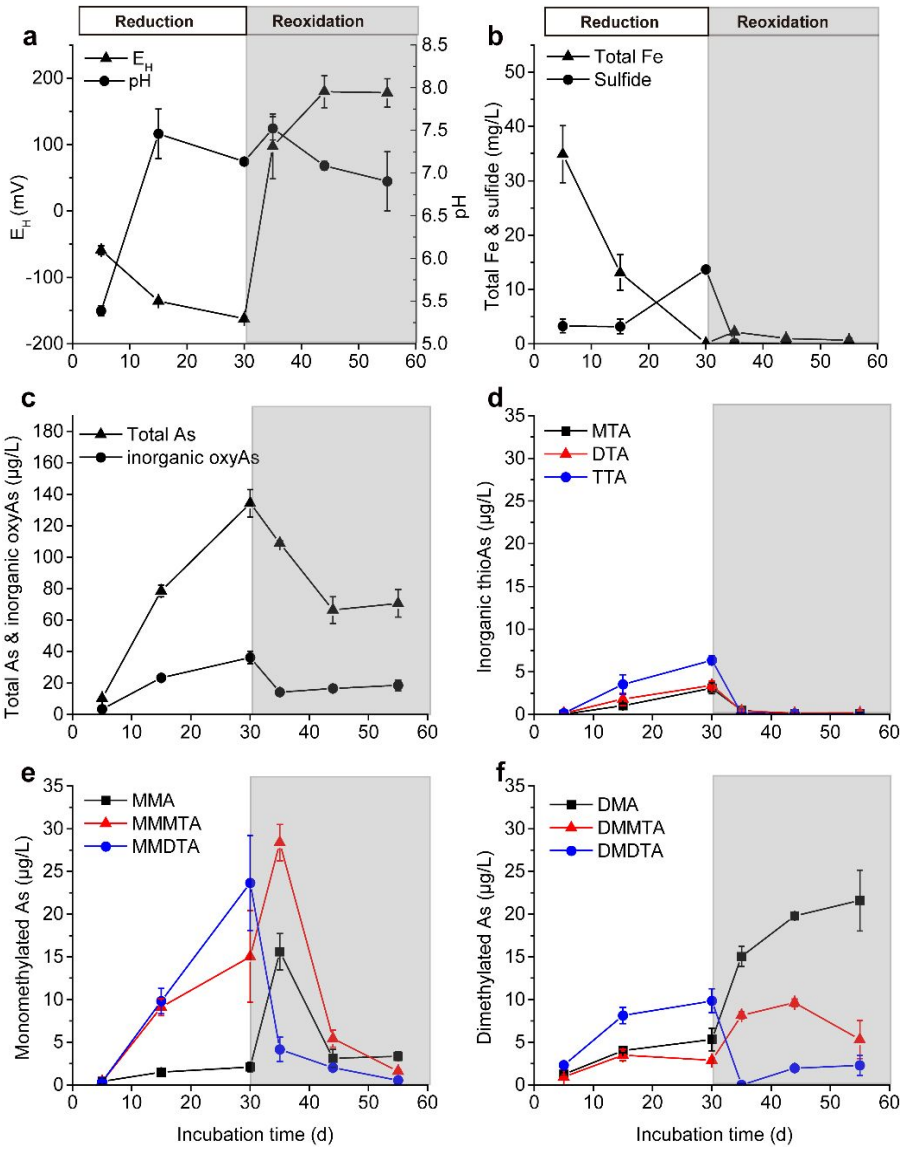

Figure 2. Pore-water chemistry (a, b) and As speciation (c, d, e, f) dynamics in reduction-reoxidation batch incubations using Veronica soil (spiked with 9 mmol L^{-1} sulfate, 100 µg L^{-1} arsenate). Bars represent standard errors (n = 3).

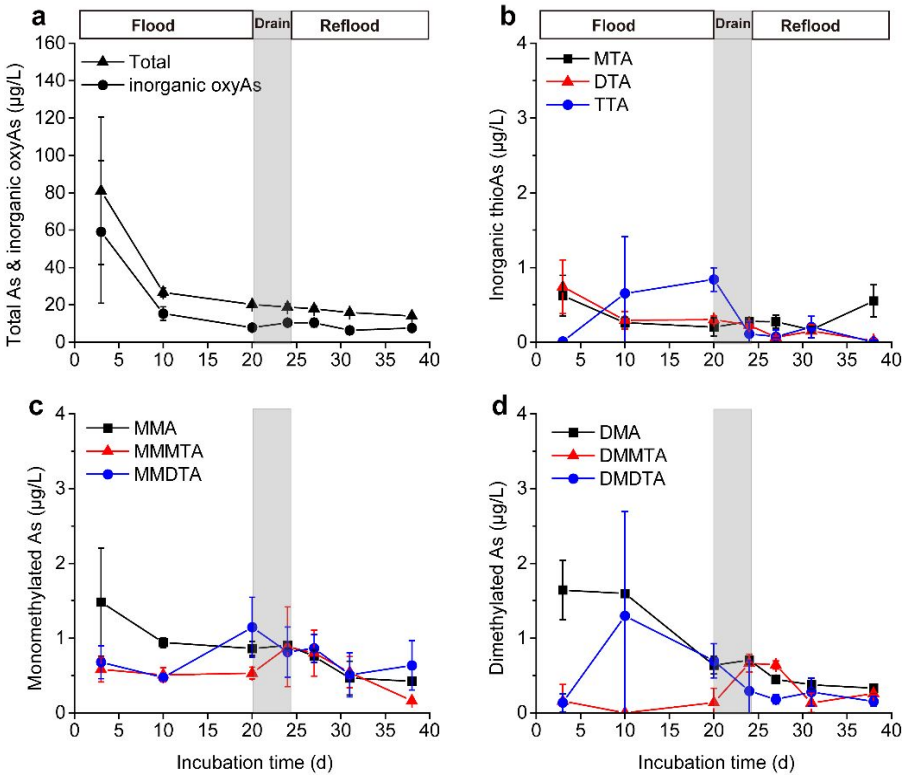

Figure 3. Arsenic speciation dynamics in flood-drain-reflood microcosm incubations using Fornazzo soil. Bars represent standard errors (n = 3). For arsenic speciation dynamics in microcosm incubations using Veronica soil see Figure S6.

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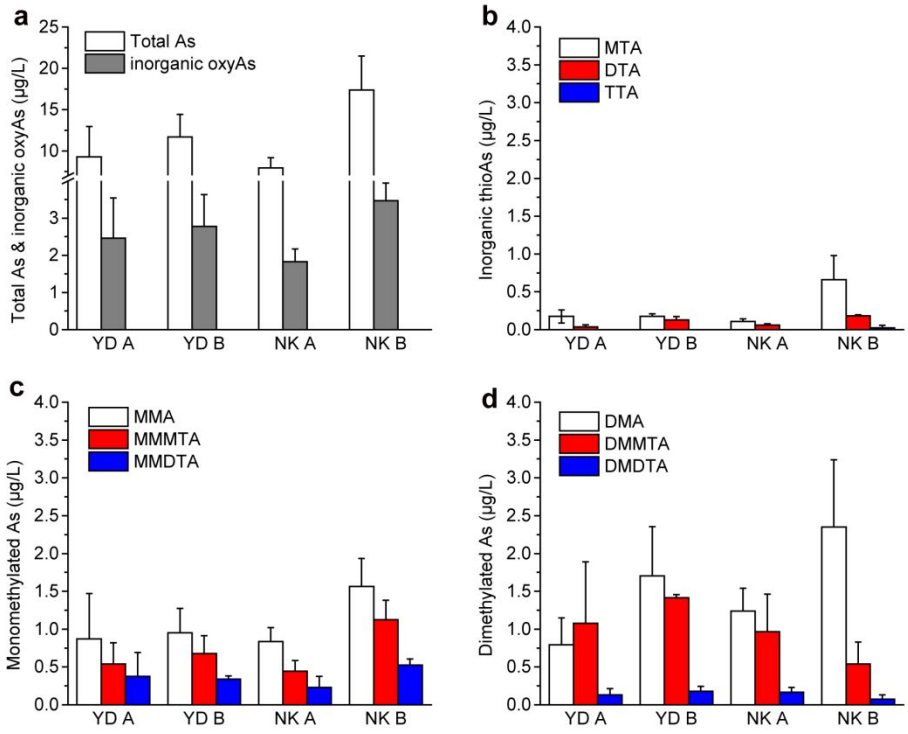

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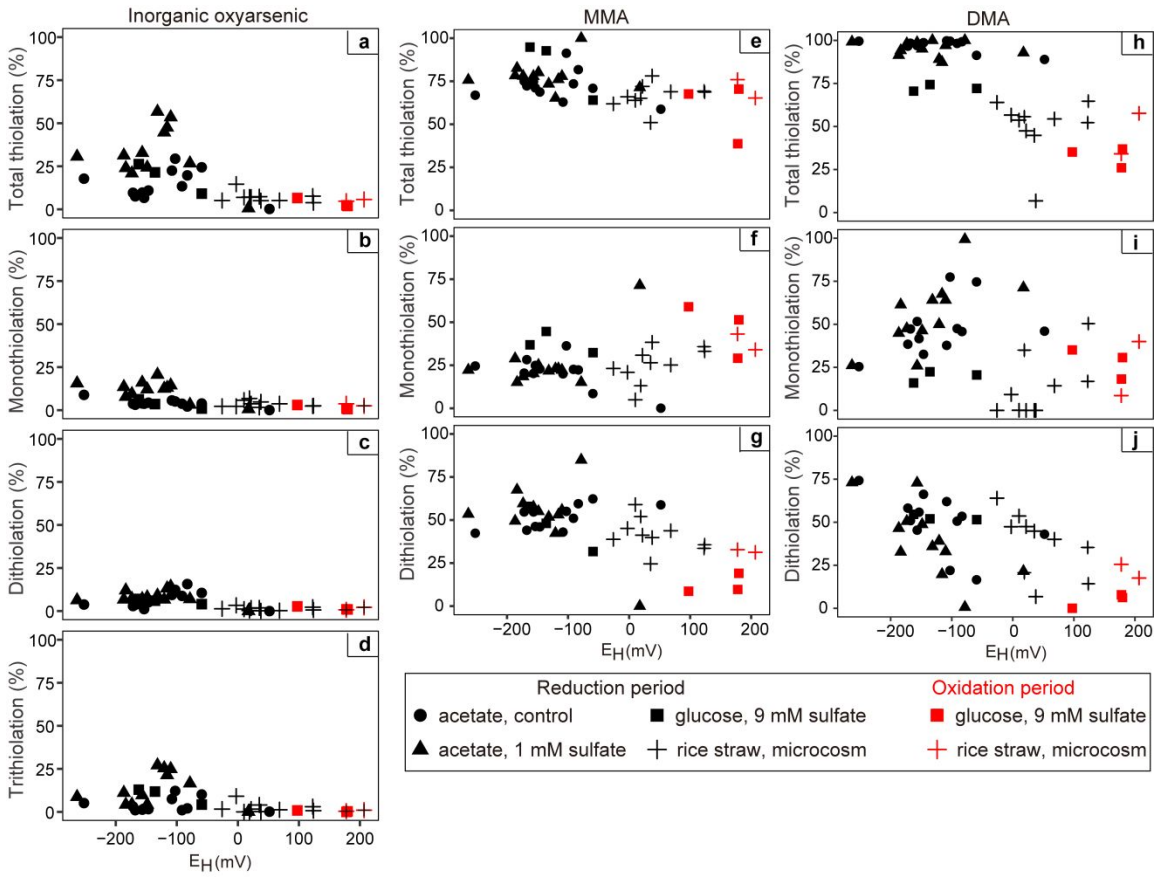
682 **Figure 4.** Arsenic speciation in paddy soil pore water of planted rhizobox experiments using
683 Fornazzo soil; YD A: rice variety Yangdao, sampled at depth A (9 cm below soil-water interface);
684 YD B:Yangdao, sampled at depth B (16.5 cm below soil-water interface); NK A: rice variety
685 Nongken, sampled at depth A; NK B: Nongken, sampled at depth B. Bars represent standard
686 errors (n = 3).



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689 **Figure 5.** Percentage of total thiolation (a, e, h), mono- (b, f, i), di- (c, g, j), and trithiolation (d) of
690 inorganic oxyarsenic species (a-d), monomethylated (e-g) and dimethylated (h-j) arsenates as a
691 function of pore-water redox potential (E_H); (calculation example: DMA dithiolation = DMDTA) /
692 (DMA+DMMTA+DMDTA)); points represent mean values of each sampling time point (n = 3;
693 standard deviation not presented for clarity of figure); data were compiled from reduction batch
694 incubations with acetate (Veronica soil), reduction-reoxidation batch incubations with glucose
695 (Veronica soil), and flood-drain-reflood microcosm incubations with rice straw (Veronica and
696 Fornazzo soil);

697