# Changes in arsenic mobility and speciation across a 2000-year-old paddy soil chronosequence

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## Highlights:

- Pedologic changes with long-term paddy use alter redox chemistry after flooding
- Arsenic (As) mobility decreases with increasing paddy soil age
- Increasing microbial activity and organic C promote As methylation in older paddies
- Long-term paddy use limits As thiolation due to high dissolved Fe and organic C
- Methylthioarsenates formed in all soils across the studied chronosequence

## Abstract

Rice accumulates arsenic (As) when cultivated under flooded conditions in paddy soils threatening rice yield or its safety for human consumption, depending on As speciation. During long-term paddy use, repeated redox cycles systematically alter soil biogeochemistry and microbiology. In the present study, incubation experiments from a 2000-year-old paddy soil chronosequence revealed that As mobilization and speciation also change with paddy soil age. Younger paddies (≤100 years) showed the highest total As mobilization, with speciation dominated by carcinogenic inorganic oxyarsenic species and highly mobile inorganic thioarsenates. Inorganic thioarsenates formed by a high availability of reduced sulfur (S) due to low concentrations of reducible iron (Fe) and soil organic carbon (SOC). Long-term paddy use (>100 years) resulted in higher microbial activity and SOC, increasing the share of phytotoxic methylated As. Methylated oxyarsenic species are precursors for cytotoxic methylated thioarsenates. Methylated thioarsenates formed in soils of all ages being limited either by the availability of methylated As in young soils or that of reduced-S in older ones. The present study shows that via a linkage of As to the biogeochemistry of Fe, S, and C, paddy soil age can influence the kind and the extent of threat that As poses for rice cultivation.


## 1. Introduction

Rice is traditionally cultivated under flooded conditions which cause porewater oxygen-depletion by microbial respiration, directly affecting the biogeochemistry and microbiology of paddy soils[1]. Oxygen depletion triggers arsenic (As) mobilization through reductive dissolution of As-bearing iron (Fe) minerals, mainly as the inorganic species arsenate[2] (Figure 1), or through direct arsenate reduction to arsenite on mineral surfaces[3]. Both

arsenate and arsenite are class I carcinogens[4], and especially the latter can be taken up by rice plants and accumulate in rice grains, making rice consumption one of the main dietary sources of As[3,5-7]. Although arsenite and arsenate typically dominate porewater speciation in paddy soils, arsenite can also be biotically transformed into the oxymethylated As species monomethylarsenate (MMA) and dimethylarsenate (DMA). Arsenic can be methylated in paddy soils by microbes with an active expression of arsenite S-adenosylmethionine methyltransferase genes (a*rsM*)[8,9], likely as detoxification mechanism or as antibiotic reaction against other microbial groups[10-12]. Chen, et al. [13] suggested that sulfate-reducing bacteria (SRB) contribute to As methylation and methylotrophic methanogens to As de-methylation, while other authors found no contribution of SRB, but fermenting bacteria to methylate As[14]. The main reasons for As methylation and the communities ruling (de)methylation in paddy soils thus remain controversial. Dimethylarsenate has a strong phytotoxic effect on rice plants and has been associated to causing straight-head disease[15] and, due to its high root-grain translocation, DMA can dominate As speciation in rice grains[16]. However, DMA shows lower toxicity for humans than inorganic As and is thus not regulated in rice products[15,17,18]. Since As speciation determines its mobility, uptake, translocation, and accumulation in rice plants as well as its toxicity, great efforts have been made to understand the dynamics of these four major oxyarsenic species (OxyAs) in paddy soil porewater, their association to soil minerals, rice plants, and rice grains[3,5,7,19].

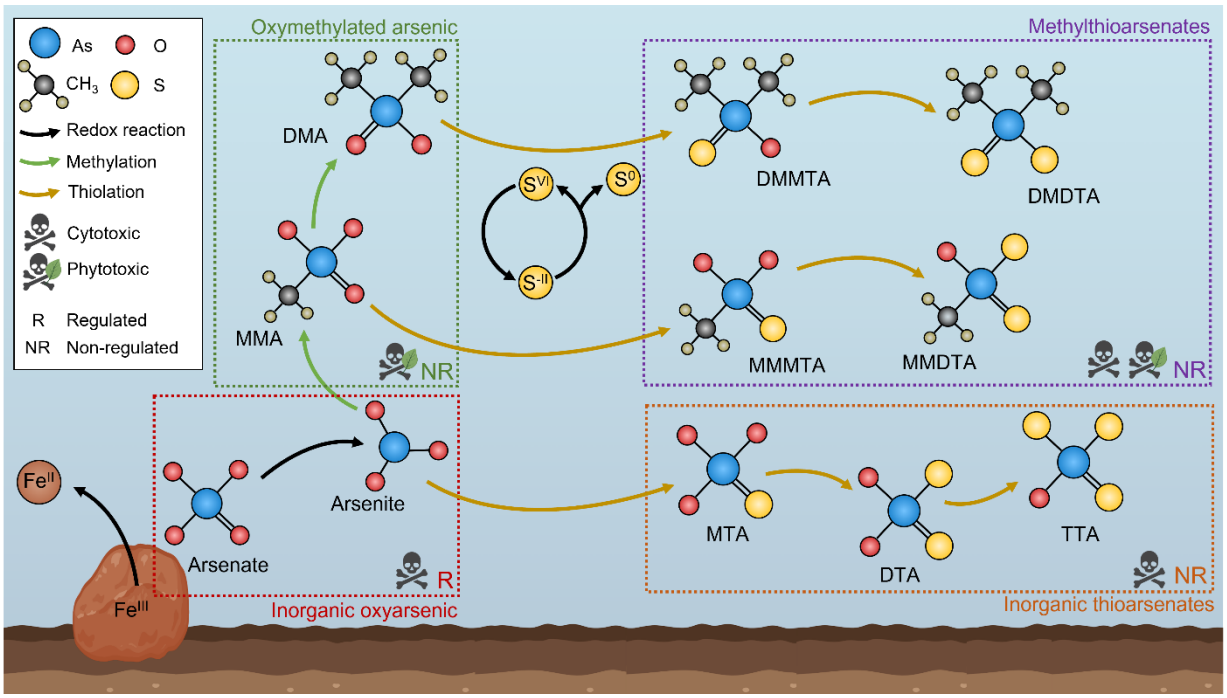



**Figure 1: Formation of the As species discussed in the present study, through reduction, methylation, and thiolation**; they are grouped into the four categories discussed here: Inorganic oxyarsenic species, inorganic thioarsenates, oxymethylated arsenic species, and methylthioarsenates. R: regulated under food guidelines, NR: non-regulated. For simplicity of the depiction, charges, oxidation states, and possible protonation of O have been omitted.

In the presence of reduced sulfur (e.g., through dissimilatory sulfate reduction, DSR, carried out by SRB)[20], both inorganic and oxymethylated As species can be abiotically thiolated to form inorganic and methylated thioarsenates, respectively. Inorganic thiolation is favored in systems with neutral to alkaline pH where arsenite reacts with $S^{-II}$ or $S^0$ in consecutive steps to form mono-, di-, or trithioarsenate (MTA, DTA, and TTA, respectively), depending on the S/As ratio[21]. Methylthiolation requires the oxymethylarsenates, MMA or DMA, as precursors. Consecutive nucleophilic reactions with $S^{-II}$ in acidic to neutral pH lead to formation of mono- or dithiolated species

(monomethylmonothioarsenate, MMMTA; monomethyldithioarsenate, MMDTA; dimethylmonothioarsenate, DMMTA; and dimethyldithioarsenate, DMDTA)[22,23]. Thioarsenates have recently been identified in paddy soils[24], rice plants[25], and rice products[26] since traditional sampling and analytical methods mask them as their respective oxyarsenic analogues[18]. Especially dimethylated thioarsenates represent a hidden risk because their toxicity is similar to or even higher than that of arsenite for rice plants and mammalian cells[18,27-29].

Besides seasonal effects on As speciation through decreases in the redox potential, chronosequence studies have confirmed that repeated flooding and draining of paddy soils triggers a characteristic and unique long-term soil development[30]. The duration of use as paddy soils (paddy soil “age”) leads to continuous changes in pedogenic properties[30,31], including accumulation of organic carbon in the topsoil, increased formation of amorphous Fe phases, decreases in soil pH, and adaptation of microbial communities with increased activity[32-36]. We hypothesize that these long-term biogeochemical changes also affect As mobilization and speciation and thus, paddy soil age can influence the kind and the extent of threat that As poses for rice cultivation.

For the present study, four topsoils of an intensively studied 2000-year-old chronosequence representing paddy soils (P) of 50, 100, 300, and 2000 years of paddy use were selected. The use of a chronosequence with a limited number of samples could carry constraints regarding representativity and potential additional effects aside from age which cannot be fully excluded, especially with a chronosequence which spans 2000 years. However, based on the results of numerous previous studies[30,33-35,37], we consider our selected soils as representative for changes that are caused primarily by typical paddy

soil aging. The selected soils wee incubated and time-resolved sampling after 1, 3, 5, 7, 10, and 35 days helped unravel changes in abiotic and biotic reaction kinetics of different redox-sensitive elements and their influence on the overall As availability and speciation in each soil and translate this to characteristic age-dependent biogeochemical trends.

## 2. Materials and Methods

### 2.1 Short description of the chronosequence

The soils used for this study come from the coastal paddy cultivation area around Cixi in the province of Zhejiang, China, located in the delta of the Yangtze River (30°10′N, 121°14′E). There, land reclamation has taken place since approximately 2000 years by river sediment deposition and by the building of dikes, allowing the transformation of coastal marshlands to agricultural fields. The construction records of these structures allow the estimation of the age of arable land and paddy soils. Details on the sampling campaign carried out in 2008 are given by Cheng, et al. [33] and Kölbl, et al. [30]. In this study, we used the ≤2 mm soil fraction of the Alp horizon of the main sites of the paddy soils (P) with 50, 100, 300, and 2000 years of rice cultivation. The soils were air-dried and stored in the dark until use for the present study. Dry storage might raise concerns regarding loss of sample representativity and potential of re-activating representative microbial communities upon incubation. However, longer dry periods are part of the characteristic conditions in paddy fields and previous research has shown that intermittent flooding of paddy soils makes their microbial communities particularly resistant to long periods of desiccation[38]. Similarly, although changes in the oxidation state of organic compounds might have occurred during storage, the same processes that allow the accumulation of

SOC with long-term paddy use[34,37] likely protect the carbon pool in the stored samples from excessive degradation.

### 2.2 Incubation experiments

Anaerobic incubations were carried out using the above-described soils. In triplicate, 10 g of soil and 20 mL of $N_2$-purged tap water were added to 120 mL serum vials inside an anaerobic chamber (95% $N_2$, 5% $H_2$, COY). The vials were sealed with butyl rubber stoppers and aluminum caps, mixed manually, and left standing in an incubator at 30 °C until sampling. Sacrificial triplicates were taken at 1, 3, 5, 7, 10, and 35 days of incubation. Similar time spans have been used before to study As speciation dynamics[13,39].

For sampling, the corresponding vials were taken out of the incubator, shaken, and allowed to reach room temperature before measuring the gas pressure inside the vial using a handheld pressure-meter (Greisinger GMH, 3100 Series). With a gas-tight syringe and a needle, 5 mL of headspace was taken from each vial, injected into an evacuated 5 mL glass vial (approx. -500 mbar), and stored in the dark until quantification of $CH_4$ and $CO_2$ via gas chromatography coupled to a Flame Ionization Detector (GC-FID, SRI Instruments 8610C) equipped with a methanizer.

After gas phase sampling, the vials were taken into the anaerobic chamber, shaken, and opened. The homogeneous mix of soil and aqueous phase was transferred to 50 mL centrifuge tubes. The tubes were closed and wrapped with parafilm before being centrifuged outside the glovebox (4000 rpm, 10 min) and brought back into the anaerobic chamber. The aqueous phase was separated from the soil using syringe and needle, filtered, and accordingly stabilized to carry out the analyses described in the following

159 section. Unless stated otherwise, all aqueous phase samples were filtered through 0.2 µm
160 acetate-cellulose syringe filters (Macherey-Nagel).

161 **2.3 Aqueous phase analyses**

162 To determine the total concentration of As and S in the aqueous phase, a 2 mL aliquot
163 was stabilized with 30 µL of 9.8 M $H_2O_2$ and 40 µL of 8 M $HNO_3$, diluted, and measured
164 by inductively coupled plasma mass spectrometry (ICP-MS, XSeries2, Thermo-Fisher)
165 after in-cell reaction with an $O_2$/He mixture (10/90%). Arsenic was determined as $AsO^+$
166 (m/z = 91) and S as $SO^+$ (m/z =48). A reference material (TMDA 62.2, Environment
167 Canada) was used for quality control, and rhodium (Rh) was used as internal standard to
168 correct signal drift.

169 Photometric determinations of Fe ($Fe_{tot}$, $Fe^{II}$) and $S^{-II}$ were carried out using the ferrozine
170 method[40,41] and the methylene blue method[42], respectively. All samples for photometric
171 determinations were diluted when required, carried out in triplicate, and measured using
172 a multiplate reader (Infinite 200 PRO, TECAN).

173 Immediately after knowing the $Fe_{tot}$ concentration, 700 µL aliquots were taken for As
174 speciation and stabilized with 100 µL of a 10 mM solution of the iron complexing agent
175 HBED (N,N'-Di(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid monohydrochloride)
176 in 10% ethanol at neutral pH (adjusted using 0.1 M NaOH). In case of Fe concentrations
177 > 1 mM, a 55 mM HBED solution was used for stabilization in the same ratio. Further
178 details on the HBED stabilization method can be found in the Supporting Information (see
179 supplementary discussion). The samples were flash-frozen on dry ice and kept at -20 °C
180 until analysis by ion chromatography (IC, Dionex ICS-200, with an AG/AS16 IonPac
181 column), using a 0.1 M NaOH gradient with a flow rate of 1.2 mL $min^{-1}$ coupled to

182 inductively coupled plasma mass spectrometry (IC-ICP-MS) [43]. Further details regarding
183 the As speciation method can be found in the Supporting Information (see supplementary
184 methods).

185 Dissolved organic carbon (DOC) was determined from a 2 mL aliquot filtered through a
186 0.45 µm polyamide syringe filter (Macherey-Nagel), stabilized with 40 µL of 6 M HCl and
187 kept at 4 °C until analysis (Multi N/C 2100 S, Analytik Jena). An additional 2 mL aliquot
188 for acetate determination was taken and kept frozen until analysis by HPLC-RID (Agilent
189 1200) with a Rezex ROA Organic Acid column[44]. Acetate analyses were carried out for
190 incubation days 1, 3, 5, and 10.

191 Redox potential and pH were measured using a multiparameter (HQ40d, Hach) with the
192 corresponding electrodes (PHC301, Ag/AgCl electrode, and MTC101, respectively).
193 Electrical conductivity was measured with a conductivity meter (Winlab Data Line,
194 Windaus) attached to an LCV 0.35/21 electrode (Meinsberger Elektroden).

195 **2.4 Solid phase analyses**

196 Total content of As and S in the original dry soil were determined by ICP-MS after
197 microwave-assisted digestion (MARS Xpress, CEM) in 10 mL aqua regia. The digested
198 samples were filtered and diluted 1:10 with ultrapure deionized water (Millipore,
199 18.2 MΩ cm) before analysis. Iron (III) phases were also characterized in the dry soils
200 before the incubations using the dithionite-citrate-bicarbonate (DCB)[45] and oxalate buffer
201 methods[46]. Iron determination after each extraction was carried out photometrically as
202 described above for aqueous phase Fe.

203 Triplicate soil samples of each incubation at 1, 3, 5, and 10 days were separated into
204 cryovials and flash-frozen in liquid nitrogen for microbial analyses. DNA and RNA were

extracted following the protocol described by Lueders, et al. [47] (described in detail in supplementary methods). The extracts were stored at -80 °C until analysis. Specific qPCR standards for each gene of interest were created from paddy soil using the In-Fusion® Snap Assembly cloning system (TaKaRa Bio Inc.) according to the manual and cloned into a pUC19 vector. Total and active bacteria were based on amplification of the respective 16S rRNA gene and transcript copy numbers in nucleic acid extracts. Total and active sulfate-reducing microbes, arsenic methylating microbes, and methanogenic microbes were approximated with respective amplification of the *dsrA* (dissimilatory sulfite reductase), *arsM* (arsenite S-adenosylmethyltransferase) and *mcrA* (methyl coenzyme M reductase) genes and transcripts (supplementary methods). Gene and transcript copies were quantified with qPCR. While 16S rRNA transcripts were quantifiable, *dsrA*, *arsM,* and *mcrA* were below quantification limit. The average copy numbers were calculated from triplicate extractions.

The remaining soil from each Incubation was stored frozen and later freeze-dried for the determination of zerovalent sulfur ($S^0$) via HPLC-UV-Vis[48]. Methodologically, soil-extracted $S^0$ in this study is operationally defined as chloroform-extractable S, which includes zerovalent S in polysulfides and S bound to solid phase, as well as $S_8$.

**2.5 Oxymethylated As-spiked incubations**

To better understand the dynamics of As (de)methylation in the chronosequence, a second batch of non-sacrificial incubations was carried out as described before. The tap water used for these incubations was amended with MMA or DMA in a 10-fold excess of the concentrations of methylated As found in the original setup. This excess was selected to have a concentration of species high enough to discern between small changes in the

As speciation. Thus, P50 and P2000 were amended with 100 µg $L^{-1}$ of MMA or DMA, while concentrations of 250 µg $L^{-1}$ were used for P100 and P300. Samples for As speciation were taken with syringe and needle from each incubation bottle after 5, 10 and 33 days of incubation in the same conditions as the original batch. Speciation stabilization and analyses were carried out as described previously.

### 2.6 Statistical analyses

Mean and standard deviation were calculated for each measured variable. Principal component analyses (PCA) were carried out for the complete dataset. Pearson correlation coefficients were used to assess the significance of all variable correlations presented. All PCA and Pearson correlation analyses were carried out using the Rstudio software (R Development Core Team, 2008).

## 3. Results and discussion

### 3.1 Arsenic content and availability through the chronosequence

Incubation experiments confirmed that paddy soil age does not only cause distinct trends in solid phase biogeochemistry, such as the well documented accumulation of soil organic carbon (SOC)[30,35] or a decrease in Fe content[30] (Figure 2a), but also in aqueous phase chemistry (Figure 2b), with general trends developing slowly during the first 300 years of paddy development but with parameters being highly distinctive from those of P2000. Principal component analyses showed that after flooding, dissolved Fe, $Fe^{II}$, and dissolved organic carbon (DOC) concentrations were the parameters most closely associated to paddy soil age (Figure SI1). Flooding triggered reductive $Fe^{III}$ mineral dissolution, releasing $Fe^{II}$ and associated SOC[37] into the aqueous phase (Figure SI2). While SOC

accumulation (due to fertilization with animal manure, and dry or charred crop residues[30]) directly translated to an increase in DOC (Figure 2b), solid-phase Fe depletion was outcompeted by the accumulation of amorphous Fe phases (Table SI2) which are easily dissolved[30,49], causing higher dissolved Fe concentrations with increasing age. These close dynamics between Fe and DOC are likely related to the stabilization of SOM due to mineral associations with amorphous Fe phases[30,34,37].

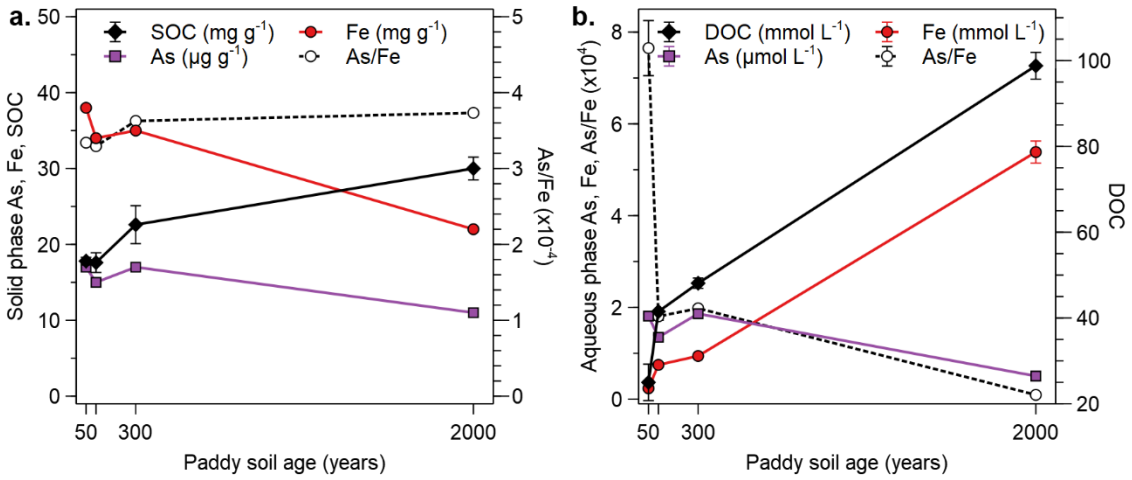



**Figure 2: Differences in solid and aqueous phase As, Fe and C in the chronosequence.** Soil content (a) and aqueous phase after 7 days of flooding (time selected to reflect peak of Fe release) (b). Data for SOC and soil Fe content are taken from Kölbl, et al. [30] for a main site and two subsites (mean values ± standard error). For DOC, As, and aqueous Fe data points represent mean values, while error bars represent the standard deviation (n=3).

Arsenic concentrations in the aqueous phase decreased with age, particularly in P2000, similar to As concentrations in the solid phase (Figure 2a and Table SI2). Previous studies on the chronosequence have shown a general mobilization of elements towards deeper soil layers or washout with soil drainage each cultivation season[30]. The higher availability of reducible Fe with increasing age caused a strong decrease of the As/Fe ratio in the

aqueous phase with paddy soil age (compared to the relatively stable values from the solid phase), meaning that more moles of As are released per mole of Fe in younger soils. Arsenic release was coupled to the dissolution of $Fe^{III}$ mineral phases, with concentrations increasing over the first 10 incubation days and decreasing after 35 days (Figure SI2), likely because of sorption to newly formed FeS phases[50,51].

In the equilibrated aqueous phase (after 35 days), As concentrations decreased with age (Figure SI2). This could be related to As in older paddies being associated with remaining Fe crystalline phases which are not so readily accessible for reductive dissolution. In this sense, although As concentrations in the solid phase showed only a relatively small overall decrease with increasing age, As mobilization decreased with increasing paddy soil age.

### 3.2 Highly toxic and mobile inorganic As dominates early stages of paddy development

The high As mobilization in younger paddies is a source of concern since As can become bioavailable for plant uptake and eventually accumulate in rice grains. For all soils and incubation times, arsenic speciation was dominated by inorganic species. Their contribution to the sum of species largely varied between 91% (P50, day 5) and 48% (P2000, day 7), overall being higher in younger paddies (Figure SI3). Inorganic oxyAs, and especially arsenate had a higher proportion, but arsenite was found in all soils at all incubation times, together with up to 9% of inorganic thioarsenates (P50, day 3). Using day 35 as a representative equilibrated system, inorganic As contribution decreased strongly with age (Figure 3a), while the contribution of inorganic thioarsenates additionally decreased with incubation time (Figure SI4).

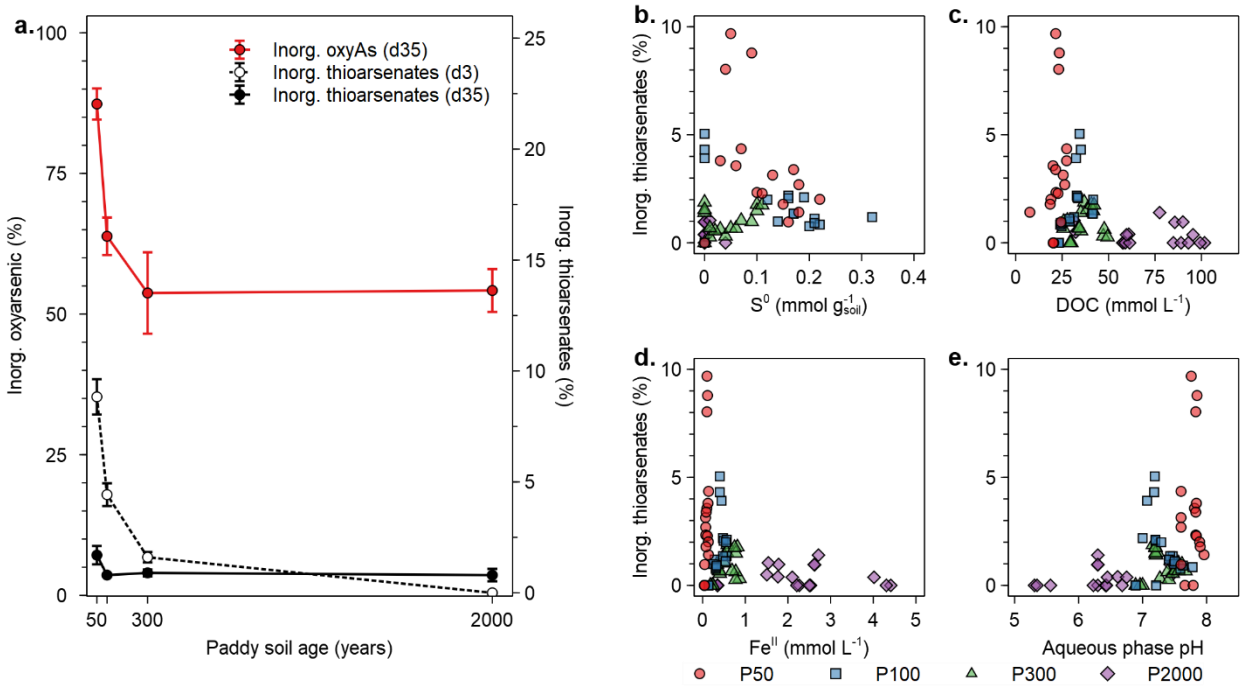



**Figure 3: Effect of paddy soil evolution on inorganic As species.** Decrease of inorganic As species with increasing age (a) and effects of different biogeochemical factors on inorganic thioarsenate formation: Accumulation of $S^0$ in the soil (b), DOC (c), $Fe^{II}$ (d), and pH (e). Data points on (a) represent mean values while error bars represent the standard deviation (n=3).

While the accumulation of inorganic oxyAs was mostly caused by the absence of biotic methylation (the second most dominant group of As species, see section 3.3), the formation of inorganic thioarsenates was regulated by geochemical factors in the aqueous phase that are strongly affected by paddy soil age.

Inorganic thioarsenate formation requires reduced sulfur, with dissimilatory sulfate reduction being its main source in paddy soil porewater[52,53]. Free $S^{-II}$ was not detected in the aqueous phase of any incubation setup, but total S concentration decreased with incubation time (Figure SI5), indicating precipitation of sulfide phases. Although observed in paddy soils of all ages, the start of DSR showed a delay with increasing age. In younger soils, it started as early as day 3 (P50), and in the older ones, as late as day 5 (P2000).

This delay is likely due to the higher availability of reducible $Fe^{III}$ (as seen in Figure SI2) in older paddies, since $Fe^{III}$ reduction has a higher energy yield and is thermodynamically preferred to S reduction[54]. Inorganic thioarsenates were detected in P2000 after day 5, coinciding with the start of DSR in this soil (Figure SI4).

Sulfate reduction was also assessed as the accumulation of $S^0$ in the soils after incubation, with younger paddies showing higher accumulation compared to older paddies (Figure SI6). Although $S^0$ has been previously reported as the best proxy for the formation of thioarsenates in paddy soil porewater[24], no direct correlation between its accumulation and inorganic thioarsenate formation was found (Figure 3b). Our results suggest that the higher availability of SOC and reduced Fe in the aqueous phase with increasing paddy soil age scavenged reduced sulfur for thiolation of organic matter[55] or precipitation of FeS phases[50], decreasing its availability for As thiolation. The quick and early formation of inorganic thioarsenates could be related to a peak in the availability of reduced sulfur during a phase of strong DSR before it was scavenged by the major release of $Fe^{II}$ into the aqueous phase taking place by day 5 or 7, depending on the soil, or due to the absence of more favorable substrates for thiolation (see section 3.3).

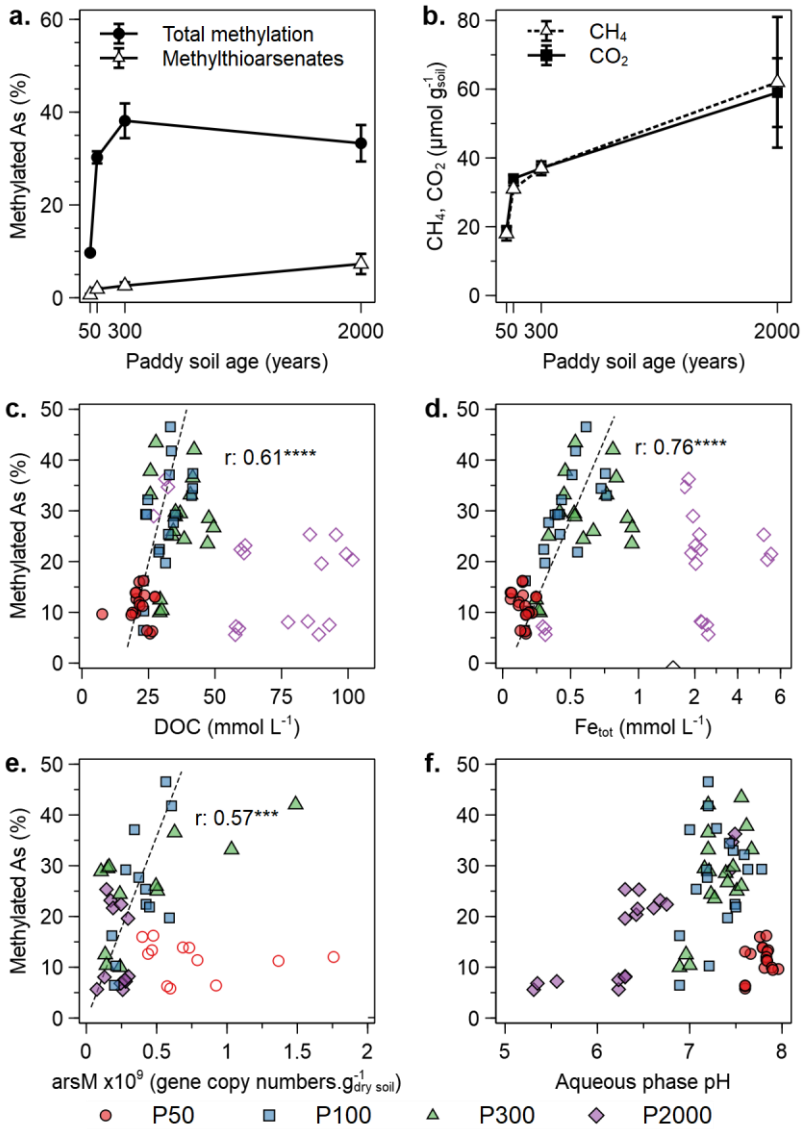
Supporting these observations, contributions to the sum of species >2% of inorganic thioarsenates were only observed under incubation conditions that had DOC concentrations < 50 mmol $L^{-1}$ and dissolved $Fe^{II}$ concentrations < 1 mmol $L^{-1}$ (Figure 3c and d, respectively). Our PCA (Figure SI1) also showed how formation of inorganic thioarsenates was negatively correlated to paddy soil age, DOC, and dissolved Fe. Another environmental factor that has been reported to strongly control inorganic thiolation is pH with more alkaline pH causing higher inorganic thiolation[20]. Contributions

>2% of inorganic thioarsenates were observed for aqueous phase pH values above 7 (Figure 3e). The accumulation of organic carbon, as well as the washout of carbonates with increasing soil age, generally decrease soil and porewater pH under paddy management[30], allowing inorganic As thiolation in younger paddies with more circumneutral pH values.

Our results suggest that the high availability of inorganic As species (including thioarsenates) in young paddy soils could represent a higher risk for the consumption of rice products coming from fields that have been under paddy use for 100 years or less. Additionally, paddy soil evolution hampers the formation of inorganic thioarsenates, which form preferentially in young paddies with lower dissolved Fe and SOC contents.

### 3.3 Higher As methylation in mature paddies

Arsenic methylation showed a sharp increase in the first 300 years of paddy evolution ( Figure 4a). After 35 days of incubation, the contribution of methylated As (which includes oxymethylated species and their thiolated analogs) to the sum of species increased from 9.6 ± 0.3 % in P50 to 38 ± 4 % in P300. These values represent the net effect of formation and consumption (i.e., de-methylation) carried out simultaneously by different microbial communities which drive contribution of methylated species to the aqueous phase speciation (Figure SI7).

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348 **Figure 4: Effect of paddy soil evolution on As methylation.** Contribution of methylated As
349 species (including MMA, DMA, MMMTA, MMDTA, DMMTA, and DMDTA) to the sum of species
350 in different soils after 35 days of incubation (a). Increased $CO_2$ and $CH_4$ release with paddy soil
351 age by day 35 of incubation (b). Data points on (a) and (b) represent mean values while error
352 bars represent the standard deviation (n=3). Parameters that are related to As methylation: DOC
353 (c), Fe (d), *arsM* gene copy numbers (e), and aqueous phase pH (f). Empty symbols indicate data
354 not taken for Pearson correlation in the case of P2000 (c, d) due to an unidentified limitation in
355 methylation and for P50 (f) because of low methylation potential. Asterisks indicate the Pearson

significance levels (p): *** = $p < 0.001$; **** = $p < 0.0001$. For (c), (d) and (f), n = 54, while for (e), n = 36.

The increase of the methylation potential with paddy soil age can be explained together with other biogeochemical parameters that showed similar trends. The general potential for microbial activity and respiration in the paddy soils strongly increased during the first 300 years (Figure SI8a and b), likely due to the accumulation of bioavailable organic substrates and, as reported before for the chronosequence, increases in the microbial biomass, alongside the establishment of specific microbial communities[30,56-58]. Additionally, DOC release and efficient microbial consumption caused increased $CO_2$ and $CH_4$ production with soil age (

Figure **4**b). On all sampling days, older soils showed a higher $CO_2$ production than younger ones (Figure SI8b). In the case of $CH_4$, its production was strongly delayed in older soils. While younger soils showed between 0.08 and 0.29% of their final $CH_4$ production after 3 days of incubation, P2000 produced only around 0.005% of its final value, being the lowest $CH_4$ producer up to day 7 (Figure SI8c). This delayed $CH_4$ production was again likely related to the high amount of reducible $Fe^{III}$ in the system with increasing age, which has been reported to suppress methanogenesis[59], possible competition with other microbial groups[60], or due to the low energy yield of methanogenesis[54]. This delay is also represented by a slower decrease of the redox potential in P2000 when compared to younger soils (Figure SI9). Nonetheless, the high accumulation of organic C and likely a well-adapted methanogenic community[38] made P2000 the highest $CH_4$ producer after 35 days of incubation.

Arsenic methylation in P50, P100, and P300 showed positive Pearson correlations with DOC ($r=0.61$, $p<0.0001$) and aqueous phase Fe ($r=0.76$, $p<0.0001$) (

Figure **4**c and d, respectively), suggesting a higher abundance of organic substrates for microbial activity from the reductive dissolution of amorphous Fe phases. A similar correlation of high methylation and DOC has been reported before[61], and other studies have seen an increase in As methylation in organic matter-amended treatments[62,63]. The oldest paddy soil, P2000, showed lower methylation values than expected if assuming a continuously increasing trend from P50 over P100 and P300. The aforementioned three soils had aqueous phase As concentrations of around 2 µmol $L^{-1}$, compared to 0.5 µmol $L^{-1}$ in P2000 (Figure SI2). P2000 also showed generally lower arsM copy numbers than the other soils, especially when normalized to 16s rRNA (Figure SI10 a and b), suggesting that microbes in this soil are less prone to methylation. This lower methylation potential could be related to several factors. On the one side, the lower As concentrations in porewater when compared to younger paddies could mean that detoxification mechanisms are not needed in P2000 as much as they would be in younger paddies. Similarly, it could also mean that the use of methylated As species as an antibiotic is less efficient in P2000 due to a lack of porewater As. On the other hand, Conrad [38] has also suggested that frequently drained soil environments show less population changes than permanently flooded ones. Long-term paddy use could result in a stable microbial community where warfare could be less necessary or methylated As could be less effective. Lastly, it has been shown that in systems with high availability of labile organic C like P2000, Carbon Catabolite Repression takes place, meaning a down-regulation of catabolic enzymes such as the aquaglyceroporin channel (GlpF). The down-regulation of this specific channel, which can also uptake arsenite, could affect the uptake of As into

the cells and thus, indirectly, decrease the methylation potential in soils with particularly high SOC[64].

While strong de-methylation due to high methanogenic activity could also be an option[13] (decreasing the net methylated As in P2000), incubation experiments with spiked MMA and DMA showed that P2000 had the lowest demethylating capacity of all soils with up to 50% of the MMA spike remaining as methylated species after 5 days, compared to 18% of the same spike in P50 (Figure SI11). These observations only highlight the difficulties of assessing (de)methylation dynamics. Having in mind the different possibilities and the still open questions regarding As methylation dynamics in paddy soils in general, it is particularly difficult to determine which exact changes in microbial dynamics are affecting the methylation capacity in P2000.

Although higher *arsM* abundances were found with higher aqueous phase As concentrations (Figure SI10a), a significant correlation between *arsM* gene counts and methylated As ($r=0.57$, $p<0.001$) was only found when excluding P50 (

Figure **4**e). It has been observed that although there may be more methylating microbes in soils with higher pH values, their activity is higher at lower pH[61]. This pH dependency was observed in P50, which had higher pH values (

Figure **4**f) and *arsM* copy numbers but showed lower methylation potential. The *arsM* copy numbers and soil pH decreased with age, while the contribution of methylated As to As speciation increased. Additional observations related to possible changes of microbial communities responsible for As methylation (from SRB-driven to fermenter-driven) with increasing paddy soil age are discussed in the supplementary discussion. Throughout all incubations and for all soils, monomethylated species had higher contributions to the sum of species than dimethylated ones. It is not clear if this difference from field observations

(where dimethylated species usually dominate the share of methylated species), is an artifact of our incubation experiments. Such dominance of monomethylated species has been previously observed and attributed to different microbial capacities and the activity of different enzyme systems carrying out the first or second methylation steps[65-67]. A recent study by Qiao, et al. [68] also identified a predominance of MMA over DMA in paddy soil extracts.

Furthermore, once formed, oxymethylated As (MMA and DMA) can lead to the formation of methylthioarsenates through their abiotic reaction with $S^{-II}$. Methylthioarsenates were detected in all soils at all incubation times except for P2000, where they were only found after 5 days of incubation (in the same way as the delayed formation of inorganic thioarsenates related to the delay in DSR) (Figure SI12). According to our data, as soon as oxymethylated As species are formed, they are readily thiolated when reduced sulfur is available in the system. Additionally, in most soils, oxymethylated species continued to be thiolated even when there was no more inorganic thiolation, especially in P100 (Figure SI4 and SI12). This suggests that in paddy soil porewater, the formation of methylthioarsenates is preferred over their inorganic homologs. The enhanced thiolation of methylated As species could be related to the pH conditions in paddy soil porewater, where acidic to neutral pH are more prevalent than the alkaline conditions that allow the thiolation of inorganic oxyAs. This preferential thiolation was also confirmed by the spiked incubations, particularly for those spiked with DMA where (even with a 10-fold excess compared to non-spiked concentrations), up to 98% of the present DMA was thiolated compared to only up to 22% of inorganic As being present as thioarsenates (Figure S13).

Methylthioarsenates contribution to As speciation through paddy soil age (Figure S14a) behaved similarly to inorganic thioarsenates in the short term (3 days of incubation), decreasing strongly with age (with the exception of P50, where there was low availability of oxymethylated precursors). The same factors decreasing the availability of reduced sulfur for the formation of inorganic thioarsenates with increasing age would also affect the formation of methylthioarsenates. Interestingly, the contribution of methylthioarsenates to As speciation after 35 days of incubation was very similar to that of oxymethylated As. This means that in the early stages of incubation, the formation of methylthioarsenates is limited by the availability of MMA or DMA as precursors, but as soon as oxymethylated species become available, their formation is limited only by the availability of reduced sulfur.

The contribution of methylthioarsenates to speciation with respect to oxymethylated As can be seen in Figure SI14b. A few data points of P50, P100, and P300 are over the 1:1 line which indicates that methylthiolated species had higher contributions than the oxymethylated ones in these paddies. These points correspond to early incubation days with relatively low methylation, but high availability of reduced sulfur, while older paddies (and later incubation times) with higher levels of methylation and limited availability of reduced sulfur for thiolation fell under the 1:1 line.

Interestingly, P2000 showed a low but constant formation of methylated and inorganic thioarsenates even by day 35 (Figure SI4 and SI12). It has been suggested that the presence of a cryptic S-cycle constantly supplies small amounts of reduced sulfur to the system for constant As thiolation and is related to the reduction of $Fe^{III}$ oxyhydroxides[24,69]. More specifically, the cryptic-S cycle involves sulfide re-oxidation to $S^0$ coupled to the

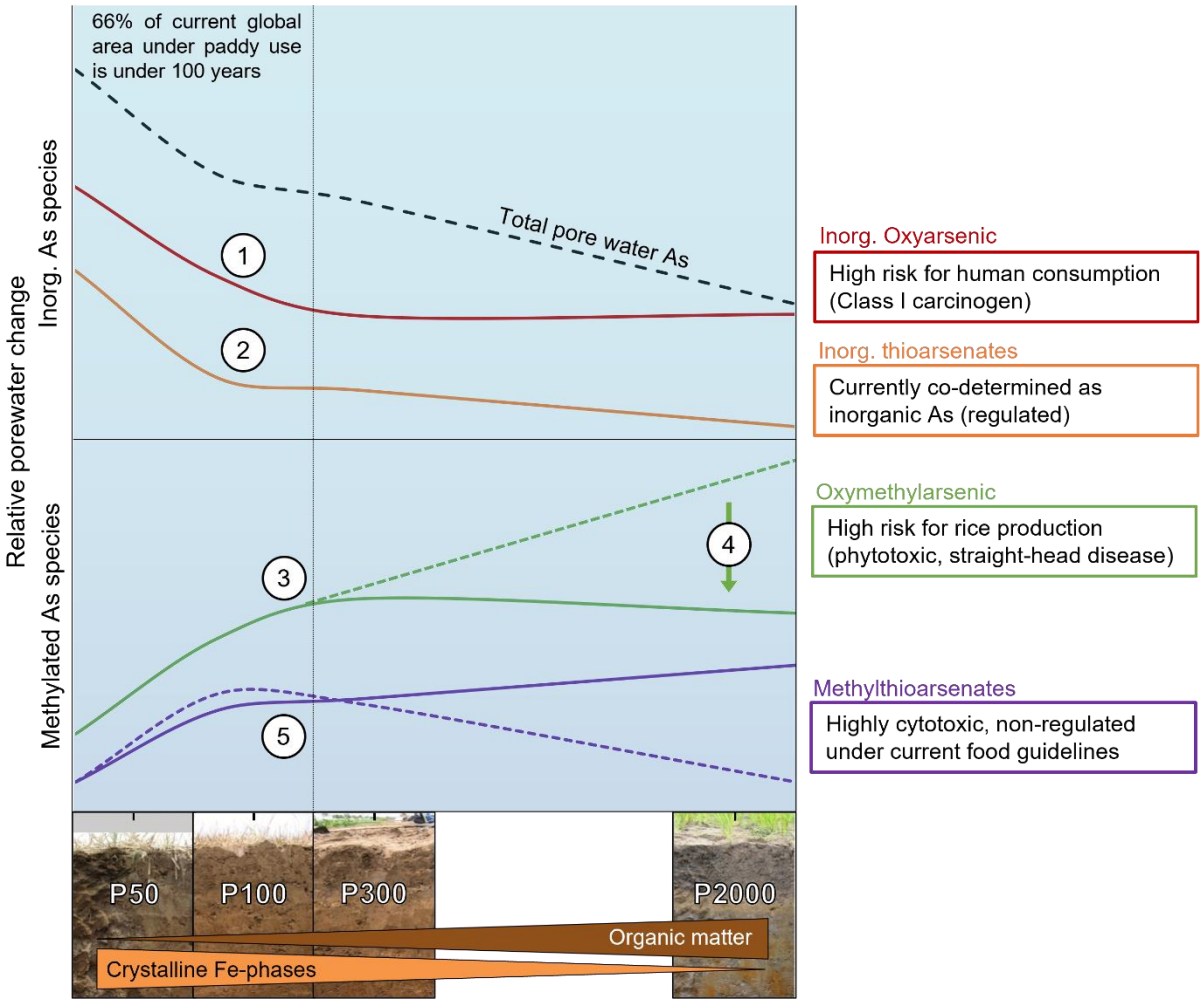
reduction of $Fe^{III}$ (oxy)hydroxides, forming mixed $Fe^{II}Fe^{III}$ minerals, pyrite, and release $Fe^{II}$ into the porewater[70]. The high availability of amorphous $Fe^{III}$ phases increasing with age could provide a sort of “S battery” for later As thiolation, especially in very old soils like P2000, where these phases are most abundant. Supporting this idea, a slight accumulation of $S^0$ in the long term took place in P2000 after 35 days of incubation.

In summary, although in early stages of incubation, younger paddies showed a higher share of methylthioarsenates with respect to their oxymethylated counterparts, older paddies offer a larger potential for methylthiolation in the long-term. Furthermore, our observations show that as soils age and As is methylated, methylthioarsenates will definitely contribute to the As speciation, to an extent depending on the environmental factors governing the availability of reduced S. This represents not only a risk for plant yield through the phytotoxicity of DMA, but also a potential risk for human consumption from DMMTA and DMDTA formation.

## 4. Conclusions

Our study presents, to the best of our knowledge, the first linkages between paddy soil age and As speciation. Although the paddy soils of the chronosequence used in this work originate from coastal marshlands[33] and the extent or rates to which paddy soil evolution affects As speciation might change according to the parent material, environmental conditions or number of harvests per year, general processes described here should be transferable to other paddy soils of different origins[71]. Following up on the age-related effects on As biogeochemistry observed in the present chronosequence, further chronosequence studies should be carried out to evaluate the extent to which these observations are transferable to other soil types and different age spans soil ages.

For our chronosequence, the high As availability in porewater of young soils (≤ 100 years), dominated by inorganic oxyAs species, suggests that these soils have an increased risk for high inorganic As concentrations in porewater (Figure 5, process 1). If porewater As speciation directly links to speciation in rice grains, cultivation on these younger soils could pose a higher risk for the consumption of the respective rice products. Currently, worldwide, 118 million ha are estimated to be under paddy use, with around 66% of these soils being younger than 100 years[72]. Based on the results from our study, there could also be a high potential for inorganic thiolation in such soils. Although currently co-determined in rice grains as inorganic As (and thus, regulated), inorganic thioarsenates (particularly MTA, the dominant inorganic thioarsenate species during most of our experiments) show lower adsorption to Fe minerals than inorganic oxyarsenic species, increasing As mobility[73,74]. In the context of rice cultivation, MTA shows lower uptake but higher root-to-shoot translocation in rice plants, which translates into a higher contribution to inorganic As enrichment in rice grains[25,75]. Our data suggests that as paddy soil development takes place, the geochemical changes in the soil decrease the potential for As thiolation (Figure5, process 2).




510

511 **Figure 5: Conceptual model representing the relative change in the contribution of**
512 **inorganic (top) and organic (bottom) As species to aqueous phase speciation with**
513 **increasing paddy soil age, together with challenges associated to each group (right).** Total
514 aqueous phase As is represented as a dashed black line for reference. Numbers represent: (1)
515 Decrease in inorganic oxyAs. (2) Decrease in inorganic thiolation due to accumulation of SOM
516 and amorphous Fe minerals. (3) Increased methylation with the accumulation of SOM and
517 increased microbial activity. (4) Decrease in measured methylated species with respect to the
518 expected value (dashed line) due to an unidentified limitation on methylation. (5) Transitioning
519 soils between young and mature, showing high potential for the formation of methylthioarsenates,

being limited by the availability of reduced sulfur (dashed line) or methylated As (solid line). Pictures from soil profiles show the upper 50 cm (taken and modified from Kalbitz, et al. [35]

Regarding methylation, we found correlation with biogeochemical parameters related to paddy soil age. However, from a microbiological perspective, formation and consumption of methylated As species is not completely clear, yet. Here, we showed how long-term paddy use enhances As methylation mainly due to the accumulation of organic matter, increased microbial activity, and decreasing soil pH (Figure 5, process 3). Furthermore, older paddies with lower As mobilization could also have a lower methylating capacity related to lower *arsM* expression or other unidentified limitations (Figure 5, process 4). Moreover, older paddies produced higher greenhouse gas emissions due to their higher microbial activity, including that of methanogens. Our results show that as soon as methylation takes place and reduced sulfur is available in the system, methylthioarsenates will form. Particularly in early stages of soil evolution (>50 years) there is enough availability of methylated As and reduced S to make methylthioarsenate species contribution highly relevant in the paddy soil porewater (up to ca. 20% for P100 at day 7) (Figure 5, process 5). Methylthiolated As species should not only be considered an issue in young paddies transitioning to mature ones but also in old ones where they were present and increasing after 35 incubation days, likely associated to cryptic S-cycling.

Observations from the present laboratory study certainly need to be verified in the field, either with the present chronosequence, others of its kind with different parent material, or in soils where the time of rice cultivation is known. Field studies will help to understand how the age-related dynamics presented here change in systems with plant-soil interactions and in the context of a cropping season, as well as how the changes in

aqueous phase As speciation observed here translate into As accumulation in rice grains. Looking at the bigger picture, these observations could help decide which agricultural practices or rice varieties to use at different sites to mitigate the most prominent risk factors for each soil age group. For example, a mature soil in which high contents of methylated species are found will profit from a rice variety with a lower yield but higher resistance to straight-head disease[76]. Similarly, rice straw can be removed from these soils to limit the methylation potential or greenhouse gas emissions, as well as applying intermittent flooding, which has been reported to decrease As accumulation in grain and $CH_4$ production[77,78]. Likewise, younger paddies could profit from a higher addition of rice straw or other forms of organic matter to enhance the methylation potential, according to previous observations[62,63]. Overall, paddy soil age is a factor that requires more attention in future studies, as it has the potential to influence the kind and the extent of threat that As poses for rice cultivation.

## Supporting Information

Appendix A: Further details on materials and methods (As speciation; DNA and RNA extraction and quantification). Biogeochemical and microbial data supporting the above-described observations and discussion. Additional discussion regarding HBED stabilization method and changes in microbial communities involved in As methylation.

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## Author contributions

This study was conceptualized by J.M.L.N. and B.P.-F.; incubation setup, and soil, aqueous phase, and gas phase analyses were carried out by J.M.L.N. with help from A.H.M. and A.N.; the assays regarding the HBED stabilization method were carried out by A.N.; DNA and RNA analyses were done by E.M.M.; $CH_4$ and $CO_2$ measurements were carried out by J.M.L.N. with assistance from B.G.; DOC analyses were carried out by J.M.L.N. with assistance from J.P.; acetate analyses were done by A.N. with assistance from T.L.; the chronosequence soils were prepared and provided by A.K. and L.U.; statistical analyses were carried out by J.M.L.N. with assistance from A.N.; the manuscript and supporting information was written by J.M.L.N. with assistance from B.P.-F.; all coauthors contributed to the manuscript revisions.

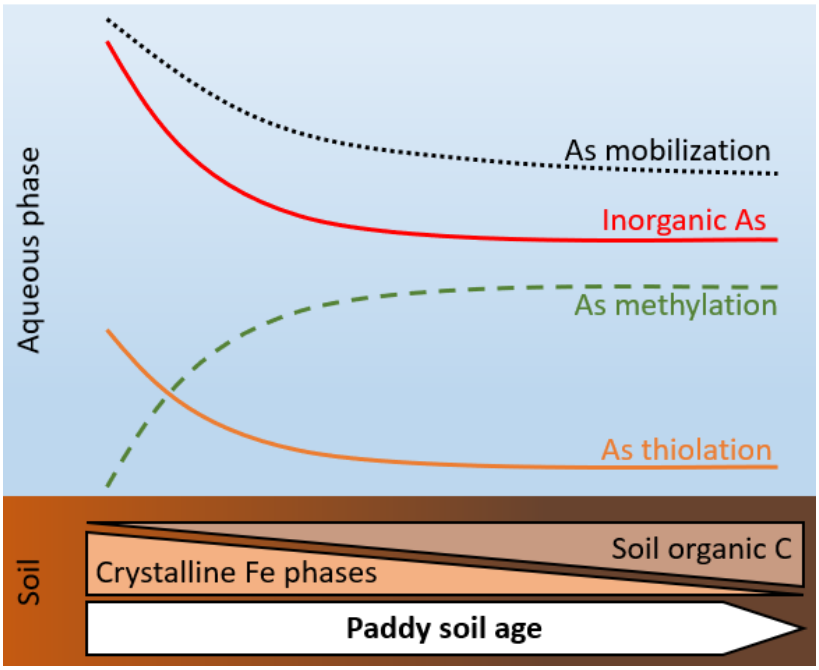
## Competing Interests

The authors declare no competing interests.

## Data availability

The data generated and analyzed for the current study are also available from the corresponding author on reasonable request.

831 **Graphical abstract**




832