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Effects of sulfur on arsenic speciation in paddy soils and rice grains

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Jiajia Wang

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Acting director: Prof. Dr. Markus Lippitz

Doctoral committee:

Prof. Dr. Britta Planer-Friedrich (reviewer)

Prof. Dr. Ruben Kretzschmar (reviewer)

Prof. Dr. Stephan Clemens (chair)

Prof. Dr. Tillmann Lüders

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Novalis, the German poet and philosopher, once said: “Die Philosophie ist eigentlich Heimweh-Trieb überall zu Hause zu sein”. The pursuit for science to me also means a gypsy lifestyle, running from city to city, moving from one country to another.

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山川异域 风月同天[a]

“Although mountain and sea set us apart

The moon and wind share our friendship and kind heart”

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May we all be blessed with health and longevity, though maybe with 6 or 7 hours of time difference, enjoying together the same moon and wind!

[a] A line from a poem written by a Japanese King (ChangWu, 長屋王) about 1300 years ago, expressing the strong desire to learn Buddhist doctrine from China.

Abstract

Arsenic (As) is a carcinogenic metalloid ubiquitously present in soil environments and its accumulation in rice grains poses a health threat to millions of people. The geochemical behavior and bioavailability of As are largely determined by its speciation. Current research on As speciation in rice paddies mainly focuses on inorganic and methylated oxyarsenic species, but does not consider thioarsenates, in which sulfur takes the place of oxygen in oxyarsenic species. The general assumption is that thioarsenates only form in environments with excess dissolved sulfide. This paradigm was recently challenged by the hypothesis that As thiolation could be mediated by reduced sulfur bound to surfaces of minerals or organic matter, suggesting thioarsenates may be more widespread than currently assumed.

Therefore, the aim of this thesis was to reveal whether and to what extent thioarsenates contribute to As speciation in paddy soil pore waters, as well as to decipher soil parameters that are important for their formation. Additionally, the effects of sulfate fertilization on As speciation in pore waters and As accumulation in rice grains were examined.

In the first study, a novel diethylenetriamine-pentaacetic acid-based sampling and analytical method was developed, enabling the simultaneous determination of thioarsenates and their respective oxyarsenic species. On the basis of field, mesocosm and soil incubation studies across various paddy soils from major rice cultivation areas in Italy, France and China, thioarsenates were revealed as important but previously overlooked contributors to As speciation in rice paddy pore waters. Thioarsenates were observed throughout the cropping season and in quantities comparable to methylated oxyarsenates. On regional scale, soil pH values represented an easy-to-measure parameter indicative for As thiolation potentials under anaerobic conditions. Inorganic thioarsenates occurred predominantly in alkaline soils, controlled by the presence of zero-valent sulfur. Methylated thioarsenates occurred predominantly in acidic soils, related to the presence of their precursors, methylated oxyarsenates.

The second study showed that for paddy soils where conditions change from anoxic during flooding to oxic during drainage, the importance and occurrence of thioarsenates in pore waters were highly dependent on soil redox potentials (E_H). Inorganic thioarsenates formed rapidly upon soil flooding with a dominance of trithioarsenate,

while they dethiolated almost completely after soil oxidation. Thiolation of methylated oxyarsenic played an important part over a wide range of soil redox potentials, with total thiolation of mono- and dimethylated As up to 70% and 100% below E_H 0 mV, respectively. Dithiolated species dominated over monothiolated species below E_H −100 mV. Among all thioarsenates, dimethylated monothioarsenate showed the least transformation upon prolonged oxidation and also represented the major thiolated As species in the oxygenated rice rhizosphere, which is especially critical since dimethylated monothioarsenate is highly carcinogenic.

While natural sulfate contents in paddy soils could support substantial As thiolation already, combined results from the first and third study showed that sulfate fertilization could further increase thioarsenate formation, especially for paddy soils with low initial zero-valent sulfur formation. Sulfate fertilization also increased As methylation, but largely decreased the bioavailability of inorganic oxyarsenic during rice cultivation. From an agronomic point of view, the third study evidenced sulfate fertilization as an effective measure to mitigate the accumulation of cancerogenic inorganic As in rice grains, irrespective of seeding practices and water managements.

Altogether, these three studies clearly illustrated the importance of sulfur in influencing As speciation in paddy soils and its accumulation in rice grains, revealing sulfur-mediated As thiolation as an important and heretofore unaccounted contributor to arsenic biogeochemistry in rice paddies. Future studies are needed to clarify whether thiolation is good or bad for producing rice low in toxic As. Furthermore, thioarsenates need to be considered in low-sulfate terrestrial environments thus to paint a more accurate picture of global As cycling.

Zusammenfassung

Arsen (As) ist ein krebserregendes Metalloid, das im Boden allgegenwärtig ist, und seine Anreicherung in Reiskörnern stellt für Millionen von Menschen eine Gesundheitsbedrohung dar. Das geochemische Verhalten und die Bioverfügbarkeit von As werden weitgehend durch seine Speziierung bestimmt. Die aktuelle Forschung zur As-Speziierung in Reisfeldern konzentriert sich hauptsächlich auf anorganische und methylierte Oxyarsen-Spezies, berücksichtigt jedoch Thioarsenate nicht, bei denen Sauerstoff den Schwefel in Oxyarsen-Spezies ersetzt. Allgemein wird angenommen, dass Thioarsenate nur in Umgebungen entstehen, die einen Überschuss an feiem Sulfid haben. Dieses Paradigma wurde kürzlich durch die Hypothese in Frage gestellt, dass Thiolierung auch durch an die Oberflächen von Mineralien oder organischer Substanz gebundenem reduzierten Schwefel ermöglich wird, was darauf hindeutet, dass Thioarsenate weiter verbreitet sein könnten als derzeit angenommen.

Ziel dieser Arbeit war es daher, herauszufinden, ob und inwieweit Thioarsenate zur As-Speziierung in Porenwässern von Reisböden beitragen, sowie Bodenparameter zu entschlüsseln, die für ihre Bildung wichtig sind. Zusätzlich wurden die Auswirkungen der Sulfatdüngung auf die As- Speziierung in Porenwässern und die As-Akkumulation in Reiskörnern untersucht.

In der ersten Studie wurde ein neuartiges Probenahme- und Analyseverfahren auf der Basis von Diethylentriamin-Pentaessigsäure entwickelt, das die gleichzeitige Bestimmung von Thioarsenaten und ihren jeweiligen Oxyarsen-Spezies ermöglicht. Auf der Grundlage von Feld-, Mesokosmos- und Bodeninkubationsstudien mit verschiedenen Reisböden aus wichtigen Reisanbaugebieten in Italien, Frankreich und China wurden Thioarsenate als wichtige, aber bisher übersehene As-Spezies in Porenwässern von Reisböden identifiziert. Thioarsenate wurden während der gesamten Anbausaison und in vergleichbaren Mengen wie methylierte Oxyarsenate gefunden. Auf regionaler Ebene stellten die pH-Werte im Boden einen leicht zu messenden Parameter dar, der Hinweise auf das As-Thiolierungspotential unter anaeroben Bedingungen gibt. Anorganische Thioarsenate traten vorwiegend in alkalischen Böden auf, wo das Vorkommen von nullwertigem Schwefel kontrolliert wurde. Methylierte Thioarsenate traten vorwiegend in sauren Böden auf, was auf die Anwesenheit ihrer Vorläufer, der methylierten Oxyarsenate, zurückzuführen ist.

Die zweite Studie zeigte, dass die Bedeutung und das Vorkommen von Thioarsenaten in Porenwässern von Reisböden, in denen sich die Bedingungen von anoxisch im gefluteten Zustand zu oxisch während der Drainage ändern, stark vom Redoxpotential (EH) im Boden abhängen. Anorganische Thioarsenate bildeten sich schnell auf gefluteten Böden, wobei Trithioarsenat dominierte, während sie nach Oxidationsphasen fast vollständig dethioliert wurden. Die Thiolierung von methylierten Oxyarsenaten spielte über einen weiten Bereich von Redoxpotentialen im Boden eine wichtige Rolle, wobei die Gesamtthiolierung von mono- und dimethyliertem As bei bis zu 70% bzw. 100% lag, wenn das Redoxpotential unter 0 mV war. Dithiolierte As-Spezies dominierten gegenüber monothiolierten Spezies, wenn das Redoxpotential unterhalb von -100 mV lag. Von allen Thioarsenaten zeigte dimethyliertes Monothioarsenat die geringste Umwandlung bei längerer Oxidation und stellte auch die wichtigste thiolierte As-Spezies in der sauerstoffhaltigen Reis-Rhizosphäre dar, was besonders kritisch ist, da dimethyliertes Monothioarsenat stark karzinogen ist.

Während die natürlichen Sulfatgehalte in Reisböden bereits eine beträchtliche As-Thiolierung unterstützen konnten, zeigten die kombinierten Ergebnisse der ersten und dritten Studie, dass eine Sulfatdüngung die Thioarsenatbildung weiter steigern konnte, insbesondere bei Reisböden mit geringer anfänglicher nullwertiger Schwefelbildung. Die Sulfatdüngung erhöhte ebenfalls die As-Methylierung, verringerte jedoch weitgehend die Bioverfügbarkeit von anorganischem Oxyarsen während des Reisanbaus. Aus agronomischer Sicht bewies die dritte Studie, dass die Sulfatdüngung eine wirksame Massnahme ist, um die Akkumulierung von krebserregendem anorganischen As in Reiskörnern zu vermindern, unabhängig von Aussaatverfahren und Wassermanagement.

Insgesamt zeigten diese drei Studien deutlich die Bedeutung von Schwefel bei der Beeinflussung der As- Speziierung in Reisböden und seiner Akkumulation in Reiskörnern und enthüllten die schwefelvermittelte As-Thiolation als einen wichtigen und bisher nicht nachgewiesenen Beitrag zur Arsen-Biogeochemie in Reisfeldern. Zukünftige Studien sind notwendig, um zu klären, ob die Thiolierung gut oder schlecht ist, um Reis mit niedrigem Gehalt an toxischen As anzubauen. Darüber hinaus müssen Thioarsenate in einer sulfatarmen terrestrischen Umgebung in Betracht gezogen werden, um ein genaueres Bild des globalen As-Kreislaufs zu zeichnen.

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List of abbreviations

ArsM	arsenite S-adenosylmethionine methyltransferase
CEC	Cation exchange capacity
D-CF	Dry seeded with continuous flooding
DIC	dissolved inorganic carbon
DMA	dimethylarsenate
DMDTA	dimethyldithioarsenate
DMMTA	dimethylmonothioarsenate
DOC	dissolved organic carbon
DTA	dithioarsenate
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
IC-ICP-MS	ion-chromatography spectrometry coupled to inductively coupled plasma mass
ICP-MS	inductively coupled plasma mass spectrometry
MMA	monomethylarsenate
MMDTA	monomethyldithioarsenate
MMMTA	monomethylmonothioarsenate
MTA	monothioarsenate
ROL	root oxygen loss
SOM	soil organic matter
TTA	trithioarsenate
W-CF	Water seeded with continuous flooding
W-WD	Water seeded with alternate wet-dry

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Extended summary

1. Introduction

1.1. Rice, paddy soils and the arsenic problem

Rice (*Oryza sativa L.*) is the staple food for about one-half of the Earth's population [1]. Total global rice consumption reaches more than 400 million metric tons each year [2]. To feed the growing population, an additional 116 million tons of rice is projected to be needed by the year 2035 [1]. Rice and rice-based foods are also widely used to feed weaning babies and young children, because of their blandness, relatively low allergic potential and nutritional benefits [3, 4].

Worldwide, rice is grown on a total area of approximately 158 million hectares, mainly in South and Southeast Asia [1]. Rice is mostly cultivated under flooded conditions in so-called paddy soils [5]. For example, nearly 80% of total rice cropping areas in Asia are managed as paddy soils [6]. However, flooding management causes excessive mobilization of arsenic (As), a notorious, toxic metalloid widely present in paddy environments, predominately as trivalent arsenite in flooded paddy soils [7].

Worse, rice is inherently accumulating As [8], mainly due to the inadvertent, yet efficient, uptake of arsenite through the silicon transport pathway [9]. Rice consumption represents the major dietary contributor to inorganic As (usually referring to the sum of arsenate and arsenite) in populations with a rice-based diet or through rice-based baby food [3, 10-12]. Inorganic As gives rise to cancers of the lung, skin, and bladder[13, 14], and has also detrimental effects on non-cancer health effects such as neurological and respiratory diseases [15]. Heath risks associated with inorganic As are particularly critical in infancy and early childhood [16], which can have lifelong health impacts. Beside inorganic As, rice contains also several methylated As species, predominantly dimethylarsenate (DMA), and to a much lesser extent monomethylarsenate (MMA). Methylated As species in grains are assumed to be of soil microbial origin since rice cannot methylate As [17]. Health risks associated with methylated As are less certain, but the level of ingestion from rice diet is generally believed

to be much lower than that of concern [17]. Therefore, current concentration limits imposed for rice and rice-based foods consider only inorganic As, but not methylated As. The limit has been set at 0.20 mg/kg for white rice in the European Union (EU) and China [18, 19], and 0.10 mg/kg for rice-based foods for infants and young children in the EU [19].

Arsenic in paddy soils originates from both natural and anthropogenic sources [20]. Typical baseline concentrations of As in soils are of the order of 5-10 mg/kg, derived from natural processes such as bedrock weathering and alluvial deposits [20, 21]. Yet, As concentrations in soils formed on top of As-rich bedrocks can reach up to hundreds of mg/kg [22]. Anthropogenic pollutions from mining and smelting, irrigation with As-rich groundwater, and the use of arsenical pesticides can lead to further elevations of As in paddy soils [20]. It should be noted that As levels in rice are problematic even where As contents in paddy soils are at background concentrations (i.e., no further anthropogenic As-pollution) [10, 23], especially for vulnerable populations such as pregnant women, infants, and young children [24]. For example, a field survey of rice grains collected from main rice cropping areas in Italy showed that one-half of the white rice (168 samples) failed the food standards (0.1 mg/kg) for infants and young children, although total As of surveyed soils (average around 9-10 mg/kg) were within background levels (the mean aqua regia As in European topsoil of 9.88 mg/kg) [25]. If rice is grown on soils with anthropogenic As-pollution, further elevation in rice As can be expected. One of the best-known examples is in Bangladeshi paddy soils, where irrigation with As-tainted groundwater results in elevated As bioavailability and its final accumulation in rice [26, 27]. Since rice is traded by dealers and governments both domestically and internationally, rice arsenic problem is a trans-boundary heath issue of global consequence [1, 28].

After the first papers that suggest rice consumption may be an important dietary source of As from 1998 [29, 30], knowledge about As biogeochemistry in paddy soils has increased enormously. Since the mobility, bioavailability and toxicity of arsenic are tightly related to its chemical speciation [7], the next section will first summarize the current understanding about As speciation in rice paddy pore waters. Another group of soluble sulfur-containing arsenic

species (thioarsenates) is then introduced, which have persistently been overlooked in paddy environments. Arsenic species, abbreviations and chemical formula throughout this thesis are summarized in Table 1.

1.2. Arsenic speciation in rice paddy pore waters as affected by redox changes

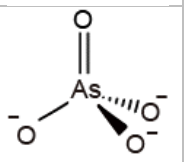
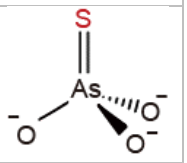
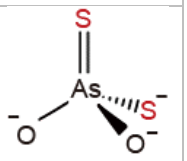
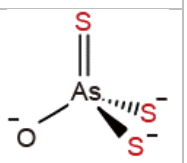
Arsenic is a redox-sensitive metalloid, thus its redox states and speciation in pore waters are strongly influenced by water-management-induced changes in paddy soil redox conditions [7]. The traditional way of rice cultivation involves puddling of flooded soils, followed by seedling transplantation [31]. Paddy soils are then kept flooded during most of the time of rice growth. Surface flood water is typically drained towards the end of tillering to control excessive rice tillering, and one to two weeks before harvest [6, 32].

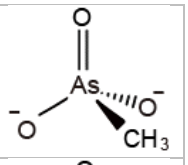
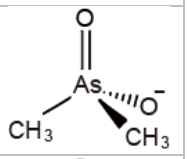
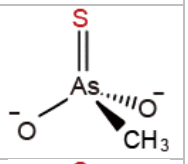
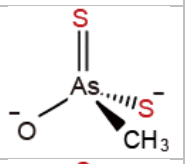
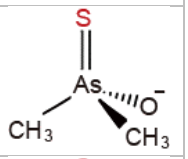
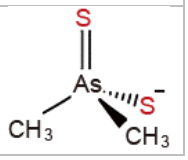
When flooding, oxygen consumption causes the soil microbial communities to use alternative terminal electron acceptors from nitrate, manganese oxides, iron (hydr)oxides, sulfate to carbon dioxide [33]. Pentavalent arsenate is a favorable terminal electron acceptor, and its microbial reduction is predicted to be more favorable over iron (hydr)oxides and sulfate under most environmental conditions [34]. In flooded paddy soils, therefore, trivalent arsenite represents the major inorganic As species owing to the combined results of the arsenate reduction and reductive dissolution of iron (hydr)oxides, which are the important mineral hosts for arsenate in oxic soils [35]. Pore-water arsenite concentrations in flooded paddy soils are reported in the range of sub μM to tens of μM [32]. Yet, considerable percentage of arsenate (typically 10-30% of the total As) is often detected in flooded paddy soils [32]. The occurrence of arsenate could be explained by the microbe-mediated anaerobic arsenite oxidation couple to nitrification [36]. Arsenite can further be methylated by a variety of microbes (e.g., sulfate-reducing bacteria, SRB) to several methylated As species in flooded paddy soils, catalyzed by arsenite S-adenosylmethionine methyltransferase (ArsM) [37, 38]. With prolonged soil flooding, degradation of methylated As species (demethylation) can happen [17], e.g. mediated by some methanogenic archaea [37]. Methylated oxyarsenates, that is MMA and DMA, are usually minor species in paddy soil pore waters, compared to the predominance of inorganic oxyarsenic species (arsenite and arsenate) [38, 39]. However, with

respect to inorganic oxyarsenic species, methylated oxyarsenates, particularly DMA, transport more readily in plant tissues [17, 40] and from leaves to grains [41, 42]. DMA, the predominant methylated As in rice grains, can account for 10-90% of total As worldwide [17].

When draining, oxygen diffusion causes the regeneration of electron acceptors, e.g. the oxidation of inorganic reductants such as ammonium, ferrous iron (Fe (II)) and sulfide [43]. The abiotic oxidation of arsenite, the dominate As species in flooded paddy soils, by oxygen is slow (e.g., with a half-life of about 9 days in air-saturated water with low iron contents and pH 7.6-8.5) [44]. However, the rate of arsenite oxidation can be largely accelerated in the presence of Fe (II) [44] and/or arsenic-oxidizing microbes [45], favoring the retention of arsenate to the solid phase together with the precipitation of newly formed Fe(III) (oxyhydr)oxides. While MMA sorption to Fe(III)(oxyhydr)oxides is similar to arsenate, DMA sorbs significantly less [46] and can survive short-term soil oxidation [47]. However, with prolonged oxidation, demethylation of DMA/MMA will happen, most possibly mediated by soil aerobic microbes [48].

Table 1 | Arsenic species and abbreviations discussed throughout this thesis

Category	Group	Individual arsenic species	Chemical formula*
Inorganic arsenic (inorg-As)	Inorganic oxyarsenic (inorganic oxyAs)	Arsenite	
		Arsenate	
	Inorganic thioarsenates (inorganic thioAs)	Monothioarsenate (MTA)	
		Dithioarsenate (DTA)	
		Trithioarsenate (TTA)	

Methylated arsenic (methyl-As)	Methylated oxyarsenates (methylated oxyAs)	Monomethylarsenate (MMA)	
		Dimethylarsenate (DMA)	
	Methylated thioarsenates (methylated thioAs)	Monomethylmonothioarsenate (MMMTA)	
		Monomethyldithioarsenate (MMDTA)	
		Dimethylmonothioarsenate (DMMTA)	
		Dimethyldithioarsenate (DMDTA)	

* Only fully deprotonated forms of all arsenic species are given for simplicity.

Besides water-management-induced changes in redox conditions, micro-oxic conditions can be formed in the rice rhizosphere due to the aeration by radical oxygen loss (ROL) from rice roots [49, 50]. The micro-oxic zone could extend to about 0.3 mm from the roots into the surrounding reducing soil as shown by a previous study using O_2 microsensors [51]. Rhizospheric aeration triggers both the abiotic and microbial oxidation of Fe(II) and arsenite, leading to the coating of amorphous Fe/Mn oxide on rhizospheric soil particles and along O_2-releasing root surfaces [52], and subsequent immobilization of As (mainly as arsenate) [53]. The effects of rhizosphere on microbial methylation are less well known and contradictory results of either inhibition [54] or promotion [38] have been reported.

Current research about pore-water As speciation mainly focuses on inorganic and methylated oxyarsenic species (Table 1). Thioarsenates are soluble pentavalent As species in which sulfur replaces oxygen (Table 1). They form upon reaction of arsenite with zero valent sulfur and sulfide (in case of inorganic thioarsenates) [55], or MMA and DMA with sulfide

(in case of methylated thioarsenates) [56, 57]. Inorganic thioarsenates include mono- (MTA), di- (DTA), and trithioarsenate (TTA). Tetrathioarsenate is only stable at pH > 12, thus generally of little environmental relevance [58]. Methylated thioarsenates include mono- (MMMTA) and dimethylated monothioarsenate (DMMTA); mono- (MMDTA) and dimethylated dithioarsenate (DMDTA). Thioarsenate formation is typically reported in aquatic environments with excess dissolved sulfide, e.g. geothermal waters [59, 60] and stagnant terminal lakes [61, 62]. It remains unknown whether and to which extent thioarsenates form in low-sulfide paddy environments. Therefore, the following section will first discuss the potential of paddy soil sulfur cycling (only focused on inorganic sulfur) to support the thioarsenate formation, then explain why thioarsenates have been persistently overlooked in previous studies.

1.3. Sulfur cycling and its potential to support thioarsenate formation in paddy soils

The standing pools of sulfate in paddy soils are usually very small (< 5 mmol/kg), except in acid sulfate soils [63]. After flooding, pore-water sulfate concentrations typically decrease from 0.4-5 mM to a steady state concentration of <100 µM [64]. Despite low concentration, sulfate reduction rates up to 310 nmol cm^{-3} d^{-1} have been measured by ^{35}S-radiotracer techniques in planted paddy soil [65]. The rates rival those measured in high-sulfate marine surface sediments [66], indicating an effective recycling mechanism in paddy soils (e.g., regeneration of sulfate and thiosulfate). The recycling of reduced sulfur compounds to sulfate is proposed as "cryptic" S-cycle in lower-sulfate freshwater systems, where sulfide reoxidation to zero-valent sulfur is coupled to reduction of Fe(III) (oxy)hydroxides and formation of mixed $Fe^{II}Fe^{III}$ minerals or pyrite ($Fe^{II}S_2$) besides ferrous ion [67]. Further oxidation to thiosulfate and sulfate is coupled to nitrate or oxygen reduction, possibly catalyzed by the sulfur-oxidizing bacteria thriving in micro-oxic rhizosphere [65, 68, 69]. Our understanding of "cryptic" S-cycle in paddy soils is still in its infancy, with the roles of soil abiotic oxidants (e.g., Fe(III) and quinones in soil organic matter [70, 71]) as well as microbial communities [64] involved in the reduction and oxidation of this cycle remain to be investigated. Nevertheless, the conceptual model of

"cryptic" S-cycle provides a fresh perspective to understanding the role of sulfur in influencing arsenic cycling in paddy soils.

Sulfur and arsenic coexist in paddy environments and their biogeochemical cycles are often interconnected [7, 37]. Although thioarsenate formation has long been recognized to play an importance role for the mobility [72, 73] and toxicity [74] of arsenic in sulfate-rich environments, soluble thioarsenates have been persistently overlooked in paddy soil pore waters. The persistent overlook is mainly due to the conceptual limitation, and secondarily the methodological shortcoming. Conceptually, the current sulfur/iron-controlled model predicts thioarsenates only form substantially in systems with excess free sulfide (e.g., in geothermal waters[59, 60] or stagnant terminal lakes [61, 62]). Methodologically, acidification, which is both used for routine sample stabilization as well as for chromatographic separation, leads to transformation of thiolated As species or/and co-elution with oxyarsenic species.

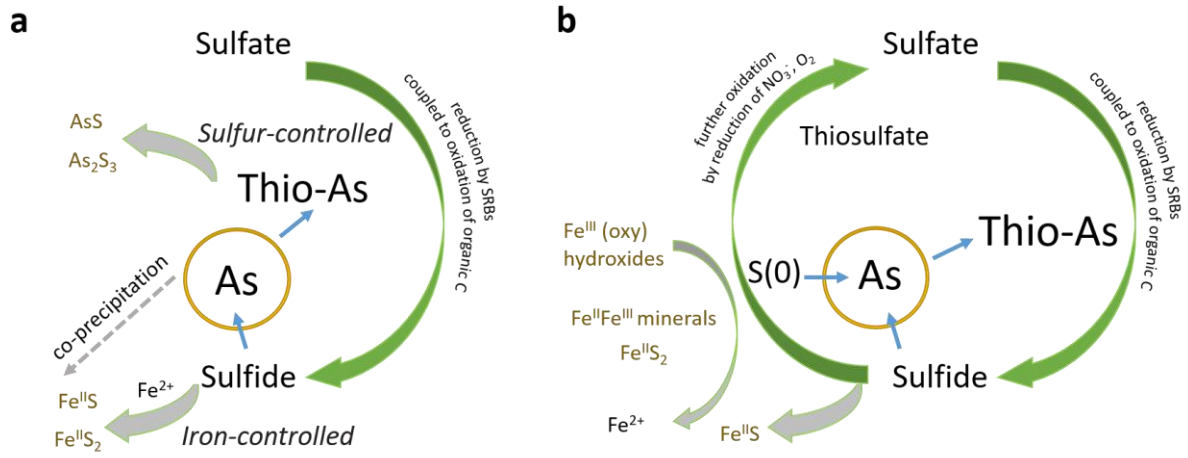


Figure 1 | Conceptual models for thioarsenate formation coupled to (a) the iron/sulfur-controlled model, and (b) the cryptic sulfur cycle model. Conceptual models are revised from O'Day et al. (2004) [75] and Wang et al., (2020) [76]. Abbreviations: thio-As=thioarsenates, S(0)= zero-valent sulfur, SRBs=sulfate-reduction bacteria, $Fe^{II}S$= mackinawite, $Fe^{II}S_2$= pyrite, AsS= Arsenic sulfide, As_2S_3=Arsenic trisulfide. Sulfur/iron-controlled model predicts thioarsenate formation only in systems with excess free sulfide (reactive Fe < sulfate in molar concentration). Cryptic sulfur cycle model predicts low but continuously replenished free sulfide and zero-valent sulfur during the rapid sulfur cycling can support thioarsenate formation in low-sulfate paddy soil, besides or instead of As scavenging on newly formed Fe-S minerals

O'Day et al. (2004) summarized a conceptual model classifying arsenic behavior as either sulfur-controlled or iron-controlled depending on iron/sulfur ratio in anoxic sediments [75]. This

conceptual model was subsequently extended by Keimowitz et al., (2005), and been used widely to explain soil-to-solution redistribution of As in both laboratory studies and field observations [77-79]. According to this model (Fig 1a), under low-iron, sulfur-controlled conditions, arsenic precipitation occurs mainly through precipitation of As sulfide minerals [75, 77], with soluble thioarsenates as potential intermediates [80]; under high-iron, iron-controlled conditions, released Fe^{2+} overwhelmingly removes sulfide from solution, preventing the formation of thioarsenates [77, 79, 81]. Paddy soil is characterized by several magnitudes higher reactive Fe (denoted as HCl-extractable Fe) relative to sulfate in molar concentration [63]. Therefore, the current geochemical model predicts that thioarsenates, even if present, are of negligible significance in "iron-controlled" flooded paddy soils (see Fig 1a *iron-controlled*) [35, 81]. This conceptual limitation greatly hinders thioarsenates research in paddy environments, but also in other low-sulfate terrestrial systems (e.g., peatlands and other wetlands).

Recent studies from our group showed that zero-valent sulfur, either in the aqueous phase or bound to the surfaces of minerals or organic matter, could react with As in solution to form thioarsenates, even when free sulfide was below detection limit [55, 82, 83]. Based on those findings, we argue that above-mentioned iron/sulfur-controlled model do not consider zero-valent sulfur, which is an important intermediate in both abiotic and biotic sulfur redox transformation [71, 84-87] and has been detected in a variety of environments including paddy soils [65, 88, 89]. From the perspective of the"cryptic" S-cycle and on the basis of our recent understanding of reduced-sulfur-mediated As thiolation [55], we therefore hypothesize that the low but continuously replenished free sulfide and zero-valent sulfur during the rapid sulfur cycling can support thioarsenate formation in low-sulfate paddy soil, besides or instead of As scavenging on newly formed Fe-S minerals (Fig 1b).

Methodologically, sample acidification is routinely used to preserve As speciation and prevent its loss with Fe (oxyhydr)oxide precipitation, as well as for chromatographic separation in previous studies. However, acidification of pore waters can result in immediate decrease or complete loss of higher thiolated As species such as TTA, DTA MMDTA and DMDTA [56, 90 56]. Monothiolated As species, i.e. MTA, MMMTA and DMMTA are relatively less acid-sensitive, but dethiolation to oxyarsenic species could happen with prolonged storage [57].

Another source of error can be chromatographic separation which is commonly done with PRP columns at acidic pH where higher thiolated species transform and DMMTA does not elute [91]. Further, if thioarsenates are not expected, all sample handling likely is done under oxic conditions, which will lead to transformation to oxyarsenic species. To unveil the role of thiolated As species, it is essential to develop a novel sample stabilization method tackling the problem of Fe (oxyhydr)oxide precipitation, and conducting chromatographic separation under alkaline conditions.

1.4. Sulfate fertilization influences As speciation in paddy soil pore waters and As accumulation in rice grains

Besides internal recycling of reduced sulfur compounds to sulfate, external input of sulfate can also affect the environmental fates in rice paddies. During rice cropping, a substantial amount of sulfate is often introduced concomitantly with the application of chemical fertilizer such as ammonium sulfate and potassium sulfate [92]. Application of sulfate-containing fertilizer (sulfate fertilization) could be more prevailing with the increasing sulfur deficiency since the reduction of sulfur dioxide emissions [93]. Sulfate fertilization has also been suggested as a mitigation measure to reduce the methane emission in rice farming owning to the substrate competitions between sulfate-reducing bacteria and methanogens [66, 94]. In this section, I will briefly review the currently understanding as well as identify knowledge gaps referring to sulfate fertilization in the context of pore-water As speciation and grain As accumulation.

Due to the high affinity between sulfur and As both in soil and rice plant, sulfate fertilization has long been suggested to decrease the As accumulation in rice [81, 95-102]. Sulfate amendment has been shown to decrease As bioavailability in previous unplanted soil incubations and pot experiments [79, 81, 102]. This is explained by the stimulation of microbial sulfate reduction and concomitant enhanced precipitation of As with newly formed Fe-As-S minerals [67, 102, 103]. Sulfate fertilization could possibly influence As methylation via stimulating

the activity of sulfate-reducing bacteria, which has very recently been proven to be involved in As methylation in flooded paddy soils [37]. Stimulated sulfate-reducing bacteria, which is involved in As demethylation [37], can then compete with methanogenic archaea for common substrates such as hydrogen and acetate [94]. Those effects could last over almost the entire rice growing seasons [104], due to the recycling of reduced sulfur to sulfate as discussed above [69]. However, previous relevant studies focused only on total As concentration and sulfate-fertilization-induced changes in pore-water As speciation have been generally ignored. The ignorance is most likely due to fact that DMA and MMA are often detected as minor species in pore waters [17]. And there exists no information about the effects of sulfate fertilization on thioarsenate formation.

Even though sulfate fertilization has long been suggested as a mitigation measure, its ultimate effect on grain As accumulation has only been evaluated in a few studies to date (summarized in appendix study 3, Table 1). Published studies often report contradictory findings: decrease in grain total As was found in four paddy soils studied [98, 100, 102], while no effects in another two paddy soils [95]. Moreover, sparse data exists about how sulfate fertilization influences grain As speciation. To the best of our knowledge, only one relevant study from Zhang et al., (2016) determined As speciation in the whole grain (with husk), showing a significant decrease in grain arsenite but no effect on DMA by thiosulfate application [100]. The overlook of As speciation is rather surprising since food guideline thresholds only address carcinogenic inorganic As (but not methylated As) [18, 19] and its percentage in grains varies greatly from 10 to 100% [10, 17]. In rice plants, sulfate amendments could stimulate the biosynthesis of thio-rich compounds (e.g. phytochelatin, PC), increase As chelation hence intracellular localization (e.g., in vacuoles), therefore decrease As translocation [97, 100, 101]. However, phytochelatin chelates effectively only arsenite but not DMA, the major methylated As species in the rice grain [17, 40]. To better understand the effect of sulfate on grain As accumulation, sulfate-fertilization-induced changes in As speciation need to be considered.

1.5. Objectives

The central aim of this thesis was to elucidate the role of sulfur for As speciation in pore waters and its accumulation in rice grains. Thioarsenates, the soluble sulfur-containing arsenic species, have not been considered in paddy soils so far due to a combination of both conceptual limitation and methodological shortcoming (see 1.3). Therefore, the current thesis sought to reveal whether and to what extent thioarsenates contribute to As speciation in paddy soil pore waters, as well as to decipher soil parameters influencing their formation. Additionally, the effects of sulfate fertilization on As speciation in pore waters and As accumulation in rice grains were examined.

The specific objectives in the present thesis were to:

(1) develop a sampling and analytical method for paddy soil pore waters to simultaneous measure thioarsenates and their respective oxyarsenic species; investigate the geochemical significance of thioarsenates in rice paddy pore waters including their field occurrence, relevance over rice growing seasons, widespread presence over regional scale and factors influencing their formation under anaerobic conditions (study 1);

(2) study how paddy soil redox potential influences the thioarsenate occurrence including their short-term dynamics under anaerobic conditions, transformation when flooded soil becomes reoxidized and occurrence in the oxygenated rice rhizosphere (study 2);

(3) study how sulfate fertilization influences the thioarsenate formation, inorganic and methylated As species bioavailability in pore waters and As accumulation in grains, under different agronomic practices (study 1 and 3).

2. Methods

2.1. Studies conducted in study 1

2.1.1 Field survey

To provide the first evidence for thioarsenate occurrence in rice paddy pore waters, we sampled 17 rice fields in Italy and 6 rice fields in France (Fig. 2a). Rice plants were in the flowering to grain filling stage. Sampling in Italy covered most of the rice cropping areas of the river Po plain, where the majority of the Italian rice is produced (soil pH 5.0-6.1, total As 5.1-16 mg/kg). Sampling in France covered the only rice cultivation area in France which is located in the coastal plain of the Camargue region, in the delta of the river Rhone (soil pH 7.5-7.6, total As 10.4-20.2 mg/kg).

2.1.2 Mesocosm rice cultivation

To study the relevance of thioarsenates over rice growing seasons, mesocosm rice cultivations (0.82 m^2, > 300 rice plants of *Oryza sativa* L. cv. Selenio, ~20 cm depth of topsoil; Fig. 2b) were conducted open air with two Italian paddy soils characterized by highest proportions of thioarsenates in pore-water during our field survey, namely Veronica (E 8°53′48″, N 45°10′39″) and Fornazzo (E 8°57′50″, N 45°13′54″). Each mesocosm was first filled with ~30 cm of gravel (ø 2-5 cm) and overlain by a soil layer of ~20 cm. Six mesocosm of each soil were fertilized with sulfate-containing N-, or K-fertilizers (ammonium sulfate and potassium sulfate; S treatments), while the other six containers of each soil were fertilized with equivalent amounts of fertilizer without sulfate (urea and potassium chloride; no S treatments). In 2017, two seeding practices were adopted under continuous flooding, namely water seeded with continuous flooding (W-CF) and dry seeded with continuous flooding (D-CF). Three containers of each soil and fertilization treatment were managed via W-CF, the other three containers via D-CF. In water seeded, soils were flooded from the day before seeding; in dry seeded, oxic soil conditions were maintained 20 days after seeding. Continuous flooding was conducted by maintaining a standing water of ~10 cm depth during the whole growing season once flooded. Pore waters were extracted at seven rice growing stages, namely tillering stage, stem elongation stage, booting stage, flowering stage, grain filling stage, dough stage, and mature stage.

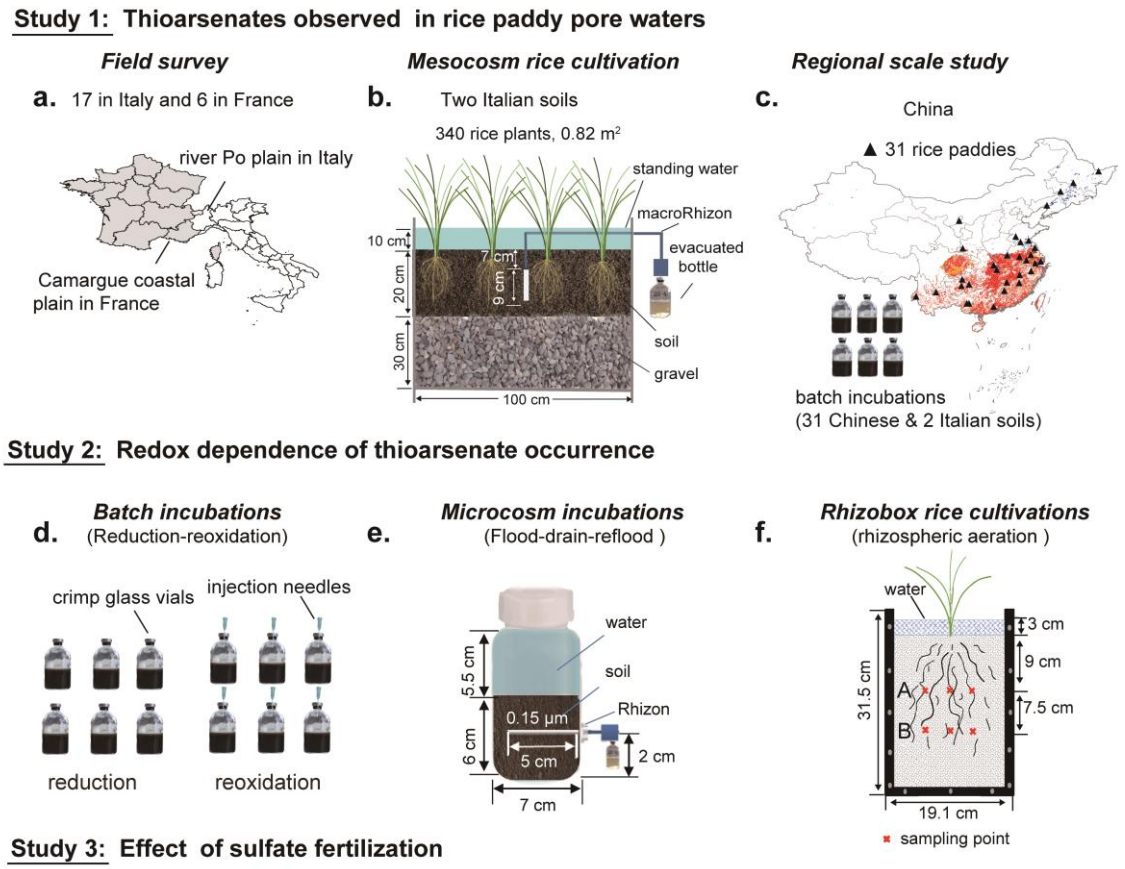


Figure 2 | Experimental setups from study 1 to 3. Study1: field survey in Italy and France (a), design of mesocosm rice cultivation (b) and sampling sites of 31 paddy fields over China (c). The colored background in Chinese map indicates the distribution of rice farming in China. Mesocosms were installed open air at the Rice Research Centre Ente Nazionale Risi in Castello d'Agogna (Pavia, Italy). Study 2: batch incubation under reduction and reoxidation (d), microcosm (e) and rhizobox (f) design. Study 3: sulfate fertilization experiments in 2017 & 2018 (g). In water seeded, soils were flooded from the day before seeding; in dry seeded, oxic soil conditions were maintained 20 days after seeding. Continuous flooding was conducted by maintaining a standing water of ~10 cm depth during the whole growing season once flooded, while wet-dry was conducted by inserting two soil drying period into continuous flooding (7 and 8 days without daily irrigation at half stem elongation and before flowering, respectively). Detailed descriptions for each setup see *Methods* 2.2-2.4.

2.1.3 Regional scale study

To study thioarsenate formation on a regional scale, we sampled paddy soils from the cultivated horizon of 31 representative paddy fields across China (Fig. 2c), one of the biggest rice cultivation countries. Twenty-nine out of the thirty-one paddy fields have total As concentrations below the Chinese risk screening values for contamination of agricultural land, and are thus considered to represent the natural background. We intentionally selected paddy soils having background As levels due to their much widespread distribution than that of anthropogenically contaminated (only 2.7% of paddy soils are As-contaminated [105]). Moreover, contaminated soils often have specific characteristics that greatly depend on contamination source and type (e.g., large input H^+, sulfate and Fe in mining-impacted area). Selected soil properties, including pH, 0.5 M HCl-extractable Fe, Soil Organic Matter (SOM), cation exchange capacity (CEC) , clay content, total As and other chalcophile metals (Cd, Pb, Cu and Zn), and soil zero-valent S content were analyzed by standard methods [106-108]. Anaerobic soil batch incubations with 31 Chinese soils plus two Italian soils (Veronica and Fornazzo) were conducted for a period of 14 days. This duration was assumed from pre-experiments and literature [39] to be sufficient for microbial growth to reach a steady state. For each incubation, 10 g air-dried soil was suspended in 20 mL of 2.5 mM glucose solution without (control, no S) or with 1.5 mM K_2SO_4 (3 mmol/kg sulfate, S) in a glovebox (N_2/H_2 95/5% (v/v)). Vials were then capped with a butyl rubber septum, incubated anaerobically in the dark at room temperature with continuous horizontal shaking (250 rotations min^{-1}).

2.2. Studies conducted in study 2

2.2.1. Batch incubations

Two sets of batch incubations were conducted, namely soil reduction batch incubations and soil reduction-reoxidation batch incubations, to study the temporal formation of thioarsenates under reducing conditions and the effect of soil reoxidation on their transformation, respectively (Fig. 2d). For reduction batch incubations, 20 g air-dried Veronica soil was suspended in 40 mL incubation solution (10 mM acetate solutions without or with the addition

of 1 mM K_2SO_4), incubated anaerobically for 20 days as described above. Acetate was selected here to create low E_H rapidly to maximize initial thiolation. For reduction-reoxidation batch incubations, 9 mmol L^{-1} sulfate and 100 µg L^{-1} arsenate was spiked, together with 2.5 mmol L^{-1} glucose as carbon source, to promote thioarsenates formation. Glucose was used here to stimulate anaerobic bacteria in general [109] and to induce a slower decrease to low E_H. The suspensions were incubated anaerobically for 30 days. Then, air was introduced into the vials by inserting injection needles (23G, Ø 0.06 × 30 mm) through the rubber septa and incubation was continued for another 25 days (reoxidation). Soil solution was obtained at different time points within anaerobic incubation and reoxidation period.

2.2.2. Microcosm incubations

To mimic the natural conditions as close as possible, unplanted microcosm incubations were conducted to simulate the water-management-induced redox fluctuations (Fig. 2e). For each microcosm, 400 g fresh soil from either Veronica or Fornazzo was premixed with 2.5 g rice straw, before adding 250 mL tap water. Microcosms were kept flooded for 20 d, drained for 4 d, and subsequently re-flooded 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 waters were extracted at different time points within flood, drainage and reflood period.

2.2.3. Rhizobox rice cultivations

To further identify the occurrence of thioarsenates in oxygenated rice rhizosphere, rhizobox experiments were performed with two rice cultivars (Yangdao 6, YD and Nongken 57, NK), selected on the basis of their different ROL (0.45 and 1 µmol O_2 per g root and h) [110]. Only Fornazzo soil was used due to its higher potential of thioarsenate formation with respect to Veronica. The rhizobox (19.1 cm × 31.5 cm × 1.5 cm) had a transparent front panel which allowed roots observation, and a rubber back panel equipped with 6 micro-rhizosamplers (two depths A and B, sampling points marked in red in Fig. 2f). One single rice seedling was transplanted into each rhizobox (900 g air-dried soil, kept flooded during whole experiments). After 15 d of pre-incubation, 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. Besides base fertilization of 0.2 g N, 0.2 g K_2O, and 0.15 g P_2O_5 per kg soil, a second fertilization was applied (one third the amount of the initial fertilization) at day 35. After a total of 100 d (15 d pre-incubation and 75 d in the climate chamber), pore waters were extracted as described in 2.4.

2.3. Sulfate fertilization experiments in study 3

To study the effect of sulfate fertilization on pore-water As speciation as well as rice As accumulation, mesocosm rice cultivations were again conducted in 2018 (Fig. 2g). Only water seeding was done. All mesocosms previously planted with dry seeding with continuous flooding (D-CF) were replaced by water seeded with alternate wet-dry irrigation (W-WD). In 2017, 200 g rice straw (cut into ~20 cm length) was mixed with the soil layer; in 2018, rice straw harvested in 2017 (range from 785-1355 g), was returned back to the corresponding mesocosm. Continuous flooding was conducted by maintaining a standing water of ~10 cm depth during the whole growing season once flooded as described above, while alternate wet-drying was conducted by inserting two soil drying periods into continuous flooding (7 and 8 days without daily irrigation at half stem elongation and before flowering, respectively). Due to the persistent rainy days after seeding in 2018, one drainage was conducted at May 19th, which could have caused fertilizer losses. Thus, more N- and K-fertilizers were added in 2018 to meet the demands of rice. Based on a typical Italian soil bulk density of 1.55 g/cm^3, the amounts of inorganic sulfur introduced were calculated to be 81 mg S/kg in 2017, and 124 mg S/kg in 2018.

After harvesting, aboveground biomass of each mesocosm were cut manually, dried in a Static Tray Dryer at 40°C for three days. Rice grain was then separated from straw, weighted as grain production. Harvested rice grain was further dried to a humidity of approximately 10%. After machinery dehusking and polishing, white rice was ground to fine powder for future As extraction and speciation analysis. Grain total As (microwave digested in concentrated HNO_3 and 30% H_2O_2, 0.2 µm filtered) was quantified by ICP-MS. Grain As speciation (extracted with 0.28 M HNO_3 for 90 min at 95°C in a heating block, 0.45 µm filter)

was analyzed by ion-chromatography (IC) (Dionex ICS-3000, Dionex Corp., USA, PRPX-100 column using a 10-40 mM $NH_4H_2PO_4$ pH 5.6 gradient) coupled to ICP-MS.

2.4. General overview of pore-water sampling, sample stabilization, and arsenic determination

For experiments using rhizosamplers (summarized in Table 2), pore waters were extracted via rhizosamplers connected to evacuated oxygen-free glass bottles, sealed with butyl rubber septum. For soil batch incubations, pore waters were obtained by centrifugation (5000 rpm, 3 min) and filtration (0.2 µm). Unstable parameters i.e. pH, E_H, Fe (the ferrozine assay), and sulfide (methylene blue method) were measured immediately. Pore waters for zero-valent S were stabilized with zinc acetate (25 µL of 200 g/L ZnAc +725 µL sample), kept at 4°C until extraction by chloroform. Soil for solid phase zero-valent S extraction was first freeze-dried, then extracted with chloroform (10 mg soil + 700 µL chloroform). Zero-valent S was measured with high performance liquid chromatography (HPLC) (Merck Hitachi L-2130 pump, L-2200 autosampler, and L-2420 UV-VIS detector; C18 column, 100% methanol eluent at 0.2 mL/min). Thus, soil-bound zero-valent S in our study is operationally defined as chloroform-extractable, reduced inorganic S.

Table 2| Rhizosamplers used in each experimental setup from study 1 to 3.

Experiments	**Rhizosampler**	**Parameter [a]**
Field survey & Unplanted microcosm incubation	Standard Rhizon	a porous part of 5 cm with an outer diameter of 2.5 mm
Mesocosm rice cultivation	MacroRhizon	a porous part of 9 cm with an outer diameter of 4.5 mm; faster and higher yield of pore-water than standard Rhizon, suitable for long-term *in situ* sampling (5 months in our study).
Rhizobox rice cultivation	MicroRhizon	a porous part of 8 mm, with an outer diameter of 1 mm; sampling of small pore-water volumes (<2 mL) near the root surfaces once the roots spread out in the rhizoboxes.

[a] All rhizosamplers have a mean pore size of 0.15 µm. Information from https://www.rhizosphere.com/products

Pore waters for total As were acidified in 0.5% H_2O_2 and 0.8% HNO_3, kept at 4°C until analyzed by inductively coupled plasma-mass spectrometry (ICP-MS, XSeries2, Thermo-Fisher). Pore-water As speciation throughout the study was analyzed using ion chromatography (IC, Dionex ICS-3000; AG/AS16 IonPac column, 4 mm, eluent gradient 2.5−100 mM NaOH at a flow rate of 1.2 mL/min) coupled to ICP-MS. Arsenic was detected as AsO^+ at m/z 91 with an O_2/He mixture (10:90%) serving as reaction gas and signal drift was corrected using Rhodium (Rh^+ m/z 103) as an internal standard added manually to each sample.

Since routine acidification cannot be used for thioarsenate preservation, flash-freezing, an As speciation preservation method successfully used in aquatic environments (e.g., hot spring [59] and groundwater [56]), was applied for pore-water stabilization in our initial field survey. An example chromatogram for simultaneous determination of inorganic and methylated thioarsenates, in addition to inorganic and methylated oxyarsenic species, in rice paddy pore waters is shown in appendix study 1, Figure S1. While revealing the occurrence of thioarsenates, this method suffered from low As recoveries (calculated as the sum of all detected As species in flash-frozen samples versus total As measured in acidified samples) in the field survey. The recoveries were generally below 50%, in many cases even below 10%. This was due to Fe (oxyhydr)oxide precipitation that is scavenging As from solution. Additionally, Fe(II)-mediated oxidation could also impact oxygen-sensitive thiolated As species (e.g., dithiolated DMDTA) [56, 62].

To solve this problem, we developed a novel preservation method using neutralized diethylenetriamine-pentaacetic acid (DTPA, 10 mM) to complex excess dissolved Fe, followed by flash-freezing to stabilized pore waters. After sample thawing in the glovebox, sample dilution (1:5) was used to avoid the matrix effect of DTPA (e.g., retention time shifts, peak splitting). An adapted eluent containing 2.4% methanol for chromatographic separation was applied to increase signal intensities of all peaks. Detection limits of the optimized method were 0.03 µg/L As and recovery rates were >80%, which is to date the best stabilization and analysis method, providing the most complete aqueous As speciation data for paddy soil pore waters (up to 11 species). The DTPA-based method was thus used in

microcosm, mesocosm, batch and rhizobox experiments. An example chromatogram for determination of pore-water As speciation using DTPA-based sampling and analytical method is shown in appendix study 1, Figure S18.

3. Results and discussion

3.1. Thiolation as an important factor to arsenic biogeochemistry in paddy soils (study 1)

During the initial survey, thioarsenates were found in 23 out of 35 pore-water samples and in 14 out of 23 paddy fields where rice was in the flowering to grain filling stage. Percentage of thioarsenates (calculated as the sum of all detected thiolated As species in flash-frozen samples versus total As measured in acidified samples) was 8.3% at maximum and 2.1% on average. Inorganic thioarsenates were detected in 11 samples (max. 7.4%, on average 3.2%) and methylated thioarsenates in 18 samples (max. 2.9%, on average 0.7%). Our field survey provided the first evidence for thioarsenate occurrence in paddy fields.

Two Italian paddy soils (one paddy field near Cascina Veronica and the other near Cascina Fornazzo) were selected for subsequent mesocosm rice cultivation. Thioarsenates were observed in all mesocosms of both soils at all seven rice growing stages (tillering, stem elongation, booting, flowering, grain filling, dough, and mature stage) both with and without sulfate fertilization (max. 19%, on average 4.1%). For comparison, maximum percentage of much-better-investigated methylated oxyarsenates was 33%, on average 6.5% (calculated as the sum of DMA and MMA in flash-frozen samples versus total As measured in acidified samples). Our mesocosm experiments confirmed the importance of thioarsenates throughout the rice cropping season.

To estimate the potential for thioarsenate formation on regional scale as well as to identify the soil parameters that are important to their formation, anaerobic soil incubations were conducted with the two above-mentioned Italian soils plus 31 representative paddy soils sampled from across China (Fig. 2c), one of the biggest rice growth countries [1]. Geographic origins of those fields range from 22.5° to 47.2° N and 98.4° to 131.6° E, spanning climate zones from sub-tropical monsoon climate (23 soils) to temperate continental climate (1 soil) and temperate monsoon climate (7 soils). Those paddy soils were developed at sites of different geology and geomorphology (see appendix study 1, Table S5b). After 14 days of incubation, thioarsenates were detected in all the soils both with and without sulfate addition (3 mmol/kg soil). Percentage of thioarsenates ranged from 0.1% to 56%, with an average of

9.6% and a median of 4.8%. For comparison, the percentage of methylated oxyarsenates ranged from 0.5% to 17%, with an average of 3.1% and a median of 1.8%.

With all soil physical and chemical properties (soil pH, total zero-valent S, total soil As, 0.5 M HCl-extractable Fe, cation-exchange capacity, soil organic matter, clay content, and soil chalcophile metals (sum of Cd, Pb, Cu. and Zn)) investigated, soil pH values (when oxic) represented an easy-to-measure parameter indicative for the potential of thioarsenate formations. Formation of inorganic thioarsenates (absolute concentrations) was predominantly related to soil pH > 6.5, while formation of methylated thioarsenates (absolute concentrations) was predominantly observed at soil pH < 7 (see appendix study 1, Figure 4). The pH dependency of thioarsenate formation was at first glance counterintuitive, since pore-water pH values of all incubations were near-neutral to slightly alkaline (6.9 to 7.9), even though soil pH ranged from 4.5 to 9.0. This is because pH of acidic soils will increase due to proton-consuming reductions of Mn(III, IV) and Fe(III) oxyhydroxides, and pH of alkaline soils will decrease due to accumulation of CO_2, making pore-water pH with prolonged flooding typically slightly acidic to near-neutral [33]. A close examination with other soil properties revealed that soil total zero-valent S (sum of aqueous and solid phase zero-valent S formed after soil incubation) represented the most important predictor for inorganic thioarsenate formation. Since zero-valent S formation increased with soil pH, pH dependency of inorganic thioarsenate formation could thus be explained by the indirect effect through zero-valent S. In most of the incubations, aqueous zero-valent S was also detected, but was generally more than one order of magnitude lower than solid phase zero-valent S. This observation supported the notion that formation of inorganic thioarsenates could be controlled by reactions with zero-valent S bound to surfaces of minerals or organic matter in low-sulfide environments (e.g., peatland and groundwater) [55, 111]. Unlike inorganic thioarsenates, formation of methylated thioarsenates was found to be positively related to the concentration of their precursors, methylated oxyarsenates. No correlation with zero-valent S was found for methylated thioarsenates, suggesting that their formation was not limited by S supply but mainly by the availability of methylated oxyarsenates. Since formation of methylated oxyarsenates decreased with soil pH, in line with previous observations in other

environments of highest methylation rates at pH 3.5 to 5.5 [112], thus could explain the predominance of methylated thioarsenates in acidic paddy soils. Negative correlation of methylated thioarsenates with soil pH also suggests that their formation in soil proceeds by nucleophilic attack of reduced S to the As atom which is facilitated at low pH [57].

In summary, on the basis of field, mesocosm and soil incubation studies across multiple paddy soils from rice cultivation areas in Italy, France and China, we highlight thiolation as an important but previously unrecognized factor to arsenic biogeochemistry in rice paddies, with soil pH representing an easy-to-measure parameter indicative for thiolation potential.

3.2. Redox-dependent thioarsenate occurrence in paddy soils and the rice rhizosphere (study 2)

We started soil reduction batch incubations with Veronica soil using acetate as supplement carbon. Acetate addition successfully promoted the rapid formation of reducing conditions (-260 mV at day 20). Pore-water As speciation analysis revealed a rapid and substantial thiolation of methylated oxyarsenates (i.e., DMA and MMA), especially to methylated dithioarsenates with prolonged soil reduction. For example, in sulfate-spiked (1 mmol L^{-1}) incubations, 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. For comparison, 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.

Dominance of methylated dithiolated As, i.e. MMDTA and DMDTA, over methylated monothiolated As, i.e. MMMTA and DMMTA, was also found in subsequent soil reduction-reoxidation batch incubations during the reduction period, yet with an overall less negative E_H (-162 mV at day 30) with glucose addition. However, fast dethiolation occurred after 5 days of soil reoxidation (E_H 97 mV). The decrease of MMDTA was accompanied by the transient rise of MMA and MMMTA, but all monomethylated species decreased to low levels with

prolonged oxidation (less than 5 μg L^{-1}). The decrease of DMDTA was accompanied by an increase of DMA and DMMTA. DMA increased slowly afterward, concurrent with a slight decrease of DMMTA. Our data confirmed the stepwise dethiolation of methylated dithiolated arsenic as suggested by Wallschläger and London (2007) in groundwater upon oxidation during sample storage.[56] The major As species that survived prolonged reoxidation of 25 d (E_H 180 mV) were, in the order DMA, inorganic oxyarsenic species, and DMMTA.

While providing important insights, we were aware that the manner of easily degradable carbon addition and batch incubations (on horizontal shaker), which was commonly used in previous paddy soil studies[113, 114], may not really reflect natural conditions. For example, easily degradable C may selectively promote the growth of r-strategy microbes that have low N/P demands [115]. To mimic natural conditions as close as possible, we designed and conducted unplanted microcosm incubations supplied with rice straw, and incubated under static flooding conditions (Fig. 2e). During incubations, E_H values did not decrease as low as in the batch incubations but were around 0 mV during the flooded period in both Fornazzo and Veronica soils. E_H increased to around 200 mV after drainage for 4 d and dropped again to around 10 mV after reflooding. Consistent with observations of batch incubations, methylated dithiolated As, i.e. MMDTA and DMDTA, were detected as the main methylated thioarsenates in the first flooding. Drainage induced a decrease of both MMDTA and DMDTA concentrations, concurrent with an increase in concentrations of MMMTA and DMMTA, respectively, in line with the results of the above-mentioned reduction-reoxidation batch incubations during the reoxidation period. After reflooding, there was a slight decrease in concentrations of MMMTA and DMMTA, but both concentrations were higher than in the first flooding.

Beside methylated thioarsenates, inorganic thioarsenates, i.e. MTA, DTA and TTA, were also detected in above-mentioned experiments, forming rapidly during reduction/flooding period in both soils, but generally with lower absolute total concentrations than that of methylated thioarsenates. Lower inorganic thioarsenate formation versus methylated thioarsenate formation was in line with Study 1, where acidic soil (oxic soil pH for Veronica and Fornazzo was 5.6 and 5.8, respectively) with a correspondingly low content of soil zero-valent sulfur

was found to be the limiting factor for inorganic thiolation. However, soil reoxidation caused nearly complete dethiolation of inorganic thioarsenates. Only monothioarsenate formed again upon short-term reflooding in the unplanted microcosm experiments.

Due to the observed lability of higher order thiolated species (e.g., DMDTA, MMDTA) in above-mentioned experiments as well as in previous oxidation studies (solution without soils) [110, 116], we hypothesized that rhizospheric aeration could act as an oxidative barrier for higher order thiolated species, while monothiolated As species could be available for root uptake. Rhizobox rice cultivations were thus performed to identify thiolated species that occur in the rice rhizosphere (design see Fig. 2f). At the day of sampling, rice roots had a macroscopically visible length of about 30 cm, so both pore-water sampling depths A and B (9 and 16.5 cm below the soil-water interface) were located within the rhizosphere, with dense and overlapping rice roots. MTA was the major inorganic thioarsenate detected in the rhizosphere (max. 0.7 ± 0.3 µg L^{-1}). MMMTA was the second-highest thiolated species (up to 1.5 µg L^{-1}), with concentrations slightly lower than its precursor MMA. DMMTA was detected as the dominant thiolated As species (up to 2.2 µg L^{-1}), with concentrations comparable to its precursor DMA. As expected, methylated dithiolated As, i.e. MMDTA and DMDTA, were detected but in much lower concentrations compared to their respective monothiolated As forms. In accordance with previous studies that indicate the potential contribution of DMMTA to rice grain As accumulation,[91, 110] here we provide the first direct evidence that DMMTA occurs in the oxygenated rice rhizosphere and is available for root uptake.

Combination of all observations from the batch and microcosm experiments based on the prevailing redox potential yielded a pattern of redox-dependent As thiolation, with E_H ranging from -260 mV (soil reduction batch incubations), -200 to -100 mV (soil reduction-reoxidation batch incubations, reduction period) and -50 to +100 mV (unplanted microcosm incubations) to + 200 mV (soil reduction-reoxidation batch incubations, reoxidation period) (see appendix study 2, Fig. 5). Total thiolation of inorganic As species was much lower than thiolation of methylated As with a maximum of 57% at E_H -132 mV and oxidation caused nearly complete dethiolation. 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. 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[17], in line with study 1. 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) [116] and in DMA-spiked landfill leachates under sulfidic conditions [117].

Due to the limited amount of sample volume, no E_H value was measured in the rhizobox experiment. However, E_H of the same soil (Fornazzo) were measured previously both directly in the planted field (+98 mV) and in mesocosm experiments with rice straw (+132 $\pm$ 45 mV, over a whole growth period, with/without sulfate fertilization, n=28) [76]. E_H in the rhizobox of the present study are assumed to be very similar (+100 to +150 mV). Little thiolation of inorganic As (median 9.2 % total thiolation, 6.1% monothiolation), little dithiolation of methylated oxyarsenates (median 16.8% for MMA, 6.1 % for DMA), a high share of monothiolation for MMA (median 32%) and DMA (median 42%), and a dominance of the methylated oxyarsenates fit well into the general observations for redox-dependent arsenic thiolation.

In summary, on the basis of batch incubations, unplanted microcosm experiments and rhizobox rice cultivations, we found a general pattern of redox-dependent thioarsenate occurrence in paddy soils from E_H -260 to +200 mV. Soil E_H values represented an easy-to-measure parameter indicative for arsenic thiolation in paddy soils under changing redox conditions. Among all thioarsenates, DMMTA showed the least transformation upon prolonged oxidation. It also was the major thiolated As species in the rhizosphere with concentrations comparable to its precursor DMA, which could be critical since dimethylated monothioarsenate is highly cytotoxic [118, 119].

3.3. Effects of sulfate fertilization (study 1 and 3)

On regional scale (31 Chinese paddy soils in study 1), sulfate amendment had less pronounced effects on arsenic thiolation in the anaerobic soil incubations, when compared with the strong effects of the different soil properties. Sulfate amendment did not generally change the relative differences in thioarsenate formation potential between different soils, but it promoted total arsenic thiolation by increasing zero-valent S contents and decreasing pore-water Fe concentrations and redox potential. For the two Italian soils, sulfate addition increased zero-valent S from 0.14 to 0.41 mmol/kg and from 0.34 to 0.39 mmol/kg for Veronica and Fornazzo, respectively. The stronger increase of zero-valent S formation in Veronica soil may explain the observed stronger increase in total As thiolation in the soil incubations (inorganic thioarsenates from 3.9 to 24%, methylated thioarsenates from 25 to 32%) in Veronica, in compared with Fornazzo soil. In the mesocosm experiments (study 1), sulfate fertilization increased thioarsenate formation over the rice growing season (average total thiolation from 2.2% to 5.9%). In line with the anaerobic soil incubation, a stronger increase in average proportions of both methylated and inorganic thioarsenates was found for Veronica compared to Fornazzo soil in mesocosm rice cultivation.

Both methylated and inorganic thioarsenates can be taken up by rice plants as shown in previous hydroponic studies [110, 120]. However, there is currently no robust method to determine thioarsenates in rice grains. Thioarsenates, if present, would be determined together with their oxyarsenic counterparts due to the hot acid digestion of rice grains in our study [110]. To evaluate how sulfate fertilization impacts grain As accumulation via its impacts on pore-water As speciation, we therefore summed inorganic oxyarsenic and inorganic thioarsenates together as inorg-As and methylated oxyarsenate and methylated thioarsenates as methyl-As (detailed information for each species see Table 1).

Sulfate fertilization decreased significantly the pore-water total As concentration over rice grow seasons in two cropping years and in both soils, irrespective of management practices. The decrease of pore-water total As was represented by the decrease of inorg-As (mainly arsenite and arsenate), resulting in a substantial decrease of inorg-As accumulation in white rice in all treatments. Decrease of As bioavailability in pore waters upon sulfate fertilization has been reported before in both unplanted soil incubations and pot experiments [79, 81, 102],

which is explained by the enhanced precipitation of As with newly formed Fe-As-S minerals [67, 102, 103]. Sulfate fertilization had no appreciable effects on pore-water methyl-As (absolute concentrations) in 2017, while a significant increase of methyl-As, especially in Veronica soil, over the rice growing season was found in 2018. A recent study by Chen et al. (2019) shows that sulfate-reducing bacteria and methanogenic archaea are involved in As methylation and demethylation, respectively [37]. The large input of sulfate in 2018 together with sulfate remaining from the previous-year fertilization (81 mg S/kg in 2017, and 124 mg S/kg in 2018) could stimulate the activity of sulfate-reducing bacteria, but simultaneously suppress activity of methanogenic archaea as indicated by previous sulfate fertilization studies [64, 102, 104, 121], therefore explain the increased methyl-As concentration in pore waters during the second cropping year. In accordance with the increased methyl-As in pore-water, sulfate fertilization slightly increased methyl-As contents in white rice harvested from Fornazzo soil in both years. However, its effect on white rice methyl-As contents harvested from Veronica soil was inconsistent, suggesting other plant-soil factors may also impact grain methyl-As accumulation.

In summary, our sulfate fertilization experiments evidenced sulfate cycling as an important factor in influencing As speciation in pore waters, as well as the accumulation of As in rice grains. Sulfate fertilization promoted both arsenic thioaltion and methylation, but decreased its bioavailability (mainly inorganic oxyarsenic species) towards rice plants. Sulfate fertilization could be an effective way to decrease the accumulation of cancerogenic inorganic As in rice. Further studies are needed to test the effectiveness of sulfate fertilization under field conditions and with different soil types, as well as to develop explicitly methods to determine thioarsenates in rice grains.

4. Conclusion

Dietary exposure of arsenic, a nonthreshold class-1 carcinogen, from rice is a trans-boundary health issue of global consequence. Comprehensive understanding of arsenic speciation is the key to understand As geochemical behaviors in paddy environments and hence its accumulation in rice grains. Current research on arsenic speciation in paddy soils has been mainly evolved around inorganic oxyarsenic (arsenite and arsenate) and methylated oxyarsenate (DMA and MMA). It remains unknown whether and to what extent sulfur-mediated thioarsenate formation influences As speciation in paddy soil pore waters. The central aim of this thesis was therefore to elucidate the role of thioarsenates in paddy soils, and to identify soil parameters that are important for its occurrence. Since there exists sparse data about how sulfate fertilization influences As speciation, we further examined the effects of external sulfate input on As speciation in paddy soil pore waters and rice grains.

To tackle the problems of acidification-induced thioarsenate loss and co-elution with oxyarsenic species, which hindered thioarsenate determination in previous studies, a novel diethylenetriamine-pentaacetic acid (DTPA)-based sampling and analytical method was developed. This method enabled the simultaneous determination of both inorganic and methylated thioarsenates (Fig. 3a Arsenic thiolation), in addition to their respective oxyarsenic species, providing the so far most complete aqueous As speciation data for paddy soil pore waters. Thioarsenates formed in various paddy soils originating from different climate zones and parent materials, throughout the whole cropping season, with concentrations comparable to the much-better-investigated methylated oxyarsenates. On regional scale, soil pH represented an easy-to-measure parameter indicative for thiolation potential under anaerobic conditions (Fig. 3b pH dependency). Inorganic thioarsenates formed predominantly in alkaline soils, controlled by the presence of zero-valent sulfur, while methylated thioarsenates were predominantly observed in acidic soils, related to the presence of their precursors, methylated oxyarsenates. For paddy soil under seasonal flooding and drainage, the importance and occurrence of thioarsenates in pore waters are highly redox-dependent (Fig. 3c redox dependency). Total thiolation of inorganic As species was much lower than thiolation of methylated As species and soil oxidation caused nearly

complete dethiolation. In contrast, thiolation of methylated As species played an important role over a wide range of soil redox potential, with total thiolation of mono- and dimethylated As up to 70% and 100% when below E_H 0 mV , respectively. Higher order thiolated species, that is DTA, TTA, MMDTA, and DMDTA, were preferentially formed over their monothiolated forms, that is MTA, MMMTA and DMMTA, with prolong soil flooding (e.g., below E_H -100 mV). However, the opposite trend occurred when flooded soil became re-oxidized. Among all thioarsenates, highly cytotoxic DMMTA showed the least transformation upon prolonged oxidation and also represented the major thiolated arsenic species in the oxygenated rhizosphere, which warrants future studies regarding its uptake, translocation and accumulation in rice.

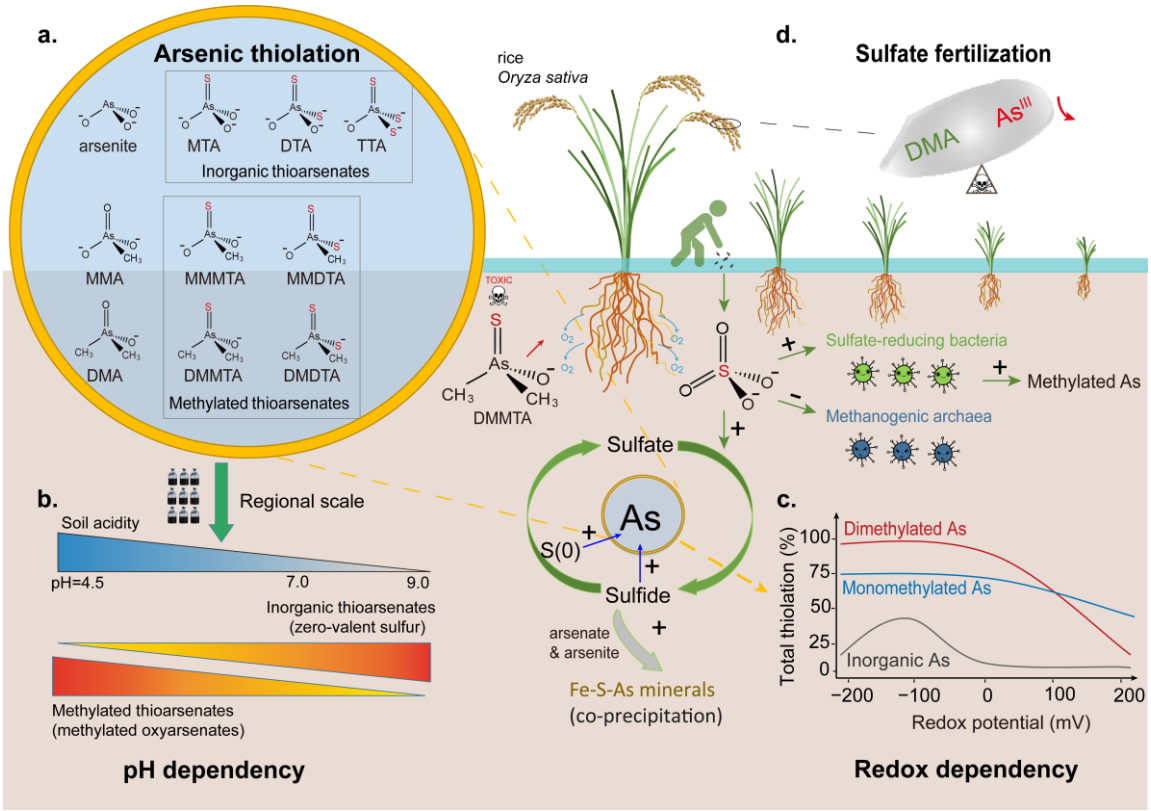


Figure 3 | Summarized and simplified conceptual model for effects of sulfur on arsenic speciation in pore waters [76] and rice grains. Signs “+” and "-" indicate positive and negative effect of sulfate fertilization on the corresponding processes, respectively. The effects of sulfate on the activities of sulfate-reducing bacteria versus methanogenic archaea are from refs [66, 104].

One of the main contributions of this thesis is to highlight thiolation as an important factor to arsenic biogeochemistry in rice paddies, advancing in general our current understanding of the role of sulfur cycling for As speciation. We challenged the current paradigm that thioarsenates only formed substantially in environments under sulfide excess, i.e. sulfur-controlled systems (reactive Fe < sulfate). Instead, we proposed that low but continuously replenished sulfide and zero-valent S during the “cryptic” S-cycle could support substantial arsenic thiolation even in low-sulfate terrestrial environments. Given the ubiquitous occurrence of arsenic and sulfur, thioarsenates need to be considered in the arsenic budget not only of paddy soils, but also in other analogue low-sulfate environments (e.g., floodplains, wetlands). Arsenic thiolation, its occurrence and importance in different environments as well as its dependence on other biogeochemical factors (in addition to soil pH/redox dependency) need to be deciphered in future studies in order to paint a more accurate picture of global arsenic cycling.

From an agronomic point of view, our study supported sulfate fertilization as an effective measure to mitigate the accumulation of cancerogenic inorganic arsenic in rice grains, irrespective of seeding practices and water managements (Fig. 3d Sulfate fertilization). Sulfate fertilization decreased the bioavailability of inorganic oxyarsenic during rice cultivation, due to the sulfate-fertilization-enhanced co-precipitation of arsenite and arsenate with newly formed Fe-As-S minerals. While natural sulfate contents were sufficient in supporting As thiolation, sulfate fertilization could further increase thioarsenate formation, especially for paddy soils with low initial zero-valent S formation. Sulfate fertilization also increased methylated As formation, owing to its opposite effects on the activity of sulfate-reducing bacteria (positive) and methanogenic archaea (negative), which are known to be involved in As methylated and demethylation in paddy soils, respectively.

This thesis clearly illustrates the importance of sulfur in influencing As speciation in paddy soils and its accumulation in rice grains, but it also raises the question of whether thiolation is good or bad for rice consumption safety. However, the routine hot acid digestion method used in this study failed to distinguish thiolated As species from their oxyarsenic counterparts (e.g., highly cytotoxic DMMTA versus less toxic DMA) in rice grains. Future studies are

needed to develop a reliable species-selective extraction and analytical methods to evaluate the contribution of thioarsenates, in particular the highly toxic, oxidation-resistant DMMTA, to grain As accumulation.

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Contribution to studies 1 to 3

Study 1: Thiolated arsenic species observed in rice paddy pore waters

Jiajia Wang	40%	initiated DTPA method development, conceived and performed all mesocosm and incubation experiments, including analyses, evaluated the results and statistics and contributed to manuscript preparation
Carolin Kerl	20%	contributed to field survey, sample analyses, data evaluation, and manuscript preparation
Pengjie Hu	3%	initiated the Chinese soil survey, selected most representative paddy soils samples, advised on incubation experiments
Maria Martin	3%	contributed to field survey and data discussion, assisted in analyses of aqueous parameters from mesocosms
Tingting Mu	2%	sampled and characterized the Chinese soils
Lena Brüggenwirth	2%	contributed to DTPA method development
Guangmei Wu	2%	sampled and characterized the Chinese soils
Daniel Said-Pullicino	3%	assisted in analyses of aqueous parameters from mesocosms and data discussion
Marco Romani	2%	assisted in design, setup and operation of mesocosms and sample collection
Longhua Wu	3%	initiated the Chinese soil survey, selected most representative paddy soils samples, advised on incubation experiments
Britta Planer-Friedrich	20%	initiated and supervised the project, carried out the field survey, conceived experiments, and wrote the manuscript

Study 2: Redox Dependence of Thioarsenate Occurrence in Paddy Soils and the Rice Rhizosphere

Jiajia Wang	65%	development of research concept, supervised the project, contribute to Flood-drain-reflood microcosm incubations , analyses and data interpretation, preparation of manuscript
Dipti Halder	3%	contributed to Reduction batch incubations and sample analyses, discussion of results
Laura Wegner	3%	contributed to Reduction-Reoxidation batch Incubations and sample analyses, data evaluation
Lena Brüggenwirth	3%	contributed to Rhizobox rice cultivations and sample analyses, data evaluation
Jörg Schaller	1.5%	assisted in Reduction batch incubations , comments on manuscript
Maria Martin	1.5%	assisted in soil sample collection and microcosm incubations design , comments on manuscript
Daniel Said-Pullicino	1.5%	assisted in soil sample collection and microcosm incubations design , comments on manuscript
Marco Romani	1.5%	assisted in design, setup and operation of mesocosms and sample collection
Britta Planer-Friedrich	20%	development of research concept, discussion of results, comments on manuscript and contribution to writing of manuscript

Study 3: Sulfate fertilization influences the transfer of inorganic and methylated arsenic from paddy soil to rice grain

Jiajia Wang	70%	development of research concept, performed mesocosm experiments, including pore-water sampling and analyses, evaluated the results and statistics and contributed to manuscript preparation
Shuai Zhang	5%	assisted in mesocosm experiments, including sampling and harvest
Carolin Kerl	2%	rice sample arsenic analyses
Umberto Roll	2%	assisted in mesocosm setup and operation, including sampling and harvest
Gianluca Beltarre	2%	assisted in mesocosm setup and operation, including sampling and harvest
Maria Martin	2%	assisted in analyses of aqueous parameters from mesocosms
Daniel Said-Pullicino	2%	assisted in analyses of aqueous parameters from mesocosms
Marco Romani	5%	assisted in design, setup and operation of mesocosms and sample collection
Britta Planer-Friedrich	10%	development of research concept, discussion of results, first comments on earlier version of the manuscrip

Appendix: Studies 1-3

Study 1

Wang, Jiajia.; Kerl, C. F.; Hu, P.; Martin, M.; Mu, T.; Brüggenwirth, L.; Wu, G.; Said-Pullicino, D.; Romani, M.; Wu, L.; Planer-Friedrich, B., Thiolated arsenic species observed in rice paddy pore waters. Nature Geoscience 2020, 13, (4), 282-287.

Study 2

Wang, Jiajia.; Halder, D.; Wegner, L.; Brüggenwirth, L.; Schaller, J.; Martin, M.; Said-Pullicino, D.; Romani, M.; Planer-Friedrich, B., Redox Dependence of Thioarsenate Occurrence in Paddy Soils and the Rice Rhizosphere. Environmental Science & Technology 2020, 54, (7), 3940-3950.

Study 3

Wang, Jiajia.; Kerl, C.; Zhang, S.; Roll, U.; Beltarre, G.; Martin, M.; Said-Pullicino, D.; Romani, M.; Planer-Friedrich, B., Sulfate fertilization influences the transfer of inorganic and methylated arsenic from paddy soil to rice grain. (draft) 2020.

Study 1: Thiolated arsenic species observed in rice paddy porewaters

Jiajia Wang; Carolin F. Kerl, Pengjie Hu, Maria Martin, Tingting Mu, Lena Brüggenwirth, Guangmei Wu, Daniel Said-Pullicino, Marco Romani, Longhua Wu, Britta Planer-Friedrich

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Arsenic in paddy field pore waters

Thiolated arsenic species observed in rice paddy pore waters

Jiajia Wang[1], Carolin F. Kerl[1], Pengjie Hu[2], Maria Martin[3], Tingting Mu[2], Lena Brüggenwirth[1], Guangmei Wu[2], Daniel Said-Pullicino[3], Marco Romani[4], Longhua Wu[2] and Britta Planer-Friedrich[1]*

[1]Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany.
[2]Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, 210008 Nanjing, China
[3]Department of Agriculture, Forest and Food Sciences, University of Turin, 10124 Turin, Italy
[4]Rice Research Centre, Ente Nazionale Risi, 27030 Castello d'Agogna, Pavia, Italy
*Phone: +49 921 55 3999. E-mail: b.planer-friedrich@uni-bayreuth.de

Abstract

The accumulation of carcinogenic arsenic in rice, the world's main staple crop, represents a health threat to millions of people. The speciation of arsenic controls its mobility and bioavailability and therefore its entry into the food chain. Inorganic and methylated oxyarsenic species have been a focus of research, but arsenic characterization in the field has largely ignored thioarsenates, in which sulfur takes the place of oxygen. Here, on the basis of field, mesocosm and soil incubation studies across multiple paddy soils from rice cultivation areas in Italy, France and China, we find that thioarsenates are important arsenic species in paddy-soil pore waters. We observed thioarsenates throughout the cropping season, with concentrations comparable to the much-better-investigated methylated oxyarsenates. Anaerobic soil incubations confirmed a large potential for thiolation across a wide diversity of paddy soil types in different climate zones and with different parent materials. In these incubations, inorganic thioarsenates occurred predominantly where soil pH exceeded 6.5 and in the presence of zero-valent sulfur. Methylated thioarsenates occurred predominantly at soil pH below 7 and in the presence of their precursors, methylated oxyarsenates. High concentrations of dissolved iron limited arsenic thiolation. Sulfate fertilization increased thioarsenate formation. It is currently unclear whether thiolation is good or bad for rice consumption safety. Nevertheless, we highlight thiolation as an important factor to arsenic biogeochemistry in rice paddies.

Main

Rice is the main staple crop for more than half of the world´s population. At the same time, it represents a major dietary source of arsenic (As), a class I carcinogen[1]. Premises for As accumulation in rice grains are its global occurrence in soils and efficient uptake by rice plants together with essential nutrients[2,3,4]. Soil-derived As becomes plant-available under flooded conditions when the reductive dissolution of iron (Fe) (oxy)hydroxides and arsenate reduction release sorbed As[5]. Pore-water As speciation is dominated by inorganic As (arsenite and arsenate). Microbe-mediated As methylation leads to formation of mono- (MMA) and dimethylarsenate (DMA)[6], which typically are minor species in pore waters[7] although DMA can contribute up to 90% of total As in the grain due to high root–shoot translocation[8]. Current research on As biogeochemistry in paddy soils has been mainly focused on these four oxyarsenic species, and it is well accepted that As speciation is responsible for its mobility and bioavailability[9].

Our objective was to reveal whether and to what extent thioarsenates contribute to As speciation in

paddy soil pore waters. Thioarsenates are pentavalent As species in which sulfur (S) replaces oxygen. They form upon reaction of arsenite with zero-valent S and sulfide (in case of inorganic thioarsenates)[10] or MMA and DMA with sulfide (in case of methylated thioarsenates)[11,12] (Fig. 1). Thioarsenates typically occur in aquatic environments with excess dissolved sulfide[13]. Just very recently they have also been detected in low-sulfide environments where thiolation is probably controlled by reactions with reduced S bound to surfaces of minerals or organic matter[10,14].

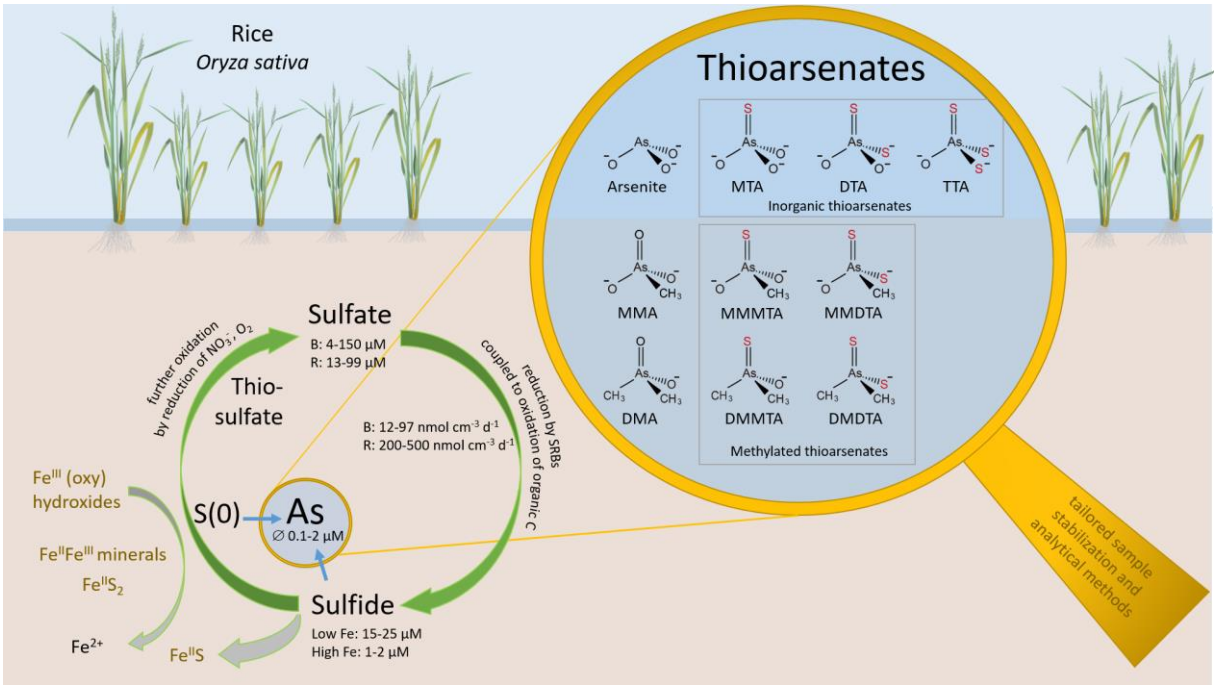

Fig. 1: Conceptual model for the formation of thioarsenates in paddy soils coupled to a cryptic S cycle. Low but continuously replenished concentrations of sulfide and zero-valent sulfur (S(0)) lead to As thiolation instead of or besides As scavenging by newly formed mixed $Fe^{II}Fe^{III}$ minerals and pyrite (FeS_2) or, at excess sulfide, mackinawite (FeS) and AsS; concentrations and rate numbers (taken from refs. [18,36]) are displayed to present typical quantities and extents of sulfate reduction rates (B, bulk soil; R, rhizosphere; SRB, sulfate-reducing bacteria): MTA, monothioarsenate; DTA, dithioarsenate; TTA, trithioarsenate; MMA, monomethylarsenate; MMMTA, monomethylmonothioarsenate; MMDTA, monomethyldithioarsenate; DMA, dimethylarsenate; DMMTA, dimethylmonothioarsenate; DMDTA, dimethyldithioarsenate. All species are displayed in their fully deprotonated form for clarity.

To date, occurrence of thioarsenates in paddy soils has never been addressed, which is only partially a methodological problem. Routine sample preservation and many chromatographic separation methods use acids that transform thioarsenates to arsenite or oxyarsenates[15] or lead to As loss by As–S precipitation[16], so thioarsenates are plainly overlooked. However, the main reason for neglect of thioarsenates is a conceptual limitation because flooded paddy soils are regarded primarily as methanogenic environments[17]. Sulfate reduction, although thermodynamically favoured relative to methanogenesis, is often considered insignificant due to typically low sulfate contents[18] (except for acid sulfate soils) and sulfide reactivity being limited by mackinawite ($Fe^{II}S$) precipitation[19]. There is, however, evidence for a 'cryptic' S cycle[18], where sulfide reoxidation to zero-valent S is coupled to reduction of Fe^{III} (oxy)hydroxides and formation of mixed $Fe^{II}Fe^{III}$ minerals or pyrite ($Fe^{II}S_2$) besides

Fe^{2+} (ref. [20]). Further S oxidation to thiosulphate and sulfate is coupled to nitrate or oxygen reduction (Fig. 1). Such an S cycle sustains high sulfate reduction rates in the bulk soil and especially the rhizosphere[18]. We hypothesized that low but continuously replenished sulfide and zero-valent S could promote thioarsenate formation besides or instead of As scavenging on newly formed Fe minerals[21]. A similar observation was made in paddy-soil incubation studies where initially sequestered As was remobilized under sulfidic conditions[22]. Thioarsenate formation was suspected, but no As–S speciation analysis was done. Sulfate fertilization, recently investigated for its potential benefits in improving nutrient uptake and rice growth[23], as well as mitigating methane emissions[24,25] and rice As accumulation[26,27], might further contribute to thioarsenate formation.

During an initial field survey, we discovered thioarsenates while sampling planted paddy fields covering the main rice cropping areas of the Po River plain in Italy (soil pH 5.0–6.1, As 5.1–16 mg kg^{-1}) and of the Camargue coastal plain in France (soil pH 7.5–7.6, As 10.4–20.2 mg kg^{-1}). Contribution of total thiolation to total As concentrations was 8.3% at maximum and 2.1% on average, numbers comparable to those observed for the much more-commonly investigated methylated oxyarsenates (for details, see Supporting Information section 1, Supplementary Table 1 and Supplementary Figs. 1–4).

Key to all further investigations was the development of a sampling and analytical method using diethylenetriamine-pentaacetic acid (DTPA, 10 mM) to complex excess dissolved Fe, followed by flash freezing for sample preservation, sample dilution and the use of an adapted eluent for chromatographic separation to avoid negative effects of high DTPA concentrations such as retention time shifts, peak splitting and poor species resolution. Detection limits of the optimized method were 0.03 µg L^{-1} As and recovery rates >80%, which is the best available stabilization and analysis method for detection of the up to 11 species of interest (see Methods for details).

We then examined the occurrence of inorganic and methylated thioarsenates in comparison with their oxyarsenic analogues over a range of scales moving from our initial field surveys to controlled mesocosm experiments and laboratory soil incubations.

Thioarsenate formation in rice cultivation mesocosms

On the basis of the field survey (Supplementary Table 1), we selected two Italian paddy soils (a eutric gleysol from a paddy field near Cascina Veronica and an umbric gleysol from a paddy field near Cascina Fornazzo), characterized by highest proportions of thiolation, for mesocosm experiments (setup, see Supplementary Fig. 5). The two soils had the same total soil As contents (5.5 mg kg^{-1}) and were relatively similar in soil pH (5.6 and 5.8 for Veronica and Fornazzo, respectively), while compared with Fornazzo, Veronica had slightly lower contents of 0.5 M HCl-extractable Fe (52 versus 71 mmol kg^{-1}), total C (2.0 versus 4.7%), and total S (2.6 versus 3.2 g kg^{-1}) (Supplementary Table 2). All mesocosms were planted with the same rice variety (*Oryza sativa* L. cv. Selenio) and managed in a randomized factorial arrangement representing (1) treated with (S) or without sulfate (no S, control) fertilizers and (2) water or dry seeded (Supplementary Figs. 5 and 6). In water-seeded treatments, soils were flooded from one day before seeding throughout the growing season, while in dry-seeded treatments, oxic soil conditions were maintained until tillering stage (20 days after seeding), after which the soils were flooded (Supplementary Fig. 6). Consequently, dry-seeded soils showed higher redox potentials and lower pore-water Fe^{II} concentrations at tillering stage compared with the water-seeded treatments (Supplementary Table 3).

Thioarsenates were observed in all mesocosms of both soils at all seven sampling stages (tillering, stem

elongation, booting, flowering, grain filling, dough and mature) under both water- and dry-seeded treatments (Fig. 2a–d). The contribution of thioarsenates to total As ranged from 0.1% to 19%, on average 4.1%. For comparison, methylated oxyarsenates ranged from below detection limit to 33%, on average 6.5%. Concentrations of inorganic thioarsenates were generally higher (maximum 6.4 µg L^{-1} or 19% of total As) than those of methylated thioarsenates (maximum 1.1 µg L^{-1} or 8.2% of total As) in both soils. No clear trend in the proportion of inorganic or methylated thioarsenates was observed over time (Fig. 2a–d). For details on trends in total As pore-water concentrations over time, see Supporting Information section 2.

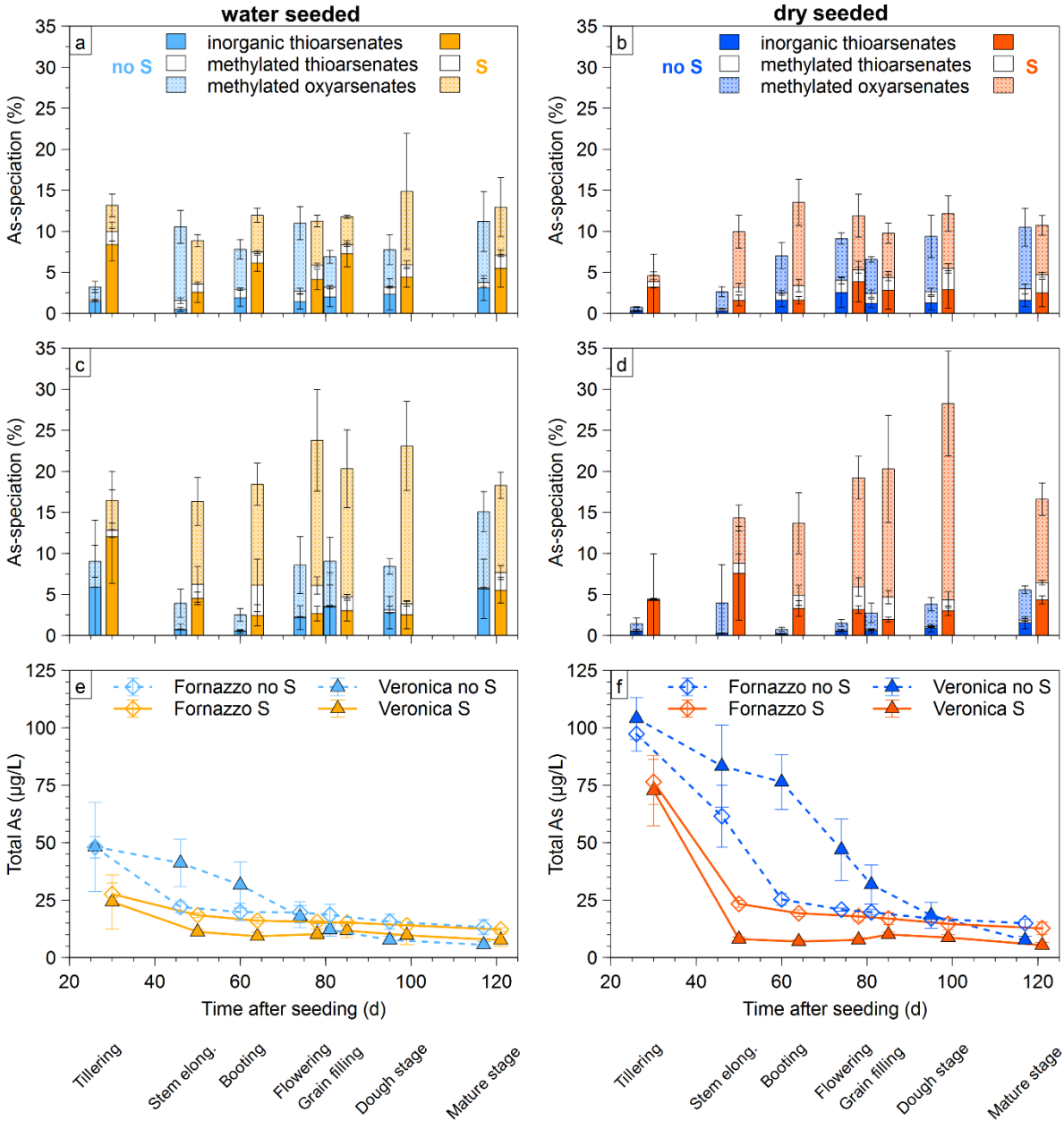

Fig. 2: Pore-water As thiolation, methylation and total As concentrations over time during rice cultivation. **a**, Fornazzo soil, water seeded. **b**, Fornazzo soil, dry seeded. **c**, Veronica soil, water seeded. **d**, Veronica soil, dry seeded. Blue colours refer to control treatments (no S), orange–red colours to sulfate addition (S); percentages refer to proportion of total As. **e**,**f**, Total As concentrations for the two soils with and without sulfate addition.

Data represent the mean; whiskers represent the standard deviation from three mesocosms (n = 3).

Sulfate fertilization substantially decreased total pore-water As concentrations (Fig. 2e,f). All S fertilized mesocosms, including dry-seeded Veronica soil, had pore-water As concentrations at or below 20 µg L^{-1} already at the stem elongation stage. The same faster decrease in As concentrations upon addition of sulfate has been reported before due to stimulation of sulfate-reducing bacteria, increased sulfide production and formation of new Fe minerals[20,28] (Fig. 1). A decrease due to re-adsorption was, however, observed mainly for inorganic oxyarsenic species, while proportions of inorganic and methylated thioarsenates, as well as methylated oxyarsenates, increased with sulfate fertilization (Fig. 3). Average total thiolation with sulfate addition was 5.9% compared with 2.2% in controls; average methylation was 8.8% compared with 4.3% in controls. Higher proportions of methylated As species upon sulfate addition are in line with previous observations[29]. Similar to the control treatment, there were no significant trends in thiolation or methylation over time (Fig. 2a–d).

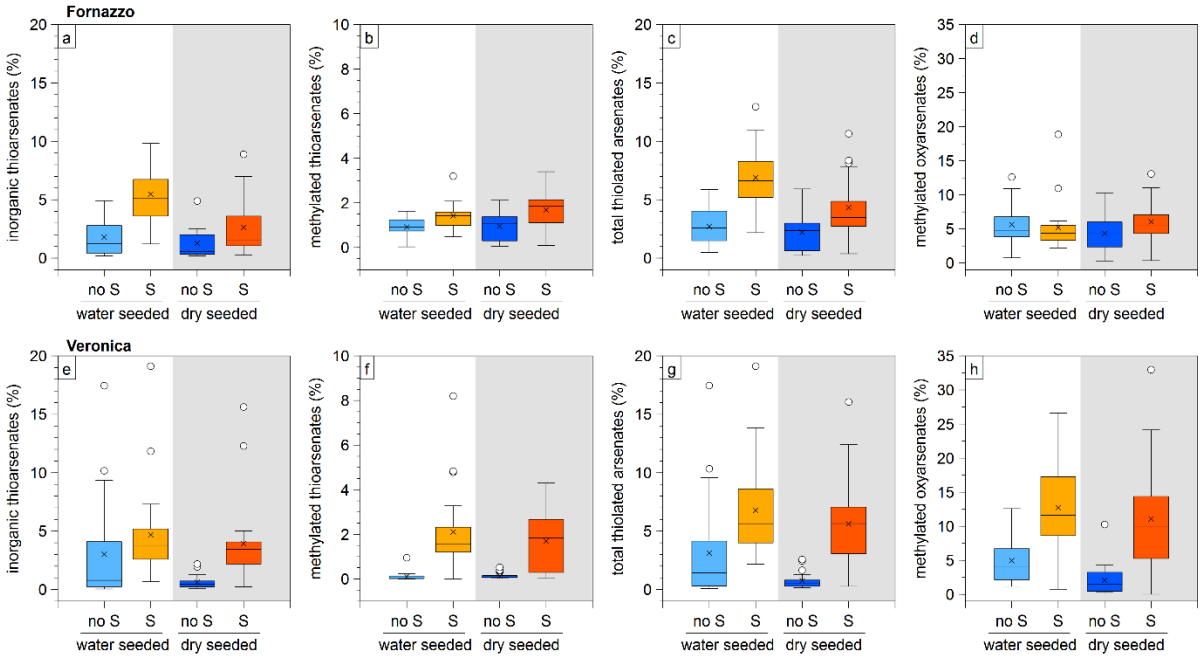

Fig. 3: Proportions of inorganic, methylated and total thioarsenates, as well as methylated oxyarsenates, integrated over time.a–h, Inorganic thioarsenates (**a**,**e**), methylated thioarsenates (**b**,**f**), total thioarsenates (**c**,**g**) and methylated oxyarsenates (**d**,**h**) integrated over all sampling times for Fornazzo and Veronica soils, respectively, water-seeded (left side of each graph) and dry-seeded (right, shaded side) treatment. Blue colours refer to control treatments (no S), orange–red colours to sulfate addition (S); percentages refer to proportion of total As. Box plots: line, median; cross, mean; box, interquartile range; whiskers, 1.5 interquartile range; data from three mesocosms over seven times ($n = 21$).

Seeding practices affected not only total As concentrations (with higher concentrations in dry- compared with water-seeded treatments) but also As thiolation. Both without and with sulfate fertilization, higher percentages were observed in water- compared with dry-seeded mesocosms due to the longer duration of anaerobic conditions in the former (Fig. 3c,g).

Comparison between the two soils showed that sulfate fertilization had a stronger impact in Veronica

compared with Fornazzo soil. We observed both a stronger decrease in total As concentrations (Fig. 2e,f) and a stronger increase in average proportions of methylated oxy- and thioarsenates in both water- and dry-seeded treatments, as well as inorganic thioarsenates in dry-seeded treatments, for Veronica compared with Fornazzo soil (Fig. 3 and Supplementary Table 4). The exact redox chemistry, especially the role of organic C, remains to be investigated, but we propose that the lower soil C content caused less-reducing conditions (reflected in higher redox potentials and less aqueous Fe^{II}; Supplementary Table 3) in Veronica soil compared with Fornazzo soil. Thereby, besides efficient removal of As on mixed $Fe^{II}Fe^{III}$ minerals, more recycling of sulfate-reducing-bacteria-produced sulfide to sulfate with formation of zero-valent S and As thiolation (see also the final paragraph of section 'Thioarsenate formation potential in soil incubations') was favoured, compared with more removal on FeS minerals and less thiolation in Fornazzo soil.

Finally, multivariate regression tree analysis comparing the relative importance of the investigated effects on pore-water As speciation in our mesocosms showed the clearest separation between sulfate and non-sulfate treatments, followed by the differences of the two selected soil types. The different rice growing stages had the least effects on pore-water As speciation (Supplementary Fig. 7).

Thioarsenate formation potential in soil incubations

To estimate the potential for thioarsenate formation on a large scale, we conducted anaerobic soil incubation experiments with the two Italian soils plus 31 soils sampled from across China (for coordinates, see Supplementary Table 5a). China is one of the biggest rice cultivation countries. The selected soils cover all major rice production regions in China, with paddy soils located in different climate zones (Supplementary Fig. 8), developed over different parent material, resulting in different soil types (Supplementary Table 5a and Supplementary Fig. 9), and at sites of different geology and geomorphology (Supplementary Table 5b). The samples cover a wide range of soil pH (4.5 to 9.0), total As contents (2.6 to 38.8 mg kg^{-1}), 0.5 M HCl-extractable Fe (30 to 184 mmol kg^{-1}) and soil organic matter (SOM; 14.0 to 104 g kg^{-1}) (Supplementary Table 5c).

After two weeks of incubation, As thiolation was detected in all paddy soils with and without sulfate addition. The proportion of total thioarsenates ranged from 0.1% to 56%, with an average of 9.6% and a median of 4.8% (Supplementary Fig. 10a). By comparison, the proportion of methylated oxyarsenates ranged from 0.5% to 17%, with an average of 3.1% and a median of 1.8% (Supplementary Fig. 10e). The dominant individual As species were DTA (>trithioarsenate (TTA) > MTA) and DMDTA (>MMMTA ≥ MMDTA > DMMTA) for inorganic and methylated thioarsenates, respectively (Supplementary Fig. 11), with different factors controlling their formation.

For inorganic thioarsenates, high absolute concentrations tended to occur at high soil pH and soil zero-valent S contents (Fig. 4a,b, confirmed by Spearman correlation (Supplementary Table 6) and principal component analysis (Supplementary Fig. 12)). The pH dependency of inorganic thioarsenates formation is at first glance surprising. Inorganic thioarsenates are known to transform to oxyarsenic species at low pH[15,16], but even though soil pH (when oxic) ranged from 4.5 to 9.0, pore-water pH values of all incubations were near neutral to slightly alkaline (6.9 to 7.9; Supplementary Fig. 13a) and should not have influenced inorganic thioarsenate (trans)formation. Linear regression analysis showed that the most important predictor for inorganic thioarsenate formation potential in our incubations was soil zero-valent S (weight factor 51%; Fig. 4e and Supplementary Table 7). Soil zero-valent S increased with soil pH (Supplementary Fig. 13c), which explains the observed correlation of

soil pH and inorganic thioarsenates as an indirect effect through zero-valent S. In most samples, we also detected aqueous zero-valent S (Supplementary Fig. 13d), but absolute concentrations were more than one order of magnitude lower than those for solid-phase zero-valent S, and even in the absence of detectable aqueous zero-valent S, inorganic thioarsenates were observed. The greater impact of solid-phase zero-valent S is consistent with previous observations in low-sulfide terrestrial environments, where inorganic thioarsenate formation was found to be controlled by reactions with S bound to surfaces of minerals or organic matter[10,14]. The concentrations of pedogenetic (0.5 M HCl-extractable) Fe had a negative but relatively low impact on inorganic thioarsenate formation potential (weight factor −6%; Fig. 4e and Supplementary Table 7), probably because little Fe dissolved at high pH (Supplementary Fig. 13e).

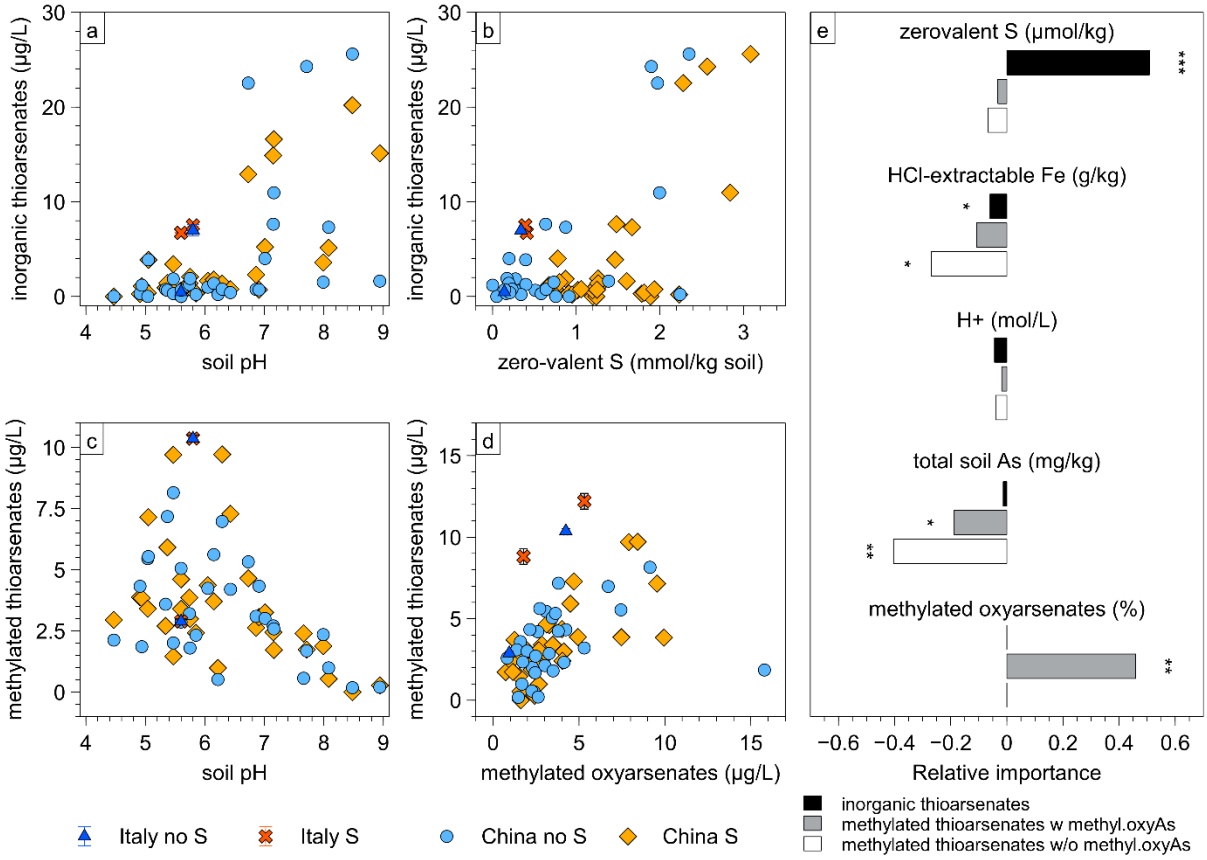

Fig. 4: Parameters that determine occurrence of inorganic and methylated thioarsenates in anaerobic soil incubations. Paddy soils were from Italy (2; experimental triplicates) and China (31, single experiments). **a**,**b**, Concentrations of inorganic thioarsenates in relation to soil pH (**a**) and solid-phase zero-valent S (**b**). **c**,**d**, Concentrations of methylated thioarsenates in relation to soil pH (**c**) and methylated oxyarsenates (**d**). Blue colours refer to control treatments (no S), orange–red colours to sulfate addition (S); standard deviation for samples from Italy reflect results from three incubations ($n = 3$). **e**, Linear regression analysis showing relative importance of selected soil parameters in control treatments on proportion of inorganic and methylated thioarsenates (with and without considering methylated oxyarsenates); asterisks indicate significance levels based on a *t*-test: $*P = 0.01–0.05$, $**P = 0.001–0.01$; $***P = 0–0.001$ (for complete list of soil parameters considered and data for sulfate addition, see Supplementary Table 7).

Methylated thioarsenates showed a different behaviour. The most important predictor for their formation was the proportion of methylated oxyarsenates (weight factor 46%; Fig. 4e and Supplementary Table 7). Methylated thio- and oxyarsenates showed a strong positive correlation (Fig. 4d and Supplementary Table 6). Negative correlation of methylated thioarsenates with soil pH (Fig. 4c and Supplementary Table 6) suggests that their formation in nature proceeds by nucleophilic attack of reduced S to the As atom, which is facilitated at low pH[12]. The higher proportion of methylated oxyarsenates observed at low pH (Supplementary Fig. 8e) is in line with previous observations in other environments of highest methylation rates at pH 3.5 to 5.5 (ref. [30]). An almost even contribution of thio- and oxyarsenates to total methylation (Fig. 4d), as well as the absence of a correlation with zero-valent S (Supplementary Table 6), suggests that thiolation of methylated species proceeds rapidly and is typically not limited by S supply but mainly by the availability of methylated oxyarsenates (in contrast to inorganic thioarsenates, where a relatively large excess of S over arsenite is required for thiolation). Examining soil properties revealed that low total soil As concentrations were the best predictor of the potential for a high (thio)methylation contribution to total As (weight factor −40%; Fig. 4e and Supplementary Table 7). We found that high soil As concentrations led only to increased inorganic As release into pore water while absolute concentrations of methylated species did not change with increasing total soil As, and therefore relative contributions decreased (Supplementary Fig. 14). Finally, pedogenetic Fe had a stronger negative impact on methylated thioarsenates compared with inorganic thioarsenates (weight factor −27%; Fig. 4e and Supplementary Table 7), probably because of the higher Fe solubility at low pH (Supplementary Fig. 13e), where methylated thioarsenates prevailed.

Compared with the strong effects that the different soil properties had on thiolation in our incubation experiments, the effect of sulfate addition was less pronounced. It promoted total thiolation (Supplementary Fig. 15a) by increasing zero-valent S contents (Supplementary Figs. 13c and 15b) and decreasing pore-water Fe concentrations (Supplementary Fig. 13e) and redox potential (Supplementary Fig. 13b), but it did not generally change the relative differences in thioarsenate formation potential between different soils. An exception was soils that had very low initial soil zero-valent S contents. Here, sulfate addition led to a strong increase in zero-valent S and total thiolation (Supplementary Fig. 15b,c). An example was Veronica soil, where an increase of zero-valent S from 0.14 to 0.41 mmol kg^{-1} compared with a much smaller increase in Fornazzo soil (from 0.34 to 0.39 mmol kg^{-1}) might explain the observed stronger increase in total As thiolation in the soil incubations (inorganic thioarsenates from 3.9 to 24%, methylated thioarsenates from 25 to 32%), which is also in line with an observed stronger increase of total thiolation on sulfate addition in the mesocosm studies (from 1.9 to 6.2%).

Environmental implications

Our combined results from field surveys, mesocosms and soil incubations reveal thioarsenates as important but previously overlooked and unforeseen contributing species to As biogeochemistry in rice paddies. Thioarsenates form in various paddy soil types, throughout the cropping season, independent of seeding practice and in quantities comparable to methylated oxyarsenates. Soil pH represented an easy-to-measure parameter indicative of thiolation potential. We suspect that in paddy soils where methylated oxyarsenates have been identified, methylated thioarsenates could have contributed comparable quantities that were, however, not distinguished from the methylated oxyarsenates due to analytical limitations in the current methodologies

adopted. Sulfate fertilization promotes thiolation, especially in soils originally low in zero-valent S.

Comparison of our anaerobic soil incubations to mesocosm experiments shows lower proportions of inorganic and especially methylated thioarsenates in the presence of rice plants (Supplementary Fig. 16). Higher-order thiolated inorganic arsenates[15] and MMMTA[31] are known to be oxygen sensitive, so root radial oxygen loss might lead to (partial) transformation in the rhizosphere. However, MTA[15] and DMMTA[32] are not oxygen sensitive. The differences in the proportion of thioarsenates between incubations and mesocosms might therefore point towards their preferential uptake. So far, uptake of thioarsenates and their efficient root–shoot translocation has been shown only in hydroponic cultures using high concentrations of pure thioarsenate standards[33,34], and only one thioarsenate species (DMMTA) has been discovered in commercial rice grains by chance during an enzymatic extraction[35]. Now that we deliver compelling support of the widespread presence of inorganic and methylated thioarsenates in paddy-soil pore waters, further transfer of methods and experiments from laboratory to field scale is required. Whether thiolation finally is boon or bane for rice safety remains to be investigated.

Methods

Aqueous As species preservation and analysis

Arsenic speciation throughout the study was done using ion chromatography (Dionex ICS-3000; AG/AS16 IonPac column, 4 mm, eluent gradient 2.5−100 mM NaOH at a flow rate of 1.2 ml min^{-1}) coupled to inductively coupled plasma–mass spectrometry (ICP-MS, XSeries2, Thermo-Fisher) at Bayreuth University. Retention times of the As species were verified by comparison with commercial standards (arsenite ($NaAsO_2$, Fluka), arsenate ($Na_2HAsO_4 \times 7H_2O$, Fluka), MMA ($CH_3AsNa_2O_3 \times 6H_2O$, Supelco), DMA ($C_2H_6AsNaO_2 \times 3H_2O$, Sigma-Aldrich)), standards synthesized according to previously published methods (DMMTA (purity 67%; 28% DMDTA, 5% DMA) and MMMTA (purity 96%; 1% MMA, 3% MMDTA)[34], MTA (purity of 98.5%; 0.5% arsenite, 1% arsenate)[33]) or by comparison with previously published retention times (MMDTA, DMDTA, DTA, TTA)[11].

For our initial field survey (see Supporting Information section 1), we used sample flash freezing, a preservation method that we previously employed successfully in other aquatic environments[10,13,14]. In contrast to sample acidification, this method revealed the occurrence of thioarsenates. However, we observed that As recoveries (calculated as the sum of all detected As species in flash-frozen samples versus total As measured in oxidized and HNO_3-acidified samples) were generally below 50%, in many cases even below 10% (Supplementary Fig. 4), especially at Fe concentrations >0.5 mM due to Fe (oxyhydr)oxide precipitation and As co-precipitation and sorption. The low recoveries prompted us to adapt the sample preservation and analysis method. Since acidification could not be used to keep Fe in solution because it changes thioarsenate speciation and could lead to AsS mineral precipitation[15], we tested different Fe chelating agents. In pretests, paddy-soil solutions derived from anaerobic incubations were preserved with different pH-neutralized chelating agents such as EDTA (ethylenediaminetetraacetic acid disodium salt solution, Sigma–Aldrich), deferoxamine mesylate salt (Sigma-Aldrich) and DTPA (diethylenetriamine-pentaacetic acid pentasodium salt, Sigma-Aldrich). Highest As species recoveries were observed when using DTPA, an octadentate ligand that can completely sequester Fe (refs. [37,38]). The better

performance compared with EDTA, for which we previously reported accelerated oxidation of arsenite and some thioarsenate artefact formation[39], might be because Fe^{II}–DTPA complexes are substantially less oxygen sensitive than Fe^{II}–EDTA complexes[40]. On the basis of expected high aqueous Fe concentrations in the sampled paddy-soil pore waters (measured values up to 6.9 mM in samples from China, see Supplementary Fig. 13e), we used 10 mM DTPA, neutralized to pH 7.5, for Fe complexation.

For a representative paddy-soil pore-water matrix ('model pore water') for method development, we used pore waters extracted from anaerobic incubations of paddy soil from Fornazzo (for details on soil properties, see Supplementary Table 2). To address the effect of DTPA on As species retention times, peak shape and species resolution, one-week-old model pore water was spiked with 100 μg L^{-1} of different As species standards, and 10 mM DTPA was added for sample preservation. DTPA had a substantial effect on peak shapes and retention times, especially for the species with short retention times (Supplementary Fig. 17). The DMA peak that eluted after 297 s in the absence of DTPA was shifted to the dead volume (142 s; Supplementary Fig. 17a). DMMTA and DMDTA were partially retained at their original retention times (376 and 446 s), but peaks became wide and small and part of the As was lost in a high-baseline background from 150 to 350 s (Supplementary Fig. 17b). The same change in peak shape and total As loss was observed for arsenite (original retention time at 406 s; Supplementary Fig. 17c). Mixes of arsenite, DMA and DMMTA also showed that species resolution between arsenite and DMMTA was lost in the presence of DTPA (Supplementary Fig. 17d). MMMTA and arsenate were less affected, but peak splitting (Supplementary Fig. 17e) and peak fronting (Supplementary Fig. 17f), respectively, were observed in the presence of DTPA as well.

Since we could not reduce the DTPA concentration because of the expected Fe concentrations but needed to decrease the negative effects of DTPA on peak separation, we tested tenfold dilution with deionized water of a fresh model pore-water sample without As spikes after addition of 10 mM DTPA (bringing DTPA concentrations down to 1 mM but also diluting Fe, As and so on tenfold). The 1/10 sample dilution increased peak separation and largely avoided As elution in the dead volume (Supplementary Fig. 18), but some peaks were close to or below detection limit. Adding 2.4% methanol to the 2.5–100 mM NaOH gradient eluent in the ion chromatography enhanced signal intensities of all peaks, except for arsenite, by a factor of 2 to 10 (Supplementary Fig. 18). A slight decrease in retention times and some arsenate fronting were observed, but all peaks could be identified and little As was lost in the dead volume.

For a quantitative evaluation, we spiked the fresh model pore-water sample with a mixed standard of 1 μg L^{-1} of DMA, DMMTA, arsenite, MMA, MMMTA and arsenate. Comparing preservation in 10 mM DTPA in deionized water versus model pore-water matrix (analysed 1/5 and 1/10 diluted), showed that the pore-water matrix itself had a minor effect on peak shifting compared with the influence of DTPA (Supplementary Fig. 19). A dilution of 1/5 resulted in peak broadening for DMA and DMMTA but no additional As loss. Quantitatively, the results of 1/10 or 1/5 dilution were comparable (Supplementary Table 8). Measured total As concentration in HNO_3 for that sample was 14.3 μg L^{-1}, and recovery from speciation analysis for the 1/10 and 1/5 dilutions with 76% and 77%, respectively, was good. Species with concentrations of 0.28 and 0.15 μg L^{-1} (equivalent to 2.5 and 1% of total As) could clearly be identified in the 1/10 and 1/5 dilutions, respectively.

Three commercial standards were routinely used for calibration (arsenate dibasic heptahydrate, disodium methyl arsonate hexahydrate, dimethylarsinic acid in 2 mM DTPA). No substantial differences were observed between using an average calibration of the three commercial standards and calibrating each

species individually using the calibration standard that was closest in retention time. Arsenite was not used for calibration because in deionized water we observed transformation of arsenite in the presence of DTPA (Supplementary Fig. 20). The arsenite transformation product eluted at the retention time of arsenate but with substantial peak fronting. Whether the species really is arsenate (obtained from arsenite oxidation) or an As(III)– or As(V)–DTPA complex is currently unclear. In spiked natural samples, we did not observe this arsenite transformation.

The final protocol for As speciation that was applied for the mesocosm and incubation experiments described in the following is summarized as follows. Samples were filtered, preserved in 10 mM DTPA, flash frozen on dry ice and stored at −20 °C. Before analysis, frozen samples were thawed under anoxic atmosphere inside a glovebox (COY, N_2/H_2 95/5% (v/v)) at room temperature. Samples were diluted 1/5 with deionized water and analysed with a 2.5–100 mM NaOH gradient containing 2.4% methanol.

We are aware that sample dilution might transform higher thiolated As species such as DMDTA, MMDTA, DTA or TTA as reported previously[41] and that we may therefore underestimate the extent of As thiolation. Further, recoveries generally were >80%, which is good considering that we calculate the sum of up to 11 species, but could still indicate a loss of As species by Fe scavenging. Therefore, where species proportions are reported in %, the reference is not the sum of species, but always total As from a sample preserved with 0.5% H_2O_2 and 0.8% HNO_3 as an independent measurement. The reported % values are therefore minimum values if a species is not affected at all by Fe scavenging. If thiolated species are scavenged by Fe similar to arsenite and arsenate, we may further slightly underestimate their importance with the currently available analytical methods. Despite some remaining shortcomings, the developed DTPA-method is the current best compromise, given the complexity of the paddy-soil pore-water samples, which are rich in organic carbon and contain high Fe that is prone to oxidation (so mere flash-freezing does not work) and As-complexed sulfide that is prone to precipitation at low pH (so acidification does not work) plus relatively low concentrations of As. The method enabled us to provide the so far most complete aqueous As speciation data for paddy-soil pore waters.

Mesocosm rice cultivation

For mesocosm experiments, we selected two paddy soils characterized by highest proportions of thiolation in pore water during our field survey in August 2016, Veronica (8° 53′ 48″E , 45° 10′ 39″N ; eutric gleysol) and Fornazzo (8° 57′ 50″E , 45° 13′ 54″N ′; umbric gleysol) (Supplementary Tables 1 and 2). A large batch of dry soil material was collected from the plough layer of the two fields in March 2017 and transported to the Rice Research Centre Ente Nazionale Risi (ENR) in Castello d'Agogna (Pavia, Italy), where we set up the mesocosms. For basic soil characterization, soil pH (measured in 2.5 ml 0.1 M $CaCl_2$ solution with 1 g soil), 0.5 M HCl-extractable Fe, total C and N (CHN analyser) and total S (determination by ICP-MS after microwave digestion in aqua regia) were determined.

Twenty-four plastic containers (0.82 m^2) were installed open air at the property of ENR. A nylon mesh rooftop protected the setup from birds and hail. Each container was filled with approximately 30 cm of gravel with a diameter of 2–5 cm overlaid by approximately 20 cm of soil. Twelve containers each were filled with the two paddy-soil types (Supplementary Fig. 5). The soil layers were mixed with 5 t ha^{-1} equivalent rice straw according to the common rice straw returning practice in this region. The rice straw was cut into pieces of approximately 20 cm length before mixing it with the soil. Six containers of each paddy soil type were either dry or water seeded. Water-seeded soils were first fertilized with 100 kg ha^{-1} of N and flooded on 16 May (Supplementary Fig. 6) before sowing with pregerminated rice seeds (*Oryza sativa* L. cv. Selenio) the following day. Dry-seeded soils were fertilized

with 100 kg ha^{-1} of N and sown on 17 May (Supplementary Fig. 6). Planted seeds germinated within ten days. Soils were kept moist until tillering stage (6 June) and subsequently flooded. All mesocosms were manually thinned to 340 rice plants per container. Irrigation water (for characteristics, see Supplementary Table 9) was supplied with a garden hose for both dry- and water-seeded treatments to maintain a standing-water level of approximately 10 cm depth during the cropping season. In addition to basal fertilization, N-, P-, or K-fertilizers were applied in the form of urea, triple superphosphate and potassium chloride as solid salts at tillering, stem elongation and booting stages (Supplementary Fig. 6). Three containers of each soil and seeding practice were additionally fertilized with sulfate fertilizer; the other three containers did not receive sulfate (control treatments). Sulfate was applied as ammonium sulfate and potassium sulfate, while equivalent amounts of urea and potassium chloride were used in the control treatment.

Sampling was done at seven rice growing stages: tillering stage (14 June), stem elongation stage (4 July), booting stage (18 July), flowering stage (1 August), grain filling stage (8 August), dough stage (22 August) and mature stage (13 September) (Supplementary Fig. 6). Pore water was extracted by micro rhizon samplers (Rhizon MOM, Rhizosphere Research Products, The Netherlands) inserted about 3–4 cm deep into the paddy soil and connected to 100 ml evacuated glass bottles. The bottles were prepared before sampling by purging them with argon (purity >99.9%) for 15 min, sealing them with a butyl rubber septum and then evacuating them to negative atmospheric pressure of ~900 mbar. Sampling took on average 40 min. An aliquot of pore water was preserved in 10 mM DTPA, flash frozen on dry ice and stored at −20 °C until analysis. Non-stable chemical parameters (pH, redox potential) were measured immediately on site. Samples for dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) were kept anoxic in the dark at 4 °C and analysed the following day at the University of Turin (VarioTOC, Elementar, Hanau, Germany). Information on pH, E_H, conductivity, DIC, DOC, Fe^{II} and total As can be found in Supplementary Table 3.

Soil sampling and anaerobic incubations

Paddy-soil samples were collected from the cultivated horizon of 31 paddy fields across China, which represent the main rice production regions in 18 Chinese provinces. The geographic origins covered an area from 22.5° to 47.2° N and 98.4° to 131.6° E, spanning climate zones from subtropical monsoon climate (23 soils) to temperate continental climate (1 soil) and temperate monsoon climate (7 soils) (Supplementary Fig. 8). On the basis of the Chinese soil taxonomic classification, all paddy soils are classified as stagnic anthrosols with both a hydragric epipedon (including cultivated horizon and plowpan) and a hydragric horizon. Those paddy soils represent three out of four key groups of stagnic anthrosols: Fe-accumuli- (15), Fe-leachi- (8), and hapli- (8) stagnic anthrosols (Supplementary Fig. 9). Detailed information regarding sampling site coordinates, soil classification, parent material, geology, geomorphology and climate zone can be found in Supplementary Table 5a,b. Twenty-nine of the 31 paddy fields had As concentrations below the Chinese risk-screening values for contamination of agricultural land (30 mg kg^{-1} when pH ≤ 6.5 (ref. [42])) and are thus considered to represent the natural background. Only two soils, Guangxi-Nanning (CH2, 34.2 mg kg^{-1}) and Jiangxi-Ganzhou (CH6, 38.8 mg kg^{-1}), exceeded the Chinese risk-screening values for paddy soil. We intentionally focused on non-contaminated paddy soils having background As concentrations because of the wider implications linked with human exposure, with respect to the less-ubiquitous anthropogenically contaminated sites (for example, only 2.7% of paddy soils in China according to a recent survey[43]) that often have specific biogeochemistries that greatly depend on contamination source and type.

Selected soil properties, including pH, 0.5 M HCl-extractable Fe, SOM, cation exchange capacity (CEC), clay content, total As and other chalcophile metals (Cd, Pb, Cu and Zn) and soil zero-valent S content were analysed by standard methods[44,45,46]. All soils were air dried and sieved to <2 mm before analysis and incubation.

For incubation, 10 g dry soil was suspended in 20 ml of 2.5 mM glucose solution without (control, no S) or with 1.5 mM K_2SO_4 (3 mmol kg sulfate, S) in a glovebox (N_2/H_2 95/5% (v/v)). The vials were incubated anaerobically, at room temperature and in the dark for 14 days. This duration was assumed from pre-experiments and literature[47] to be sufficient for microbial growth to reach a steady state. For sampling, soil suspensions were centrifuged and filtered (0.2 µm). Aqueous phase parameters (pH, redox potential, dissolved free sulfide and aqueous zero-valent S, total As) were measured as described in the preceding. Another aliquot was preserved in 10 mM DTPA, flash frozen on dry ice and stored at −20 °C for As speciation analysis. Aqueous total Fe was measured immediately by the ferrozine test[48]. Soil for solid-phase zero-valent S extraction was first freeze-dried, and then extracted with chloroform (10 mg soil +700 µL chloroform) and analysed by high-performance liquid chromatography[49]. Thus, soil-bound zero-valent S in our study is operationally defined as chloroform-extractable, reduced inorganic S.

Statistical analyses

All statistical analyses were performed via R statistical computing environment. Spearman's correlation was calculated using the 'Hmisc' package. Principal component analysis of As species (DMA, MMA, DMDTA, MMDTA, MMMTA, DMMTA, MTA, DTA and TTA) in the batch incubations was calculated using the 'vegan' package. Multiple linear regression analysis between inorganic and methylated thioarsenates (%), respectively, and soil physical and chemical properties (soil pH, total zero-valent S, total soil As, 0.5 M HCl-extractable Fe, CEC, SOM, clay content and soil chalcophile metals (sum of Cd, Pb, Cu and Zn)) was done using the 'MASS' package (with default parameters). For methylated thioarsenates, two models were calculated, one with, a second without, considering methylated oxyarsenates. Relative importance of variables in multiple regression was calculated using the 'relaimpo' package (type = 'Img')[50]. Residuals were checked for normal distribution, which is a prerequisite for multiple linear regression. Multivariate regression tree analyses were done using the 'mvpart' package (with default parameters)[51].

Data availability

The datasets generated during and/or analysed during the current study are also available from the corresponding author on reasonable request.

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Author information

Contributions

J.W. initiated DTPA method development, conceived and performed all mesocosm and incubation experiments including analyses, evaluated the results and contributed to manuscript preparation; C.F.K. contributed to field survey, sample analyses, data evaluation and manuscript preparation; L.B. contributed to DTPA method development; P.H. and L.W. initiated the Chinese soil survey and advised on incubation experiments; P.H., T.M., G.W. and L.W. sampled and characterized the Chinese soils; M.R. assisted in design, setup and operation of mesocosms and sample collection; M.M. and D.S.-P. assisted in analyses of aqueous parameters from mesocosms; M.M. contributed to field survey and data discussion; B.P.-F. initiated and supervised the project, carried out the field survey, conceived experiments and wrote the manuscript; all authors contributed to revising the manuscript.

Corresponding author

Correspondence to Britta Planer-Friedrich.

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Supporting information

Thiolated arsenic species observed in rice paddy pore-waters

Jiajia Wang[1], Carolin F. Kerl[1], Pengjie Hu[2], Maria Martin[3], Tingting Mu[2], Lena Brüggenwirth[1], Guangmei Wu[2], Daniel Said-Pullicino[3], Marco Romani[4], Longhua Wu[2] and Britta Planer-Friedrich[1]*

[1]Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany.

[2]Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, 210008 Nanjing, China

[3]Department of Agriculture, Forest and Food Sciences, University of Turin, 10124 Turin, Italy

[4]Rice Research Centre, Ente Nazionale Risi, 27030 Castello d'Agogna, Pavia, Italy

*Phone: +49 921 55 3999. E-mail: b.planer-friedrich@uni-bayreuth.de

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1. Thioarsenates discovered in planted paddy fields in Italy and France

Methods. For an initial field survey, we sampled 23 different paddy fields in Italy (17 fields) and France (6 fields) (all located in the Mediterranean climate zone, for coordinates see Table S1) during the cropping season in August 2016 when the rice plants were in the flowering to grain filling stage. Sampling in Italy covered most of the rice cropping areas of the river Po plain, where the majority of the Italian rice is produced. The paddy fields were located in the alluvial plain, the river valley, and the lower river plain. All Italian soils developed on recent clastic deposits with mixed lithology (e.g., noncalcareous gravels, silty sands), and are mostly classified as luvisols, gleysols, and a few fluvisols and cambisols (Soil Atlas of Europe, https://esdac.jrc.ec.europa.eu/content/soil-atlas-europe). Paddy fields in France covered the whole extent of the only rice cultivation area in France which is located in the coastal plain of the Camargue region, in the delta of the river Rhone. The soils there developed on recent deposits of the Rhone river and are classified as gleyic fluvisols. In total, 35 pore-water samples were collected. At most paddy fields only one pore water sample was taken, at five paddy soils we took replicates. Pore-water was extracted by micro rhizon samplers (Rhizon MOM, Rhizosphere Research Products, The Netherlands) inserted about 3-4 cm deep into the paddy soil and connected to evacuated 100 mL glass bottles. The bottles were first sealed with a butyl rubber septum in an anoxic glovebox (N_2/H_2 95/5% (v/v)), then evacuated to negative atmospheric pressure of ~900 mbar. During sampling, glass bottles were shielded with aluminum foil to avoid potential photooxidation [1]. To retrieve enough volume (minimum 10 mL) for all analyses, minimum sampling time was 4 hours, maximum sampling time up to 24 hours. After retrieving the pore water samples, one soil sample from the plow layer was collected at each site.

After collection, pore-water samples were filtered through 0.2 µm cellulose-acetate filters. Samples for As speciation analysis were immediately flash-frozen on dry ice, and stored at -20 °C before being analyzed by ion chromatography (IC, Dionex ICS-3000) coupled to inductively coupled plasma-mass spectrometry (ICP-MS, XSeries2, Thermo-Fisher) at Bayreuth University following a previously established method for analysis of inorganic and methylated thioarsenates [2].

Retention times of the As species were verified by comparison with commercial standards (arsenite ($NaAsO_2$, Fluka), arsenate ($Na_2HAsO_4 \times 7H_2O$, Fluka), MMA ($CH_3AsNa_2O_3 \times 6H_2O$, Supelco), DMA ($C_2H_6AsNaO_2 \times 3H_2O$, Sigma-Aldrich)), standards synthesized according to previously published methods (DMMTA (purity 67%; 28% DMDTA, 5% DMA) and MMMTA (purity 96%; 1% MMA, 3% MMDTA) [3], MTA (purity of 98.5%; 0.5% arsenite, 1% arsenate)) [4]

or by comparison with previously published [5] retention times (MMDTA, DMDTA, DTA, TTA). Calibration standard solutions were made from arsenate dibasic-heptahydrate, sodium (meta)arsenite, disodium methyl arsonate hexahydrate, and dimethylarsinic acid. All other As species were quantified by peak area comparison to the standard closest in retention time. Validity of this method has been proven previously [2].

An example of the chromatographic separation of the different As species is reported in Fig. S1. Samples for total As and Fe were acidified in 0.5% H_2O_2 and 0.8% HNO_3 and kept at 4°C until analysis by ICP-MS. Samples for zero-valent S were stabilized with zinc acetate (25 µL of 200 g/L ZnAc +725 µL sample), kept at 4°C until extraction by chloroform in the laboratory, then measured with high performance liquid chromatography (HPLC) (Merck Hitachi L-2130 pump, L-2200 autosampler, and L-2420 UV-VIS detector; C18 column, 100% methanol eluent at 0.2 mL/min) as described before [6]. Sulfide was measured photometrically on-site using the methylene blue method (HACH procedure No. 8131). Redox potential, pH, and conductivity were measured directly on-site by a WinLab redox micro-electrode, a WinLab 423 combination pH electrode, and a Mettler Toledo TetraCon 325 electrode.

Soil samples were analyzed for soil pH (measured in 2.5 mL 0.1 M $CaCl_2$ solution with 1 g soil), 0.5 M HCl-extractable Fe, total C and N (CHN analyzer), and total As and S (determination by ICP-MS after microwave digestion in aqua regia).

Results. Soil pH ranged from 5.0-6.1 and 7.5-7.6, total soil As contents from 5.2-16 and 10.4-20.2 mg/kg, HCl-extractable Soil Fe contents from 50-198 and 105-181 mmol/kg, and total C from 0.8-4.7 and 4.5-6.0 %, for the Italian and French paddy soils, respectively (Table S1a). Thioarsenates were determined in 23 out of 35 pore-water samples and in 14 out of 23 different fields (Table S1b). The contribution of total thiolation to total As concentrations was 8.3% at maximum and 2.1% on average. These numbers are comparable to those observed for the much more-commonly-investigated methylated oxyarsenates which we detected in 31 samples from 20 fields (max. 10.4%, on average 1.3%). Inorganic thioarsenates (monothioarsenate (MTA) and dithioarsenate (DTA)) were detected in 11 samples (max. 7.4%, on average 3.2%) and methylated thioarsenates (monomethylmonothioarsenate (MMMTA), DMMTA, dimethyldithioarsenate (DMDTA)) in 18 samples (max. 2.9%, on average 0.7%). Seven samples taken within the same paddy field (Veronica, Table S1b) showed large heterogeneity in the proportion of thioarsenates (2.9-8.3%) without any obvious relation to pore-water chemistry, such as dissolved sulfide concentrations (Table S1b). Inorganic thioarsenates were observed in large quantities only at pore-water Fe concentrations < 0.5 mmol/L suggesting that Fe concentrations above a threshold value

could limit their formation (Fig. S2a). Methylated thioarsenates, in contrast, occurred over a wider range of dissolved Fe concentrations (Fig. S2b) and Spearman´s correlation test showed positive correlation with methylated oxyarsenates ($r = 0.60$, $P<10^{-4}$; Fig. S2b, S2c, S3). There was no correlation between inorganic and methylated thioarsenates.

For these first field surveys, we used relatively long pore-water sampling times (4-24 hours) to obtain enough volume for analyses (minimum 10 mL) and, for species preservation, we used just flash-freezing, without adding stabilizing agents. Even though all As chromatographic peaks were clearly distinguishable (Fig. S1), high Fe concentrations (up to 2.3 mmol/L) caused Fe precipitation and, by co-precipitation and sorption, low As recoveries (calculated as the sum of all detected As species in flash-frozen samples versus total As measured in oxidized and acid-stabilized samples; Fig. S4).

All species proportions are reported with respect to total As (not the sum of species). As such, a partial precipitation or sorption of thiolated As species on any Fe (hydr)oxides [7] formed during sample storage could have contributed to an underestimation of the true proportions reported here.

For all later analyses, short sampling times (0.5-1 hour) and an optimized DTPA-sample stabilization and analysis were chosen. For details on the DTPA method development and evaluation see main manuscript and Fig. S15-S18.

Table S1a | Coordinates and basic soil chemistry from 17 Italian paddy fields (IT) and 6 French paddy fields (FR)

Paddy fields	Longitude	Latitude	Soil pH	HCl extract-able soil Fe	total soil As	total soil S	total soil C
				mmol/kg	mg/kg	g/kg	%
ITALY							
IT_Vignarello	8°44′42.82″	45°20′39.77″	5.5	62.6	8.9	3.4	2.0
IT_Barbavara	8°47′09.49″	45°21′19.41″	5.5	67.6	10.5	3.2	2.7
IT_Gambarana	8°46′30.88″	45°01′38.88″	5.8	197.7	16.0	6.0	1.4
IT_Breme	8°37′06.19″	45°08′38.34″	5.8	177.0	13.8	5.4	1.3
IT_Langosco	8°32′43.84″	45°12′34.24″	5.7	165.5	12.9	8.4	1.3
IT_Vercelli	8°15′12.88″	45°18′36.59″	5.7	75.9	11.4	9.5	1.1
IT_Cascina Oschiena	8°45′03.84″	45°18′36.58″	5.7	109.9	6.1	4.2	1.3
IT_Fontanetto Po	8°11′59.46″	45°12′15.50″	6.0	144.9	9.1	4.8	3.4
IT_Terranova	8°30′02.95″	45°11′53.56″	6.1	125.3	11.8	5.3	1.4
IT_Rovasenda	8°16′40.00″	45°32′34.05″	5.8	97.2	14.3	4.3	1.0
IT_Cascina Albera	8°56′48.18″	45°10′49.49″	5.8	52.0	5.9	2.7	1.4
IT_Lomello	8°47′55.87″	45°07′26.71″	5.0	120.6	8.9	4.1	1.4
			5.6	90.2	7.3	3.9	1.5
IT_Cascina Fornazzo	8°57′49.97″	45°13′53.76″	5.8	71.7	5.6	3.2	4.7
IT_Cascina Veronica	8°53′47.60″	45°10′39.30″	5.6	50.0	5.2	2.2	2.1
IT_Castello d´Agogna (ENR) field 1	8°41′56.50″	45°14′51.30″	5.5	53.0	5.8	2.6	2.0
			5.4	50.6	5.8	2.4	1.9
			5.5	145.9	16.0	4.3	0.9
			5.5	152.3	15.6	4.3	0.8
IT_Castello d´Agogna (ENR) field 2			5.5	146.7	15.2	4.4	0.8
			5.5	144.1	15.6	4.2	0.8
IT_Castello d´Agogna (ENR) field 3			5.6	181.0	15.8	4.2	0.9
			5.5	159.3	14.9	4.2	0.9
FRANCE							

FR_Seyne	4°42′57.24″	43°34′23.63″	7.5	166.0	18.2	22.1	5.4
FR_Vedeau	4°42′24.53″	43°26′04.63″	7.6	143.0	14.7	23.1	6.0
FR_Adrien	4°33'40.93''	43°42'07.42''	7.5	109.5	10.4	18.0	4.9
FR_Furane	4°31′06.35″	43°41′11.58″	7.5	134.1	20.2	19.8	4.9
FR_Boismeaux	4°28′14.10″	43°35′54.44″	7.5	138.9	13.1	18.8	4.5
FR_Signore	4°29′46.35″	43°37′10.51″	7.6	104.7	10.6	22.6	5.2

Table S1b | Pore water chemistry including As speciation for samples from 17 Italian paddy fields (IT) and 6 French paddy fields (FR)

Paddy fields	pore water pH	E_H	Conductivity	Sulfide	Zerovalent sulfur	Fe	DMA	DMMTAa	Arsenite	DMDTA[b]	MMA	MMMTA[c]	Arsenate	MTA[d]	DTA[e]	Total Thiolated As	Sum As species	Total As
		mV	µS/cm	µmol/L		mmol/L	µg/L											
ITALY																		
IT_Vignarello	6.1	194	310	<0.3	4.6	0.3	0.2		2.8				1.0				3.9	38.1
IT_Barbavara	6.6	184	305	<0.3	2.4	0.2	0.04		0.5				0.2				0.7	10.6
IT_Gambarana	6.9	71	1154	<0.3	<1	0.3	0.1		3.1		0.3		2.5				6.1	86.2
IT_Breme	6.6	-21	1340	<0.3	4.3	2.6	0.4		1.1		0.4	0.2	0.3			0.2	2.4	82.0
IT_Langosco	6.4	145	575	<0.3	5.4	0.9	0.2	0.2	0.5		0.1	0.1	0.2			0.3	1.3	37.3
IT_Vercelli	6.3	339	209	<0.3	<1	0.0			1.9				1.6				3.4	7.8
IT_Cascina Oschiena	6.5	72	1092	1.2	<1	1.5		0.1	0.5				0.2			0.1	0.9	68.2
IT_Fontanetto Po	7.0	377	1831	1.2	<1	<0.1	0.1		2.7		0.1		1.0				3.9	9.0
IT_Terranova	7.0	68	2490	1.3	5.8	0.2	1.5	0.1	11.8				2.3			0.1	15.8	84.2
IT_Rovasenda	6.6	116	370	1.2	<1	0.1	0.6	0.1	0.3				0.4			0.1	1.4	6.0
IT_Cascina Albera	6.7	93	315	1.8	1.2	0.6	0.1		1.4			0.04	0.3			0.04	1.8	31.3
IT_Lomello	6.8	92	280	0.9	2.7	0.1			1.4				0.4				1.8	25.9
	7.0	97	362	<0.3	5.2	0.0	0.2		2.6		0.1		2.3	0.2		0.2	5.5	15.2
IT_Cascina Fornazzo	7.1	98	673	<0.3	5.2	0.3	0.5	0.1	1.3		0.2	0.3	0.5	0.1	0.2	0.8	3.3	14.9
IT_Cascina Veronica	6.4	90	541	<0.3	<1	0.5	0.6	0.4	1.8	0.1	0.1	0.03	0.6	0.2	0.4	1.2	4.4	28.2
	6.2	122	341	<0.3	<1	0.3		0.1	0.9			0.1	0.7	0.2	0.2	0.5	2.1	9.1
	6.4	83	n.a	3.0	<1	0.3			1.6		0.04	0.0	3.8	0.4	0.3	0.8	6.2	17.7

Paddy fields	pore water pH	E_H	Conductivity	Sulfide	Zerovalent sulfur	Fe	DMA	DMMTAa	Arsenite	DMDTA[b]	MMA	MMMTA[c]	Arsenate	MTA[d]	DTA[e]	Total Thiolated As	Sum As species	Total As
		mV	µS/cm	µmol/L		mmol/L	µg/L											
IT_Cascina Veronica	6.3	117	285	1.1	<1	0.3			2.8		0.2	0.1	1.8	0.3	0.6	1.0	5.8	11.9
	n.a.	n.a.	n.a.	<0.3	<1	0.3			2.3		0.1		6.9	0.5	0.2	0.8	10.1	20.6
	6.5	169	n.a.	<0.3	1.8	0.3			0.7		0.05		1.2	0.2	0.3	0.5	2.4	8.0
	6.2	105	400	<0.3	<1	0.3	0.03		0.4		0.1		0.7	0.1	0.1	0.2	1.5	7.1
IT_Castello d´Agogna (ENR) field 1	6.8	-13	1183	1.0	1.5	1.8	0.1	0.3	0.6		0.5		0.1			0.3	1.6	105
	6.6	85	723	<0.3	<1	0.8	0.1		0.6				0.3				1.0	44.4
IT_Castello d´Agogna (ENR) field 2	6.7	83	n.a.	1.1	2.4	0.6	0.1	0.03	0.7				0.2			0.03	1.0	30.0
	6.8	1	1346	1.0	<1	1.8	0.1	0.1	0.5		0.3	0.1	0.1			0.2	1.2	58.5
	6.8	44	1181	<0.3	<1	0.8	0.2	0.1	0.5		0.5	0.2	0.1			0.3	1.6	24.8
	6.7	70	890	<0.3	<1	1.2	0.1	0.1	0.4		0.2		0.1			0.1	0.9	36.7
IT_Castello d´Agogna (ENR) field 3	6.7	83	792	<0.3	3.8	0.7	0.1		1.2		0.1		0.2				1.6	45.4
	6.8	117	364	<0.3	2.8	0.3	0.1		2.0				0.6				2.6	47.1
FRANCE																		
FR_Seyne	7.2	37	1030	<0.3		0.5			1.9				0.2				2.1	72.8
FR_Vedeau	6.8	32	2200	<0.3		0.7	1.4		6.7				0.5				8.6	165
FR_Adrien	7.3	91	1340	<0.3		0.2	0.2	0.1	21.9		0.04		2.8	0.2	0.1	0.4	25.3	86.7
FR_Furane	7.2	-16	1854	<0.3		1.4	1.0	0.1	11.7				2.5			0.1	15.3	335
FR_Boismeaux	7.3	16	2010	<0.3		1.2	1.1	0.4	16.8	0.1	0.9	0.2	2.5	0.1		0.8	22.2	268
FR_Signore	7.5	37	1509	<0.3		0.6	0.3		4.4		0.1		0.4				5.3	69.5

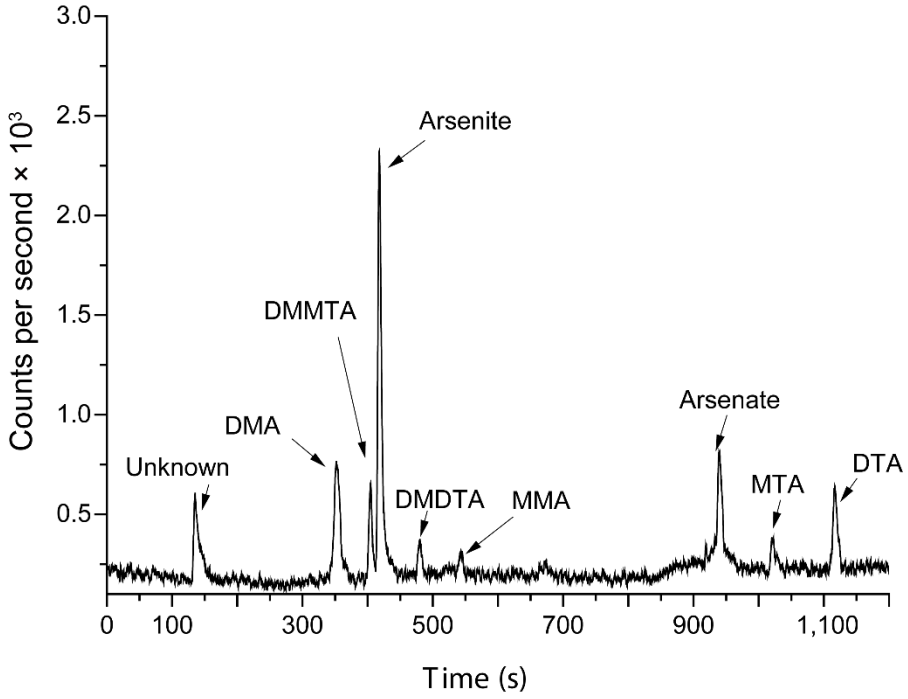

Figure S1 | Example chromatogram for determination of inorganic and methylated thio and oxy As species in paddy field pore-water by IC-ICP-MS. The presented sample is IT_Cascina Veronica (pH 6.36, Table S1).

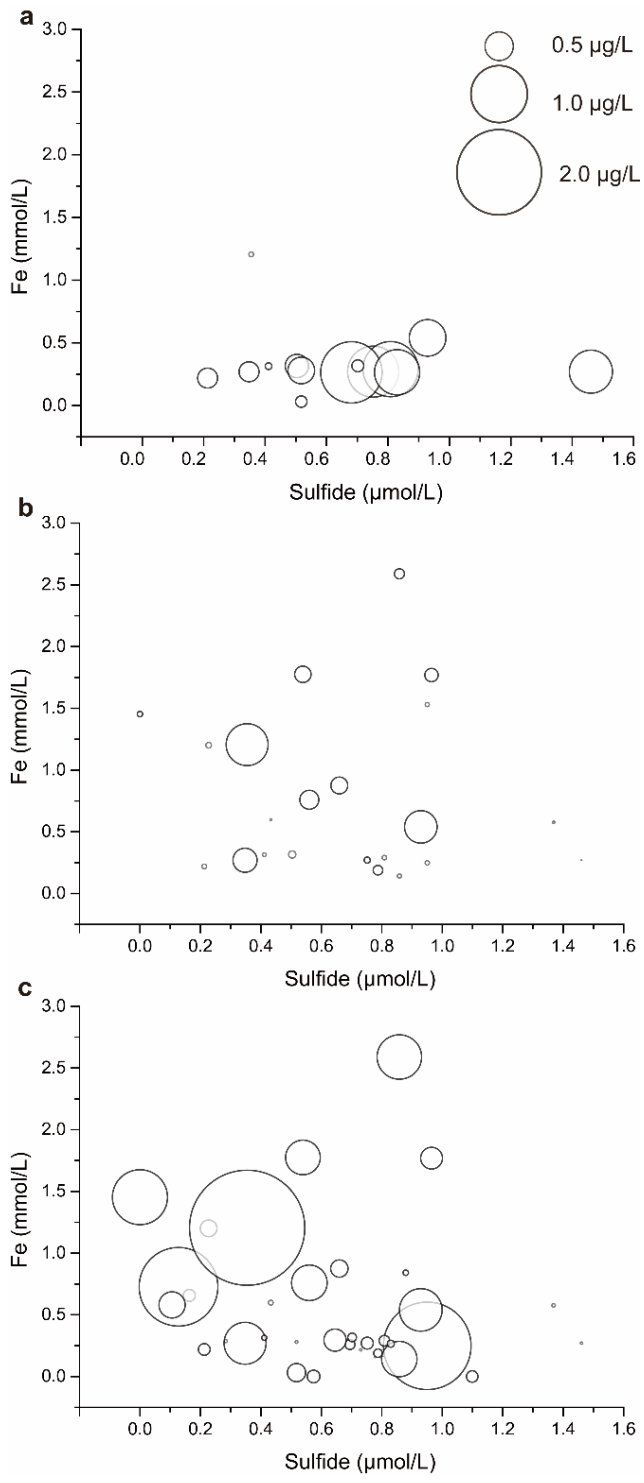

Figure S2 | Arsenic speciation in paddy field pore-waters from Italy and France in relation to aqueous Fe and sulfide. Contribution of a) inorganic thioarsenates, b) methylated thioarsenates and c) methylated oxyarsenates to total As; bubble size represents concentration of As species; bubbles are only displayed where concentrations of As species were above detection limit.

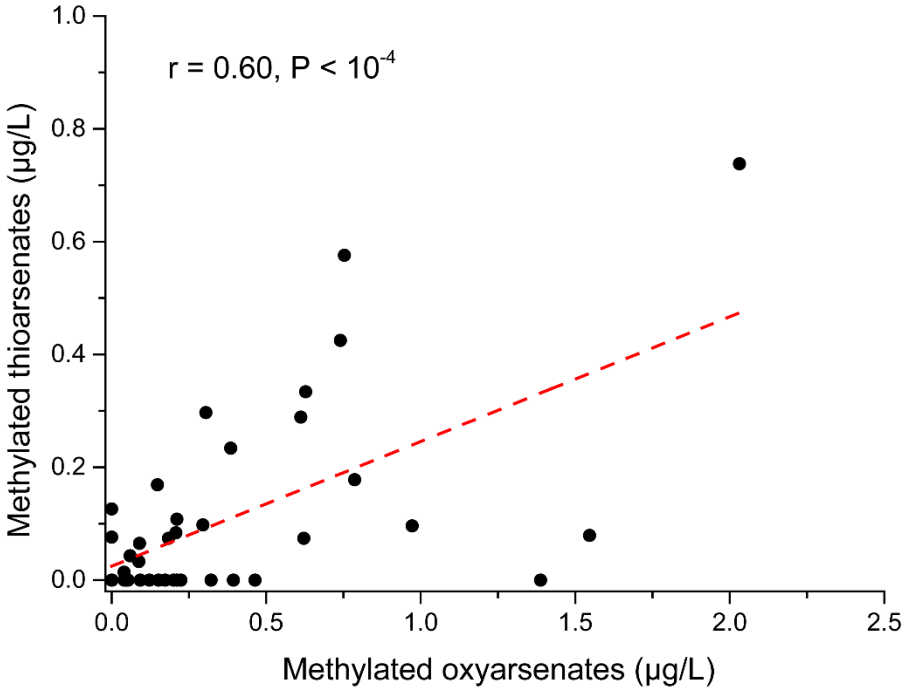

Figure S3 | Correlation of methylated thioarsenates with methylated oxyarsenates in paddy field pore-waters from Italy and France.

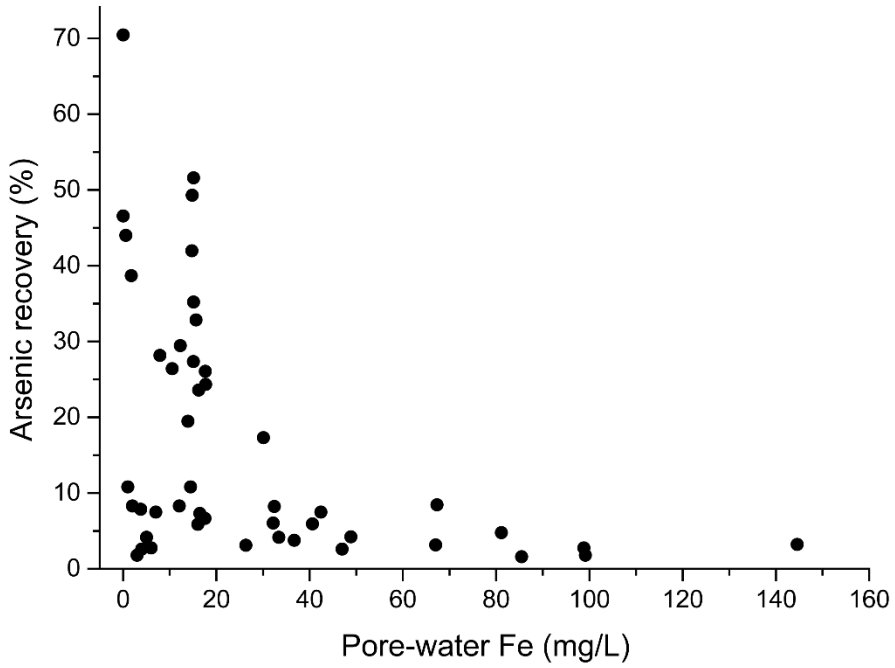

Figure S4 | Effect of Fe on As recovery for samples from paddy fields in Italy and France. Arsenic recovery is calculated as sum of As species from flash-frozen samples versus total As in H_2O_2-HNO_3-stabilized samples.

2. Mesocosm rice cultivation experiments – effects of sulfate-fertilization, seeding-practice, and soil type on thioarsenate formation

Looking at the pore-water total As concentrations during rice cultivation in the mesocosm experiments (Fig 2), one can see that over time, pore-water total As concentrations in control treatments decreased from approximately 100 and 50 µg/L at tillering stage in the dry and water seeded mesocosms, respectively, to <20 µg/L around flowering stage (except for the dry seeded Veronica soil where concentrations only dropped after the dough stage, Fig. 2e, f). Highest pore-water As concentrations occurred within days after flooding due to rapid As mobilization by reductive dissolution of Fe(III)-(oxy)hydroxides, and then decreased due to As re-adsorption on or precipitation with newly formed Fe minerals in line with previous reports [8, 9]. The higher As concentrations in dry vs. water seeded treatments at the same sampling date could be explained by an overall less As mobilization in water seeded treatments because they are flooded in May when microbially catalyzed As mobilization is still partially limited by lower temperatures while flooding of dry seeded treatments in June leads to higher As mobilization due to enhanced microbial activity. In addition, the difference could reflect the time it takes for As concentrations after flooding and initial mobilization to drop again due to re-adsorption and precipitation reactions (4 weeks later in dry than in water seeded treatments). No clear trend in the proportion of inorganic or methylated thioarsenates was observed over time (Fig. 2a-d).

Table S2 | Soil classification and basic chemical parameters for Veronica and Fornazzo soil

Parameters	Veronica	Fornazzo
Geology/geomorphology	Lower river plain	Valley of the Ticino river
Parent material	Pleistocene alluvium	Olocene alluvium
Soil classification		
USDA	Aeric Endoacquepts coarse-loamy over sandy, mixed, mesic	Histic Humaquepts coarse-loamy, mixed, mesic
FAO	Eutric Gleysoil	Umbric Gleysoil
Texture		
gravel (%weight)	nd	23.9
clay %	2.1	1.4
fine silt %	11.5	6.2
coarse silt %	8.8	6.8
fine sand %	18.2	23.7
coarse sand %	59.3	61.8
Cation exchange capacity (cmol /kg)	9.52	14.39
Base saturation (%)	0.24	0.24
Effective base saturation (%)	0.84	0.91
soil pH	5.6	5.8
0.5 M HCl-extractable Fe (mmol/kg)	52	71
Oxalate-extracted Fe (mmol/kg)	9.9	19.2
C (%)	2.0	4.7
N (%)	0.6	0.5
Total As (mg/kg)	5.8	5.6
Oxalate-extracted As (mg/kg)	1.4	1.5
S (g/kg)	2.6	3.2

Table S3 | Pore water chemistry at different rice growing stages in the mesocosm experiments (2017) separated by soil type, fertilization (non-sulfate/sulfate), and seeding practice (dry-seeding/wet-seeding) (n=3)

Pore water parameters	Soil type & Seeding practice	Fertili-zation	Jun 14th	Jul 4th	Jul 18th	Aug 1th	Aug 8th	Aug 22th	Sep 13th
			Tillering	Stem elongation	Booting	Flowering	Grain filling	Dough	Mature
pH	**Veronica**								
	water-seeding	non-sulfate	6.9 ± 0.1	6.8 ± 0.1	6.7 ± 0.2	6.5 ± 0.1	6.5 ± 0.1	6.8 ± 0.1	6.8 ± 0.1
		sulfate	6.9 ± 0.1	6.9 ± 0.2	6.6 ± 0.1	6.8 ± 0.1	6.7 ± 0.1	7.1 ± 0.1	6.8 ± 0.2
	dry-seeding	non-sulfate	6.8 ± 0.3	6.5 ± 0.4	6.5 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	6.8 ± 0.3	6.6 ± 0.1
		sulfate	6.8 ± 0.2	6.9 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.8 ± 0.2	7.0 ± 0.1	6.8 ± 0.1
	Fornazzo								
	water-seeding	non-sulfate	7.2 ± 0.2	7.3 ± 0.1	7.2 ± 0.1	7.1 ± 0.1	7.2 ± 0.1	7.2 ± 0.2	7.3 ± 0.2
		sulfate	7.0 ± 0.1	7.1 ± 0.1	6.9 ± 0.1	7.1 ± 0.1	7.1 ± 0.1	7.5 ± 0.1	7.1 ± 0.1
	dry-seeding	non-sulfate	7.0 ± 0.1	7.0 ± 0.3	7.2 ± 0.1	7.0 ± 0.1	7.1 ± 0.1	7.2 ± 0.1	7.4 ± 0.1
		sulfate	7.2 ± 0.1	7.2 ± 0.2	7.3 ± 0.1	7.0 ± 0.04	7.1 ± 0.1	7.3 ± 0.1	7.4 ± 0.1
E_H (mV)	**Veronica**								
	water-seeding	non-sulfate	181 ± 85	168 ± 62	185 ± 56	122 ± 5	161 ± 9	179 ± 19	160 ± 26

		sulfate	123 ± 25	131 ± 36	159 ± 24	92 ± 16	121 ± 11	149 ± 18	126 ± 16
	dry-seeding	non-sulfate	212 ± 44	163 ± 9	162 ± 11	180 ± 98	163 ± 5	165 ± 11	167 ± 24
		sulfate	207 ± 59	135 ± 30	127 ± 47	89 ± 30	130 ± 17	131 ± 31	129 ± 16
	Fornazzo								
	water-seeding	non-sulfate	181 ± 81	163 ± 15	148 ± 59	116 ± 19	103 ± 19	130 ± 36	118 ± 26
		sulfate	96 ± 2	112 ± 50	87 ± 7	69 ± 10	77 ± 20	80 ± 11	84 ± 13
	dry-seeding	non-sulfate	287 ± 6	211 ± 4	132 ± 43	111 ± 37	126 ± 11	135 ± 5	130 ± 29
		sulfate	163 ± 61	143 ± 37	193 ± 75	109 ± 75	119 ± 35	146 ± 20	143 ± 51
Conductivity (µS/cm)	**Veronica**								
	water-seeding	non-sulfate	533 ± 92	325 ± 30	504 ± 83	580 ± 54	777 ± 13	667 ± 263	601 ± 170
		sulfate	745 ± 374	382 ± 165	455 ± 51	412 ± 34	493 ± 111	386 ± 59	567 ± 21
	dry-seeding	non-sulfate	805 ± 184	324 ± 41	554 ± 153	630 ± 52	835 ± 89	693 ± 38	651 ± 76
		sulfate	614 ± 52	335 ± 72	434 ± 59	631 ± 424	414 ± 64	345 ± 37	478 ± 66
	Fornazzo								
	water-seeding	non-sulfate	956 ± 54	932 ± 94	1128 ± 95	1171 ± 118	1362 ± 120	1392 ± 211	1469 ± 223
		sulfate	1026 ± 116	781 ± 32	922 ± 60	1038 ± 208	955 ± 64	1051 ± 147	1188 ± 10

	dry-seeding	non-sulfate	723 ± 113	909 ± 63	1102 ± 24	1133 ± 49	1281 ± 37	1397 ± 125	1416 ± 130
		sulfate	988 ± 124	846 ± 79	1483 ± 731	876 ± 119	841 ± 65	866 ± 69	1016 ± 102
TIC (mg/L)[a]	**Veronica**								
	water-seeding	non-sulfate	14.2 ± 2.79	19.5 ± 1.75	15.7 ± 0.96	16.7 ± 2.62	10.4 ± 1.14	15.8 ± 5.37	20.4 ± 6.69
		sulfate	15.8 ± 4.27	15.4 ± 4.62	20.3 ± 7.46	25.4 ± 5.94	33.6 ± 10.4	28.7 ± 3.37	31.5 ± 3.61
	dry-seeding	non-sulfate	21.9 ± 8.47	18.0 ± 0.8	14.1 ± 0.9	12.8 ± 1.39	13.3 ± 9.72	10.5 ± 1.57	15.1 ± 0.65
		sulfate	19.2 ± 1.69	22.3 ± 7.27	23.8 ± 6.24	25.2 ± 5.07	23.5 ± 5.28	24.8 ± 3.58	25.7 ± 3.73
	Fornazzo								
	water-seeding	non-sulfate	43.1 ± 12.9	88.9 ± 7.44	90.4 ± 8.63	101 ± 12.11	82.7 ± 7.89	97.0 ± 18.0	108 ± 22.4
		sulfate	50.4 ± 10.5	76.1 ± 3.13	77.1 ± 0.79	89.3 ± 0.76	84.9 ± 9.04	99.8 ± 5.25	107 ± 5.75
	dry-seeding	non-sulfate	66.7 ± 24.6	79.0 ± 6.86	75.8 ± 6.10	81.6 ± 12.7	62.0 ± 38.6	77.9 ± 1.59	85.6 ± 1.77
		sulfate	86.3 ± 10.8	85.1 ± 9.16	79.5 ± 6.38	83.9 ± 6.11	98.3 ± 14.6	85.6 ± 6.21	89.1 ± 7.56
TOC (mg/L)[b]	**Veronica**								
	water-seeding	non-sulfate	23.0 ± 2.46	94.3 ± 25.9	102 ± 63.2	91.6 ± 60.9	73.4 ± 51.0	64.9 ± 41.3	59.9 ± 32.5
		sulfate	30.6 ± 5.57	85.8 ± 33.0	93.5 ± 44.7	95.5 ± 47.8	91.6 ± 50.6	95.0 ± 53.4	92.0 ± 49.4

	dry-seeding	non-sulfate	59.1 ± 21.0	58.7 ± 3.22	64.2 ± 6.93	61.3 ± 1.29	49.1 ± 5.30	46.0 ± 3.51	47.3 ± 3.09
		sulfate	47.9 ± 6.44	57.3 ± 6.04	57.8 ± 6.06	57.9 ± 15.7	65.8 ± 12.2	57.3 ± 25.2	51.2 ± 25.6
	Fornazzo								
	water-seeding	non-sulfate	73.9 ± 44.2	136 ± 21.0	147 ± 48.5	131 ± 49.7	103 ± 24.2	96.4 ± 35.5	94.2 ± 34.2
		sulfate	56.5 ± 11.0	116 ± 26.0	111 ± 21.8	105 ± 10.8	95.8 ± 3.61	104 ± 10.1	99.9 ± 18.2
	dry-seeding	non-sulfate	96.5 ± 17.5	106 ± 6.54	122 ± 9.10	118 ± 17.3	104 ± 7.21	81.0 ± 5.29	81.2 ± 10.6
		sulfate	96.7 ± 10.3	108 ± 2.00	113 ± 20.6	110 ± 28.6	134 ± 38.8	106 ± 36.4	108 ± 42.6
Fe^{II} (mmol/L)[c]	**Veronica**								
	water-seeding	non-sulfate	0.23 ± 0.02	0.25 ± 0.01	0.29 ± 0.04	0.31 ± 0.07	0.45 ± 0.03	0.34 ± 0.14	0.28 ± 0.07
		sulfate	0.22 ± 0.03	0.27 ± 0.02	0.25 ± 0.06	0.28 ± 0.07	0.34 ± 0.06	0.29 ± 0.07	0.25 ± 0.06
	dry-seeding	non-sulfate	0.07 ± 0.04	0.20 ± 0.01	0.29 ± 0.03	0.35 ± 0.03	0.48 ± 0.04	0.43 ± 0.01	0.33 ± 0.00
		sulfate	0.08 ± 0.02	0.25 ± 0.02	0.26 ± 0.01	0.26 ± 0.04	0.31 ± 0.04	0.30 ± 0.04	0.24 ± 0.04
	Fornazzo								
	water-seeding	non-sulfate	0.32 ± 0.07	0.43 ± 0.04	0.43 ± 0.03	0.43 ± 0.04	0.48 ± 0.03	0.41 ± 0.06	0.40 ± 0.03
		sulfate	0.34 ± 0.05	0.40 ± 0.08	0.36 ± 0.05	0.37 ± 0.06	0.40 ± 0.03	0.39 ± 0.03	0.35 ± 0.04
	dry-seeding	non-sulfate	0.11 ± 0.04	0.34 ± 0.01	0.40 ± 0.01	0.42 ± 0.04	0.49 ± 0.04	0.47 ± 0.03	0.42 ± 0.04

		sulfate	0.14 ± 0.09	0.37 ± 0.05	0.37 ± 0.04	0.37 ± 0.07	0.44 ± 0.08	0.40 ± 0.08	0.37 ± 0.05
Total As (µg/L)	**Veronica**								
	water-seeding	non-sulfate	48.2 ± 19.5	41.2 ± 10.3	31.6 ± 10.1	17.8 ± 4.89	12.1 ± 2.7	7.67 ± 1.55	5.57 ± 1.30
		sulfate	24.2 ± 11.8	11.2 ± 1.07	9.26 ± 1.73	10.2 ± 2.27	11.7 ± 2.98	9.69 ± 4.03	7.58 ± 2.62
	dry-seeding	non-sulfate	104 ± 9.1	83.3 ± 17.9	76.4 ± 12.0	46.9 ± 13.4	31.8 ± 8.53	18.4 ± 5.70	7.57 ± 1.73
		sulfate	72.6 ± 15.2	8.04 ± 0.84	6.97 ± 0.86	7.69 ± 1.05	10.0 ± 1.10	8.71 ± 1.27	5.41 ± 1.25
	Fornazzo								
	water-seeding	non-sulfate	48.0 ± 4.66	22.0 ± 2.44	19.8 ± 3.80	19.7 ± 4.60	18.6 ± 4.58	15.6 ± 3.34	13.3 ± 3.22
		sulfate	27.6 ± 4.80	18.5 ± 1.07	16.0 ± 1.38	15.5 ± 0.75	15.2 ± 0.55	14.1 ± 0.41	12.2 ± 0.74
	dry-seeding	non-sulfate	97.3 ± 7.49	61.5 ± 13.5	25.3 ± 2.57	20.9 ± 0.66	19.7 ± 1.27	17.0 ± 1.62	14.9 ± 1.82
		sulfate	76.5 ± 9.82	23.4 ± 1.56	19.3 ± 1.46	17.9 ± 2.19	17.0 ± 2.51	14.7 ± 2.69	12.7 ± 2.78

[a] Total inorganic carbon; [b] Total organic carbon;[c] Fe^{II} was spectrophotometricaly determinated via 1,10-phenanthroline method.

Table S4 | Mean values for thiolation and methylation (integrated over the seven sampling times) for comparison of factors of increases by sulfate-addition (ratio S/no S) for the two different soil types (Veronica/Fornazzo) and water- vs. dry-seeding

	water-seeding			**dry-seeding**	
Inorganic thioarsenates	Veronica	Fornazzo		Veronica	Fornazzo
contribution to As speciation - no S [%]	3.0	1.8		0.6	1.3
contribution to As speciation - S [%]	4.7	5.5		3.9	2.6
ratio S/noS	1.5	3.1		6.4	2.1
Methylated thioarsenates	Veronica	Fornazzo		Veronica	Fornazzo
contribution to As speciation - no S [%]	0.1	0.9		0.2	1.0
contribution to As speciation - S [%]	2.1	1.4		1.7	1.7
ratio S/noS	19.8	1.6		11.6	1.8
Total Thiolation	Veronica	Fornazzo		Veronica	Fornazzo
contribution to As speciation - no S [%]	3.1	2.7		0.8	2.2
contribution to As speciation - S [%]	6.8	6.9		5.6	4.3
ratio S/noS	2.2	2.5		7.4	2
Methylated oxyarsenates	Veronica	Fornazzo		Veronica	Fornazzo
contribution to As speciation - no S [%]	5.0	5.6		2.1	4.4
contribution to As speciation - S [%]	12.7	5.2		11.1	6.1
ratio S/noS	2.6	0.9		5.3	1.4

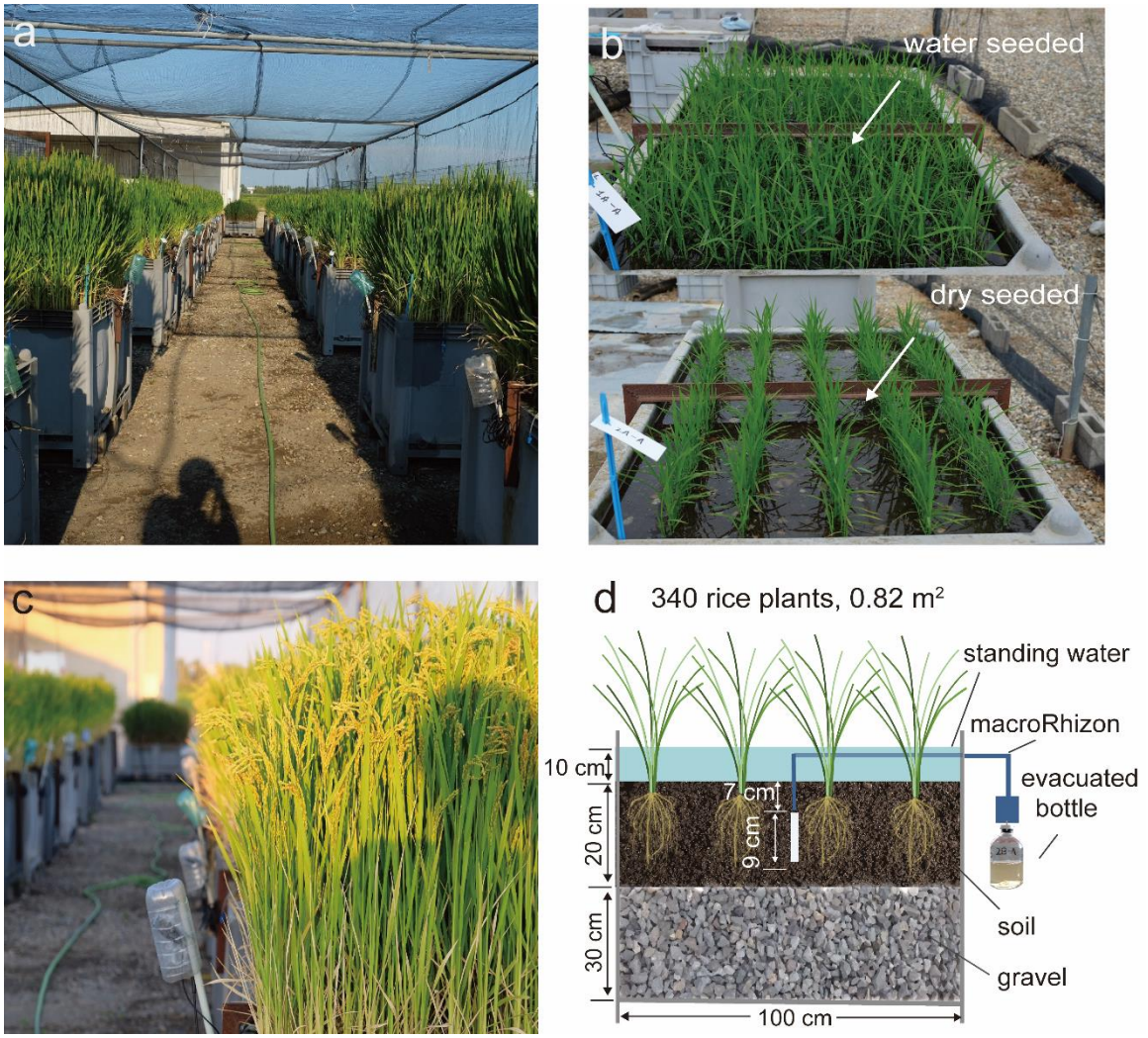

Figure S5 | Photos and schematic design of the mesocosm rice cultivation experiments. A total of 24 mesocosms with 2 different soil types, water and dry seeded, with and without sulfate fertilization (each setup conducted in triplicates) were installed at the Rice Research Centre Ente Nazionale Risi in Castello d'Agogna (Pavia, Italy); a) flowering stage; b) seeding practices; c) mature stage; d) scheme of the setup.

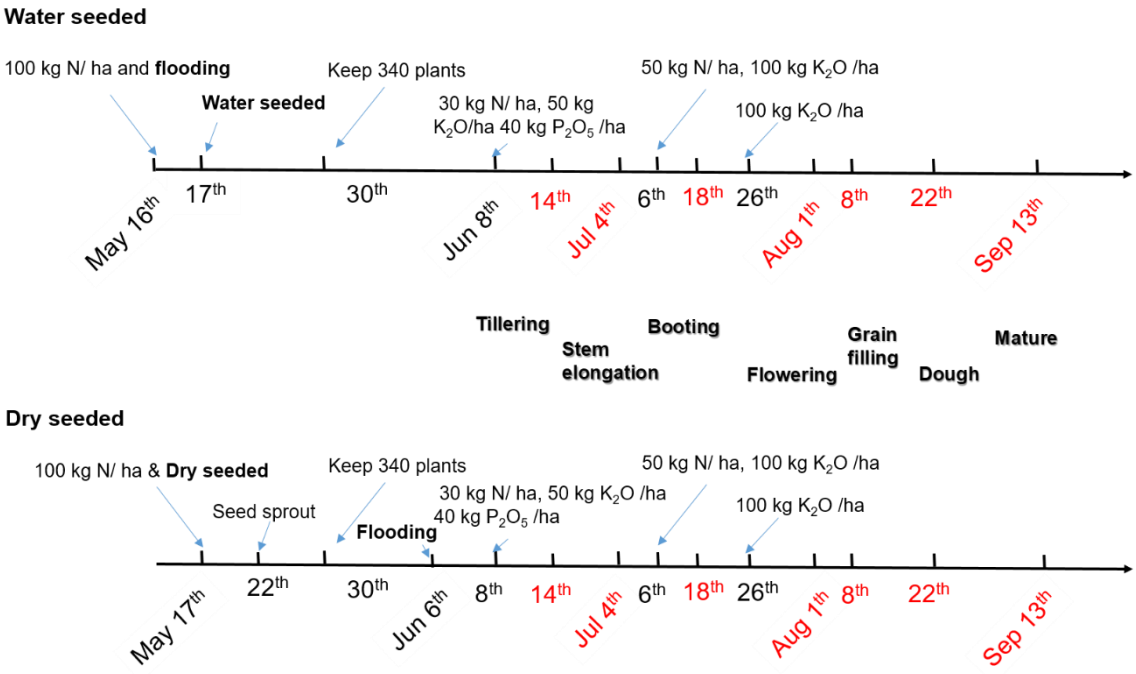

Figure S6 | Agronomic management of water and dry seeded mesocosm rice cultivation experiments in 2017. For sulfate treatments, ammonium sulfate and potassium sulfate were applied, while urea and potassium chloride were used equivalent in N and K for control treatments. Dates in red indicate pore-water sampling dates.

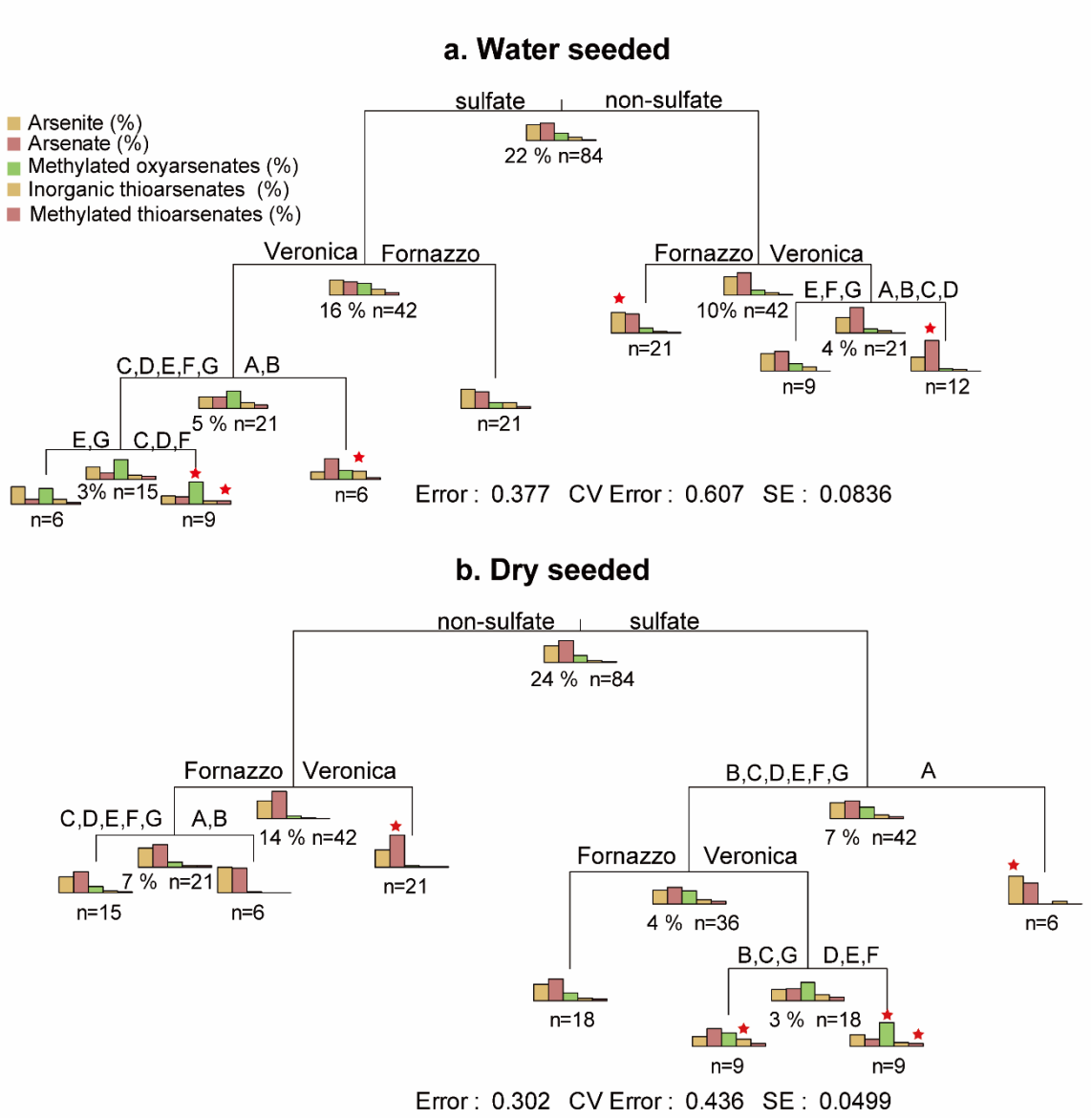

Figure S7 | Multivariate regression tree for pore-water As speciation in the mesocosm experiments for a) water seeded and b) dry seeded treatments. Multivariate regression tree analyses were done following previously published methods[10]. Capital letters A-G on the node represent the seven rice growth stages from tillering stage to maturity. Indicator species, based on relative abundance and relative frequency of occurrence of As species, are denoted by stars. Pre-separation in water and dry seeded was done because different redox regimes lead to an offset of growth stages in dry seeded compared to water seeded treatments by about 7-10 days.

3. Incubation experiments revealing soil properties that determine the potential for thioarsenate formation

Table S5a | Location, parent material, and soil classification of 31 paddy soils sampled in China.

No.	Location	Longitude	Latitude	Parent material	Soil classification (within the suborder Stagnic Anthrosols[a])
CH1	Jiangmen, Guangdong	112º31′09.2″	22º30′33.9″	river alluvium	Fe-accumuli-
CH2	Nanning 2, Guangxi	108º17′12.6″	23º06′22.4″	limestone	Fe-accumuli-
CH3	Nanning1, Guangxi	108º16′52.0″	23º06′27.6″	limestone	Fe-accumuli-
CH4	Dehong, Yunnan	98º25′55.1″	24º26′31.11″	river alluvium	Fe-accumuli-
CH5	Chuxiong, Yunnan	102º03′5.71″	25º11′01.3″	river alluvium (sandy)	Fe-leachi-
CH6	Ganzhou, Jiangxi	114º27′11.8″	25º26′31.8″	river alluvium	Fe-accumuli-
CH7	Guiyang, Guizhou	106º40′17.6″	26º20′12.7″	limestone	Fe-leachi-
CH8	Qiandongnan, Guizhou	108º03′43.19″	26º36′46.71″	limestone	Fe-leachi-
CH9	Sanming, Fujian	117º10′30.44″	26º50′56.14″	river alluvium	Fe-accumuli-
CH10	Ji'an, Jiangxi	114º54′36.0″	26º58′21.4″	river alluvium	Fe-accumuli-
CH11	Zunyi, Guizhou	106º48′14.5″	27º30′13.15″	limestone	Hapli-
CH12	Yingtan, Jiangxi	117º15′21.1″	28º20′31.1″	river alluvium	Fe-accumuli-
CH13	Yueyang, Hunan	113°5'43.8''	29°14'23.0''	lacustrine deposits	Fe-accumuli-
CH14	Jinhua, Zhejiang	119º20′34.1″	29º01′04.9″	river alluvium	Hapli-
CH15	Jingzhou, Hubei	112º 31′49.03″	30º 5′53.10″	river alluvium	Fe-accumuli-
CH16	Hangzhou, Zhejiang	120º05′13.3″	30º28′05.6″	river alluvium	Fe-accumuli-
CH17	Jiaxing, Zhejiang	121º07′01.26″	30º48′06.3″	marine sediment	Fe-leachi-
CH18	Zhenjiang, Jiangsu	119º18′15.47″	31º57′58.38″	river alluvium	Fe-leachi-
CH19	Xuancheng,Anhui	118º29′55.7″	31º00′02.6″	river alluvium	Hapli-
CH20	Hefei, Anhui	117º13′28.1″	31º35′54.1″	lacustrine deposits	Fe-accumuli-

CH21	Xinyang, Henan	114º59′03″	31º54′13″	river alluvium (loess)	Fe-accumuli-
CH22	Hanzhong, Shaanxi	106º54′36.15″	33º09′37.77″	loess	Fe-leachi-
CH23	Yancheng, Jingsu	120º04′10.6″	33º16′12.5″	marine sediment	Fe-accumuli-
CH24	Xuzhou, Jiangsu	117º24′38.49″	34º17′46.59″	river alluvium	Fe-leachi-
CH25	Lianyungang, Jiangsu	119º20′06.1″	34º32′58.0″	river alluvium	Fe-leachi-
CH26	Jining, Shandong	116º33′32.5″	35º18′47.7″	river alluvium	Fe-accumuli-
CH27	Yinchuan, Ningxia	106º16′0.97″	38º10′04.78″	river alluvium	Hapli-
CH28	Panjin, Liaoning	122º13′46.6″	40º58′26.09″	marine sediment	Hapli-
CH29	Siping, Jilin	124º42′29.1″	43º28′47.8″	river alluvium	Hapli-
CH30	Wuchang, Heilongjiang	127º02′12.9″	45º03′42.1″	river alluvium	Hapli-
CH31	Jiamusi, Heilongjiang	131º36′34.2″	47º11′12.8″	river alluvium	Hapli-

[a] Based on the Chinese Soil Taxonomic Classification (adopted by WRB in 1998) all paddy soils used here are classified as Stagnic Anthrosols. Stagnic Anthrosols are anthrosols that have an anthrostagnic moisture regime, and both a hydragric epipedon (including a cultivated horizon and a plowpan) and a hydragric horizon.

Table S5b | Geology/geomorphology and climate zones of the 31 paddy fields sampled in China.

No.	Geology/Geomorphology	Climate Zone
CH1	The lower hilly and wide valley basin of Jiangmen	Sub-tropical Monsoon
CH2	The valley plain area of Wuming Basin	Sub-tropical Monsoon
CH3	The valley plain area of Wuming Basin	Sub-tropical Monsoon
CH4	The lower hilly and wide valley basin of Dehong	Sub-tropical Monsoon
CH5	The low mountain and hilly area in Lufeng Basin	Sub-tropical Monsoon
CH6	The lower hilly and wide valley basin of Ganzhou	Sub-tropical Monsoon
CH7	The lower hilly and wide valley basin of Guiyang	Sub-tropical Monsoon
CH8	The lower hilly and wide valley basin of Qiandongnan	Sub-tropical Monsoon
CH9	The lower hilly and wide valley basin of Sanming	Sub-tropical Monsoon
CH10	The lower hilly and wide valley basin of Ji'an	Sub-tropical Monsoon
CH11	The lower hilly and wide valley basin of Zunyi	Sub-tropical Monsoon
CH12	The valley plain area of Xinjiang Basin	Sub-tropical Monsoon
CH13	The low-lying land of lakeshore plain of Dongting Lake	Sub-tropical Monsoon
CH14	The first grade terrace of Xinlixi River in the valley plain area of Jinqu Basin, Eastern China	Sub-tropical Monsoon
CH15	The alluvial and lacustrine plain area of Jianghan Plain, Central China	Sub-tropical Monsoon
CH16	The alluvial-marine plain area in North Zhejiang Plain, Eastern China	Sub-tropical Monsoon
CH17	The alluvial-marine plain area of the Yangtze River Delt	Sub-tropical Monsoon
CH18	The hilly and low-lying area of Zhenjiang in Jiangsu Province, Eastern China	Sub-tropical Monsoon
CH19	The first grade terrace in the valley plain of QingYijiang River	Sub-tropical Monsoon
CH20	The low-lying land of lakeshore plain of Chaohu Lake in Anhui Province, Eastern China	Sub-tropical Monsoon
CH21	The low foothill area of Xinyang in southern margin of North China Plain	Sub-tropical Monsoon
CH22	The first grade terrace of Bao River in alluvial and lacustrine plain area of the Hanzhong	Sub-tropical Monsoon

	Basin, in southern Shaanxi Province, China	
CH23	The alluvial-marine plain area of Yancheng in North Jiangsu Plain, Eastern China	Sub-tropical Monsoon
CH24	The alluvial and lacustrine plain area of Xuzhou in North China Plain	Temperate Monsoon
CH25	The coastal plain area in North Jiangsu Plain, Eastern China	Temperate Monsoon
CH26	The low-lying land of lakeshore plain of Weishan Lake in North China Plain	Temperate Monsoon
CH27	The first grade terrace of the Yellow River in Yinchuan Plain, Northwest China	Temperate Continental
CH28	The coastal plain area in Liaohe Plain, Northeast China	Temperate Monsoon
CH29	The first grade terrace of East Liao River in Liaohe Plain, Northeast China	Temperate Monsoon
CH30	The interchannel zone between Lalin River and Mangniu River in Song Nen Plain, Northeast China	Temperate Monsoon
CH31	The first grade terrace of Songhua River in Sanjiang Plain, Northeast China	Temperate Monsoon

Table S5c | Basic soil chemistry of the 31 paddy soils sampled in China.

No.	soil pH	CEC[a]	Clay	SOC[b]	Total As	Chalcophile metals				HCl-extractable Fe	Total zerovalent sulfur[c]	
						Cd	Pb	Cu	Zn		control	3 mmol/kg sulfate
		cmol/kg	%	g/kg	mg/kg					mmol/kg	mmol/kg	
CH1	5.1	4.0	16.5	27.9	6.7	0.1	9.9	3.3	35.3	31.9	0.4	1.5
CH2	5.6	9.2	20.3	56.5	34.2	0.3	33.3	15.7	82.3	74.7	0.9	1.9
CH3	6.1	8.2	24.4	36.7	15.5	0.6	23.2	28.5	108.4	94.6	0.2	0.7
CH4	6.2	9.2	33.0	25.9	5.9	0.1	24.6	11.7	87.3	84.1	2.2	2.2
CH5	6.9	20.0	29.8	61.6	8.2	0.7	25.3	74.4	103.4	113.3	0.6	1.9
CH6	4.9	5.5	14.7	20.7	38.8	0.4	53.9	36.7	111.3	78.1	0.0	0.7
CH7	6.9	15.7	13.2	104.4	15.5	0.5	26.6	31.0	142.8	69.7	0.1	1.2
CH8	5.8	8.2	37.5	39.7	10.3	0.2	21.1	17.5	83.7	30.0	0.2	1.3
CH9	5.3	4.2	9.6	42.9	2.6	0.2	31.7	20.0	126.4	41.5	0.3	0.7
CH10	4.9	5.6	17.0	25.5	8.4	0.1	19.1	14.0	63.8	106.3	0.2	0.9
CH11	8.0	16.6	28.6	46.8	11.1	0.6	23.0	25.3	100.1	99.4	0.7	0.8
CH12	4.5	4.2	18.5	39.3	7.5	0.6	19.7	74.3	52.8	60.4	0.1	1.2
CH13	5.6	9.8	17.0	14.0	15.9	0.3	29.9	21.3	89.3	129.1	0.2	1.0
CH14	5.5	10.9	16.9	21.4	8.0	0.2	23.2	7.9	57.4	57.2	0.3	0.9
CH15	8.1	11.8	11.4	25.7	12.4	0.3	22.3	30.9	110.2	130.2	0.9	1.7
CH16	6.4	16.6	43.2	35.8	6.1	0.2	24.6	24.4	79.7	98.1	0.2	1.8
CH17	7.0	18.3	31.1	33.7	11.0	0.2	22.8	26.6	110.5	110.7	0.2	0.8
CH18	7.2	9.7	16.6	28.2	7.9	0.1	18.7	16.9	58.9	110.3	0.6	1.5
CH19	5.0	10.7	24.8	34.5	9.9	0.2	23.6	14.3	62.0	90.3	0.8	1.2
CH20	5.7	15.7	20.6	27.6	7.0	0.1	14.8	12.5	44.1	99.5	0.4	1.3
CH21	5.4	10.4	18.3	34.3	5.8	0.1	16.3	12.7	55.4	132.1	0.5	1.2
CH22	5.5	12.9	21.0	38.8	9.5	0.3	20.4	19.5	77.6	142.3	0.6	1.8

CH23	6.2	6.4	25.1	33.3	5.9	0.1	15.0	13.5	67.4	105.7	0.2	1.3
CH24	7.7	15.6	13.1	32.5	11.8	0.2	16.9	19.5	76.2	115.1	1.3	2.3
CH25	8.9	16.3	28.3	27.2	16.5	0.2	27.9	27.1	119.6	120.4	1.4	1.6
CH26	7.7	17.9	28.7	42.7	13.0	0.3	35.0	23.3	83.1	113.9	1.9	2.6
CH27	8.5	4.7	10.6	17.9	11.3	0.2	12.4	15.8	61.9	79.2	2.4	3.1
CH28	7.2	20.6	15.8	28.4	7.9	0.1	17.9	18.1	71.4	69.5	2.0	2.8
CH29	6.7	16.6	13.8	30.4	8.6	0.1	15.4	16.9	67.2	130.5	2.0	2.3
CH30	6.3	18.8	25.1	45.1	9.6	0.1	17.3	19.9	68.3	146.3	0.2	1.1
CH31	5.9	19.0	25.3	39.5	10.0	0.1	14.9	14.0	44.8	184.7	0.3	1.0

[a] Cation exchange capacity; [b] Soil organic carbon; [c] Total zerovalent sulfur formation after 14 days of soil incubation as described in section 4, which was calculated as sum of aqueous and solid phase zerovalent sulfur for control treatments and 3 mmol/kg sulfate addition treatments, respectively.

Table S6 | Spearman's correlation analyses for soil and pore water parameters for anaerobic soil incubations of 31 paddy soils from China and 2 paddy soils from Italy; marked in green are R values with a P-value < 0.05

R (control, no S)	methylated thioarsenates [%]	total thiolation [%]	soil pH	soil-bound zerovalent S [µmol/kg]	HCl-extractable Fe [g/kg]	soil total As [mg/kg]	soil organic carbon [g/kg]	CEC [cmol/kg]	Clay [%]	Total chalcophile metals [mmol/kg]	porewater pH	methylated oxyarsenates [%]	porewater zerovalent S [µmol/l]	porewater As [µg/L]	porewater Fe [mg/L]
inorganic thioarsenates [%]	0.22	0.85	0.58	0.46	-0.10	-0.18	-0.10	0.17	-0.25	-0.04	0.44	-0.01	0.25	-0.48	-0.89
methylated thioarsenates [%]		0.54	-0.31	-0.21	-0.36	-0.63	0.19	-0.01	0.00	-0.17	-0.46	0.69	0.03	-0.63	-0.04
total thiolation [%]			0.28	0.30	-0.21	-0.34	0.02	0.06	-0.33	-0.16	0.16	0.29	0.26	-0.58	-0.66
soil pH				0.54	0.33	0.32	0.06	0.54	0.08	0.21	0.87	-0.54	-0.03	-0.12	-0.79
soil-bound zerovalent S [µmol/kg]					0.28	0.10	-0.04	0.30	-0.09	-0.16	0.69	-0.19	0.31	-0.24	-0.51
HCl-extractable Fe [g/kg]						0.27	0.00	0.54	0.16	-0.09	0.37	-0.31	-0.09	0.24	0.02
soil total As [mg/kg]							0.00	0.12	-0.07	0.46	0.37	-0.56	-0.33	0.45	0.02
soil organic carbon [g/kg]								0.30	0.36	0.32	0.04	0.12	-0.02	-0.24	0.06
CEC [cmol/kg]									0.37	0.08	0.57	-0.17	0.08	0.00	-0.33
Clay [%]										0.10	0.05	0.22	-0.16	-0.09	0.13
Total chalcophile metals [mmol/kg]											0.23	-0.27	-0.26	-0.15	-0.16
porewater pH												-0.59	0.17	-0.02	-0.71
methylated oxyarsenates [%]													0.07	-0.41	0.23
porewater zerovalent S [µmol/L]														0.06	-0.20
porewater As [µg/L]															0.33

P-Value (control, no S)	methylated thioarsenates [%]	total thiolation [%]	soil pH	soil-bound zerovalent S [µmol/kg]	HCl-extractable Fe [g/kg]	soil total As [g/kg]	soil organic carbon [g/kg]	CEC [cmol/kg]	Clay [%]	Total chalcophile metals [mmol/kg]	porewater pH	methylated oxyarsenates [%]	porewater zerovalent S [µmol/l]	porewater As [µg/L]	porewater Fe [mg/L]
inorganic thioarsenates [%]	0.22	0.00	0.00	0.01	0.57	0.32	0.58	0.35	0.17	0.85	0.01	0.97	0.17	0.01	0.00
methylated thioarsenates [%]		0.00	0.08	0.23	0.04	0.00	0.30	0.95	1.00	0.36	0.01	0.00	0.86	0.00	0.85
total thiolation [%]			0.11	0.09	0.24	0.05	0.93	0.75	0.07	0.39	0.38	0.10	0.15	0.00	0.00
soil pH				0.00	0.06	0.07	0.74	0.00	0.69	0.26	0.00	0.00	0.89	0.52	0.00
soil-bound zerovalent S [µmol/kg]					0.12	0.56	0.82	0.10	0.64	0.38	0.00	0.29	0.08	0.20	0.00
HCl-extractable Fe [g/kg]						0.14	0.98	0.00	0.40	0.64	0.04	0.07	0.62	0.19	0.91
soil total As [mg/kg]							1.00	0.52	0.72	0.01	0.04	0.00	0.06	0.01	0.90
soil organic carbon [g/kg]								0.10	0.05	0.08	0.85	0.50	0.93	0.20	0.75
CEC [cmol/kg]									0.04	0.65	0.00	0.37	0.68	1.00	0.07
Clay [%]										0.60	0.79	0.23	0.38	0.61	0.49
Total chalcophile metals [mmol/kg]											0.22	0.15	0.16	0.42	0.40
porewater pH												0.00	0.36	0.92	0.00
methylated oxyarsenates [%]													0.69	0.02	0.19
porewater zerovalent S [µmol/L]														0.74	0.25
porewater As [µg/L]															0.07

R (Sulfate Treatment)	methylated thioarsenates [%]	total thiolation [%]	soil pH	soil-bound zerovalent S [µmol/kg]	HCl-extractable Fe [g/kg]	soil total As [mg/kg]	soil organic carbon [g/kg]	CEC [cmol/kg]	Clay [%]	Total chalcophile metals [mmol/kg]	porewater pH	methylated oxyarsenates [%]	porewater zerovalent S [µmol/l]	porewater As [µg/L]	porewater Fe [mg/L]
inorganic thioarsenates [%]	0.08	0.91	0.59	0.35	-0.07	-0.14	-0.16	0.18	-0.17	-0.03	0.63	-0.06	-0.19	-0.39	-0.86
methylated thioarsenates [%]		0.40	-0.41	-0.33	-0.35	-0.67	0.21	-0.02	0.02	-0.21	-0.34	0.72	-0.13	-0.46	0.27
total thiolation [%]			0.38	0.21	-0.22	-0.34	-0.06	0.11	-0.20	-0.11	0.52	0.18	-0.23	-0.50	-0.73
soil pH				0.49	0.33	0.32	0.06	0.54	0.08	0.21	0.66	-0.47	-0.07	-0.05	-0.90
soil-bound zerovalent S [µmol/kg]					0.21	0.12	-0.04	0.25	-0.04	-0.15	0.44	-0.30	0.25	-0.26	-0.54
HCl-extractable Fe [g/kg]						0.26	0.00	0.54	0.15	-0.09	0.39	-0.38	0.07	0.29	-0.07
soil total As [mg/kg]							0.00	0.12	-0.07	0.46	0.09	-0.67	-0.17	0.47	-0.08
soil organic carbon [g/kg]								0.30	0.36	0.32	-0.15	0.09	0.05	-0.21	-0.01
CEC [cmol/kg]									0.37	0.08	0.38	-0.26	0.11	0.07	-0.39
Clay [%]										0.10	-0.18	0.17	0.25	-0.09	0.11
Total chalcophile metals [mmol/kg]											-0.02	-0.22	-0.12	-0.14	-0.16
porewater pH												-0.36	-0.03	-0.04	-0.69
methylated oxyarsenates [%]													-0.14	-0.46	0.34
porewater zerovalent S [µmol/L]														-0.04	0.03
porewater As [µg/L]															0.34

P-Value (Sulfate Treatment)	methylated thioarsenates [%]	total thiolation [%]	soil pH	soil-bound zerovalent S [µmol/kg]	HCl-extractable Fe [g/kg]	soil total As [mg/kg]	[g/kg]	CEC [cmol/kg]	Clay [%]	Total chalcophile metals [mmol/kg]	porewater pH	methylated oxyarsenates [%]	porewater zerovalent S [µmol/l]	porewater As [µg/L]	porewater Fe [mg/L]
inorganic thioarsenates [%]	0.65	0.00	0.00	0.04	0.68	0.44	0.37	0.34	0.35	0.86	0.00	0.74	0.28	0.03	0.00
methylated thioarsenates [%]		0.02	0.02	0.06	0.05	0.00	0.25	0.90	0.90	0.26	0.06	0.00	0.46	0.01	0.16
total thiolation [%]			0.03	0.23	0.22	0.05	0.73	0.56	0.27	0.56	0.00	0.32	0.20	0.00	0.00
soil pH				0.00	0.06	0.07	0.74	0.00	0.69	0.26	0.00	0.01	0.68	0.79	0.00
soil-bound zerovalent S [µmol/kg]					0.25	0.49	0.81	0.18	0.82	0.41	0.01	0.09	0.16	0.15	0.00
HCl-extractable Fe [g/kg]						0.14	0.99	0.00	0.41	0.62	0.03	0.03	0.71	0.12	0.72
soil total As [mg/kg]							1.00	0.52	0.72	0.01	0.65	0.00	0.33	0.01	0.69
soil organic carbon [g/kg]								0.10	0.05	0.08	0.41	0.63	0.77	0.27	0.95
CEC [cmol/kg]									0.04	0.65	0.03	0.16	0.56	0.71	0.04
Clay [%]										0.60	0.33	0.36	0.17	0.64	0.57
Total chalcophile metals [mmol/kg]											0.89	0.23	0.51	0.46	0.41
porewater pH												0.05	0.87	0.82	0.00
methylated oxyarsenates [%]													0.42	0.01	0.08
porewater zerovalent S [µmol/L]														0.81	0.88
porewater As [µg/L]															0.07

Table S7 | Relative importance of predictor values for the occurrence of inorganic and methylated thioarsenates (%) using multiple linear regression analysis with soil physical and chemical properties separated by control (no S) and sulfate-addition (S) incubations; for methylated thioarsenates two models were used, one including the share of methylated oxyarsenates in the pore water and one only using soil parameters; significance levels (sig. level) are indicated as *** (0-0.001), ** (0.001-0.01), * (0.01-0.05), and . (0.1-1)

Control (no S)	**weight factor %**	**lower 95% range [%]**	**upper 95% range [%]**	**sig. level**
inorganic thioarsenates				
CEC [cmol/kg]	13.6	1.7	30.2	**
Clay [%]	-18.3	-3.3	-40.6	**
HCl-extractable Fe [g/kg]	-6	-1.1	-16.6	*
{H+} [mol/L]	-4.4	-2.8	-14.5	
zerovalent S [µmol/kg]	50.8	17.8	74.9	***
SOC [g/kg]	-4.5	-0.9	-11.2	
total soil As [mg/kg]	-1.3	-0.4	-8.1	
Total chalcophile metals [mmol/kg]	-1.1	-0.5	-8.2	
$r^2 = 0.7352$, $p = 7.1*10^{-5}$				

methylated thioarsenates + oxyarsenates				
CEC [cmol/kg]	**3.2**	1.6	24.5	
Clay [%]	-9.8	-1.1	-21.6	*
HCl-extractable Fe [g/kg]	-10.8	-3.3	-29.1	
{H+} [mol/L]	-1.8	-0.9	-10.9	
zerovalent S [µmol /kg]	-3.2	-0.8	-20.4	
SOC [g/kg]	-0.8	-0.7	-9.4	
total soil As [mg/kg]	-18.8	-8.8	-30.3	*
Total chalcophile metals [mmol/kg]	5.7	2.3	23	*
methylated oxyarsenates [%]	45.9	8.2	55.7	**
$r^2 = 0.6902$, $p = 8.9*10^{-4}$				

methylated thioarsenates - oxyarsenates				
CEC [cmol/kg]	-6	-1.6	-25.7	
Clay [%]	-10.1	-1	-23.8	.

HCl-extractable Fe [g/kg]	-26.9	-3.9	-45.4	*
{H+} [mol/L]	-4	-1.3	-19.6	
zerovalent S [µmol /kg]	-6.7	-0.9	-30.3	
SOC [g/kg]	-1	-0.8	-15.6	
total soil As [mg/kg]	-40.4	-12.3	-49.1	**
Total chalcophile metals [mmol/kg]	4.9	2.6	37.1	
$r^2 = 0.5022$, $p = 2.8*10^{-2}$				

Sulfate treatment	**weight factor %**	**lower 95% range [%]**	**upper 95% range [%]**	**sig. level**
inorganic thioarsenates				
CEC [cmol/kg]	20.6	3	30.6	**
Clay [%]	-12.6	-1.6	-31.5	*
HCl-extractable Fe [g/kg]	-8.8	-1.8	-19.4	*
{H+} [mol/L]	-4.2	-2.7	-17.3	
zerovalent S [µmol /kg]	46.1	21	68.6	**
SOC [g/kg]	-6.3	-1.1	-15.5	*
total soil As [mg/kg]	-0.8	-0.4	-10.4	
Total chalcophile metals [mmol/kg]	-0.8	-0.5	-7.7	
$r^2 = 0.674$, $p = 5.6*10^{-4}$				

methylated thioarsenates + oxyarsenates				
CEC [cmol/kg]	4.3	1.5	14.6	
Clay [%]	-1.5	-0.5	-17.5	
HCl-extractable Fe [g/kg]	-12.9	-3.2	-21	
{H+} [mol/L]	-0.8	-0.5	-15.4	
zerovalent S [µmol /kg]	-3.7	-0.6	-13.6	
SOC [g/kg]	-0.3	-0.4	-8.2	
total soil As [mg/kg]	-11.2	-4.7	-23.6	
Total chalcophile metals [mmol/kg]	1.5	0.6	19.9	
methylated oxyarsenates [%]	63.8	21.2	65.8	***
$r^2 = 0.8304$, $p = 2.8*10^{-6}$				

methylated thioarsenates - oxyarsenates				
CEC [cmol/kg]	-8.3	-1.8	-19.6	
Clay [%]	-1.9	-0.6	-29.4	
HCl-extractable Fe [g/kg]	-39.1	-3.6	-47.3	*
{H+} [mol/L]	-1.7	-1.2	-27.5	
zerovalent S [µmol /kg]	-10.9	-0.9	-28	.
SOC [g/kg]	-0.8	-0.8	-14.9	
total soil As [mg/kg]	-33.1	-9.3	-49.7	*
Total chalcophile metals [mmol/kg]	4.1	1.1	40.9	
$r^2 = 0.5151$, $p = 2.2*10^{-2}$				

Note: Implications of HCl-extractable Fe, pH, zerovalent S, total soil As, and methylated oxyarsenates are discussed in the main manuscript; the reasons for the sig. negative impact of clay on both inorganic and methylated thioarsenates and the sig. positive impact of CEC (Cation Exchange Capacity) on inorganic thioarsenates are currently unclear. The correlation with CEC might be in line with previous observations of high ionic strengths increasing the kinetics of inorganic thioarsenate formation from arsenite and reduced sulfur in solution. [11, 12]

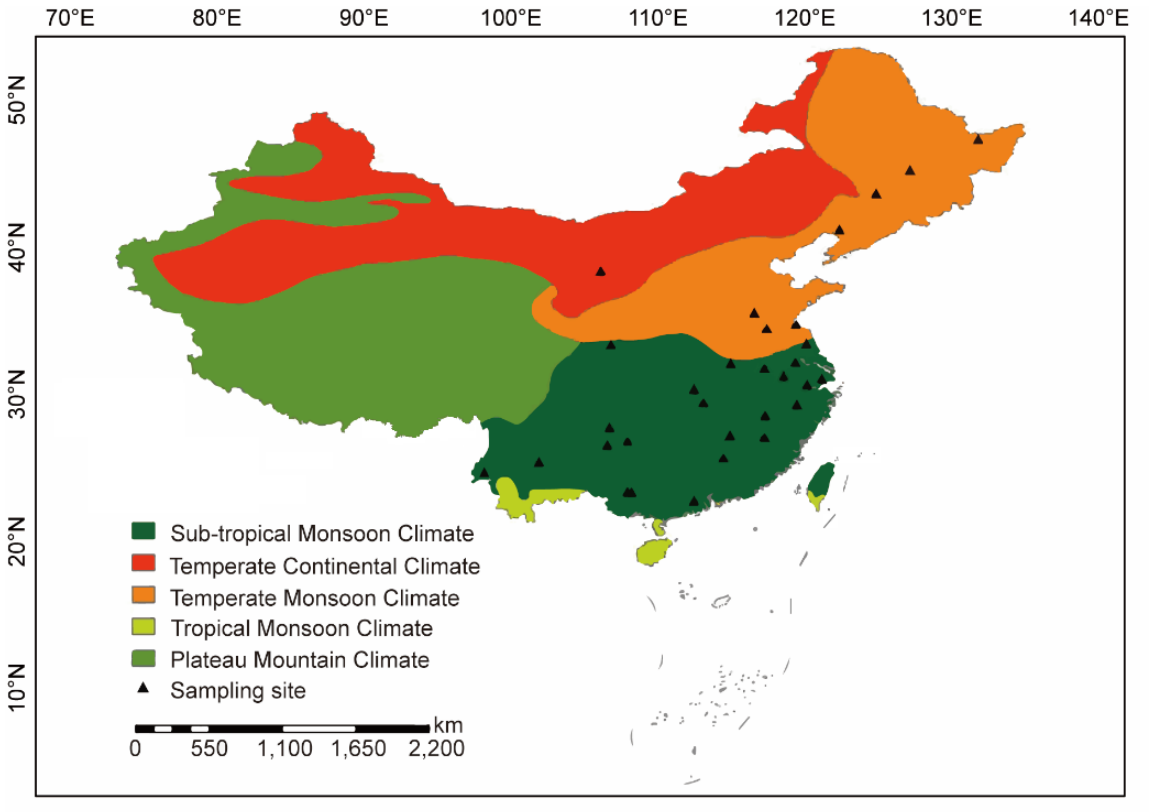

Figure S8 | Sampling sites of 31 paddy fields in different climate zones over China. The geographic origins covered an area from 22.5° to 47.2° N and 98.4° to 131.6° E, spanning climate zones from sub-tropical monsoon climate (23 fields) to temperate continental climate (1 fields) and temperate monsoon climate (7 fields).The base map used is from the National Fundamental Geographic Information System of China.

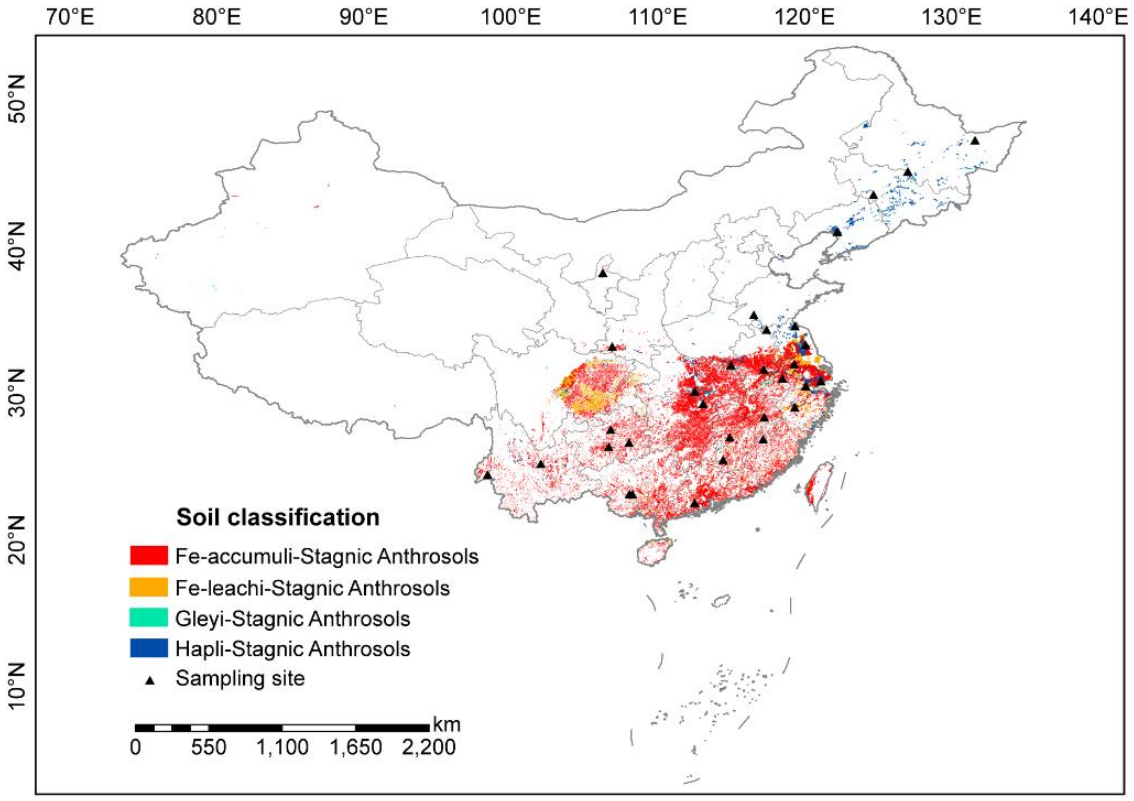

Figure S9 | Sampling sites of 31 paddy fields based on soil classification over China. The colored background indicates the distribution of Stagnic Anthrosols in China. Based on Chinese Soil Taxonomic Classification (adopted by WRB in 1998), all paddy soils used here are classified as Stagnic Anthrosols, including Fe-accumuli- (15 soils), Fe-leachi-(8 soils), Hapli- (8 soils) Stagnic Anthrosols. Stagnic Anthrosols are anthrosols that have an anthrostagnic moisture regime and have both a hydragric epipedon (including a ultivated horizon and a plowpan) and a hydragric horizon. The base map used is from the National Fundamental Geographic Information System of China.

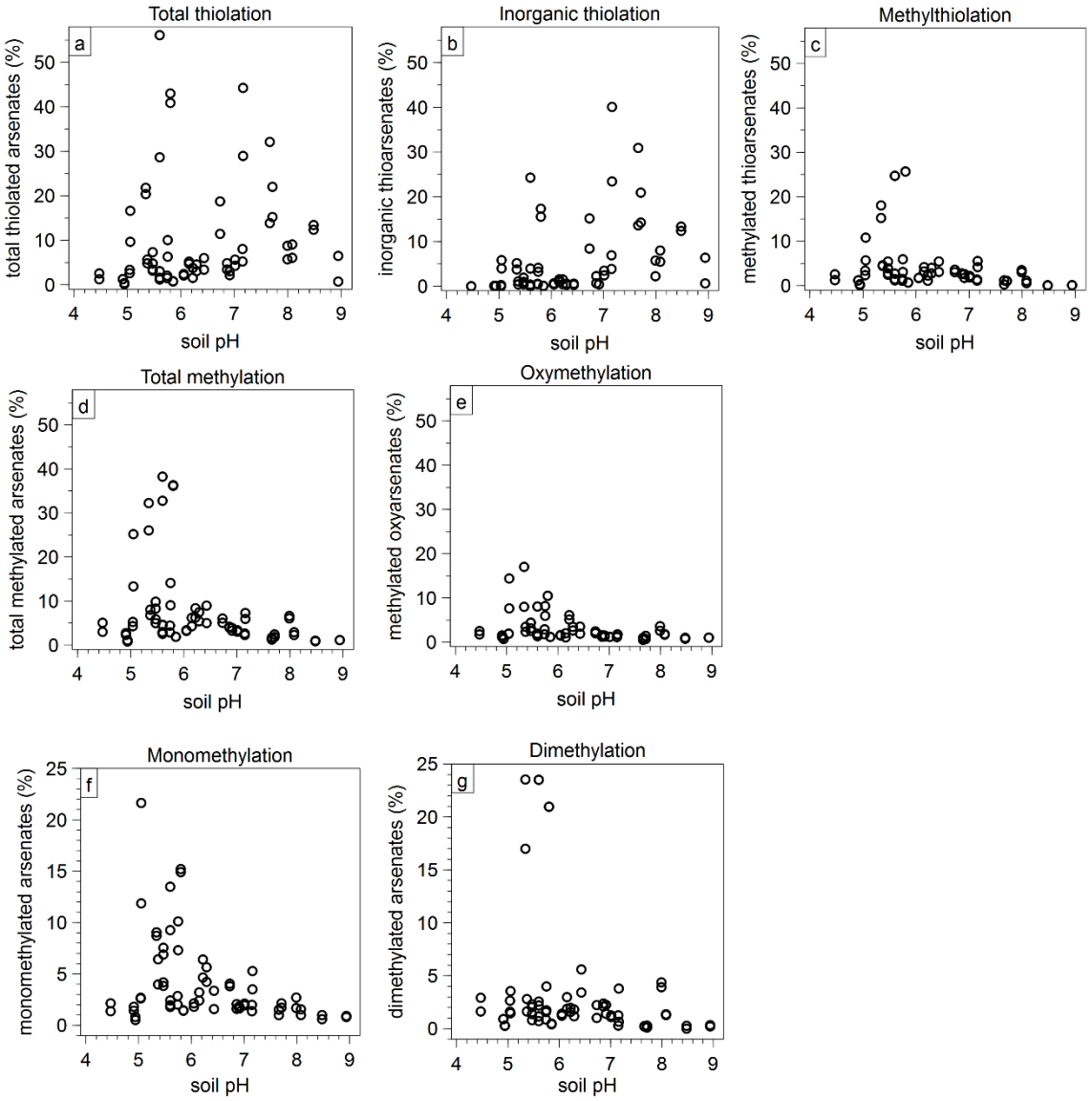

Figure S10 | Summarized As speciation determined in anaerobic soil incubations in relation to soil pH. Data from control and sulfate addition for 31 paddy soils from China and 2 paddy soils from Italy were combined. a) total thiolation which is the sum of b) inorganic thiolation and c) methylthiolation; d) total methylation which is the sum of e) oxymethylation and c) methylthiolation; f) all mono- and g) all dimethylated arsenates (integrating oxy and thio species).

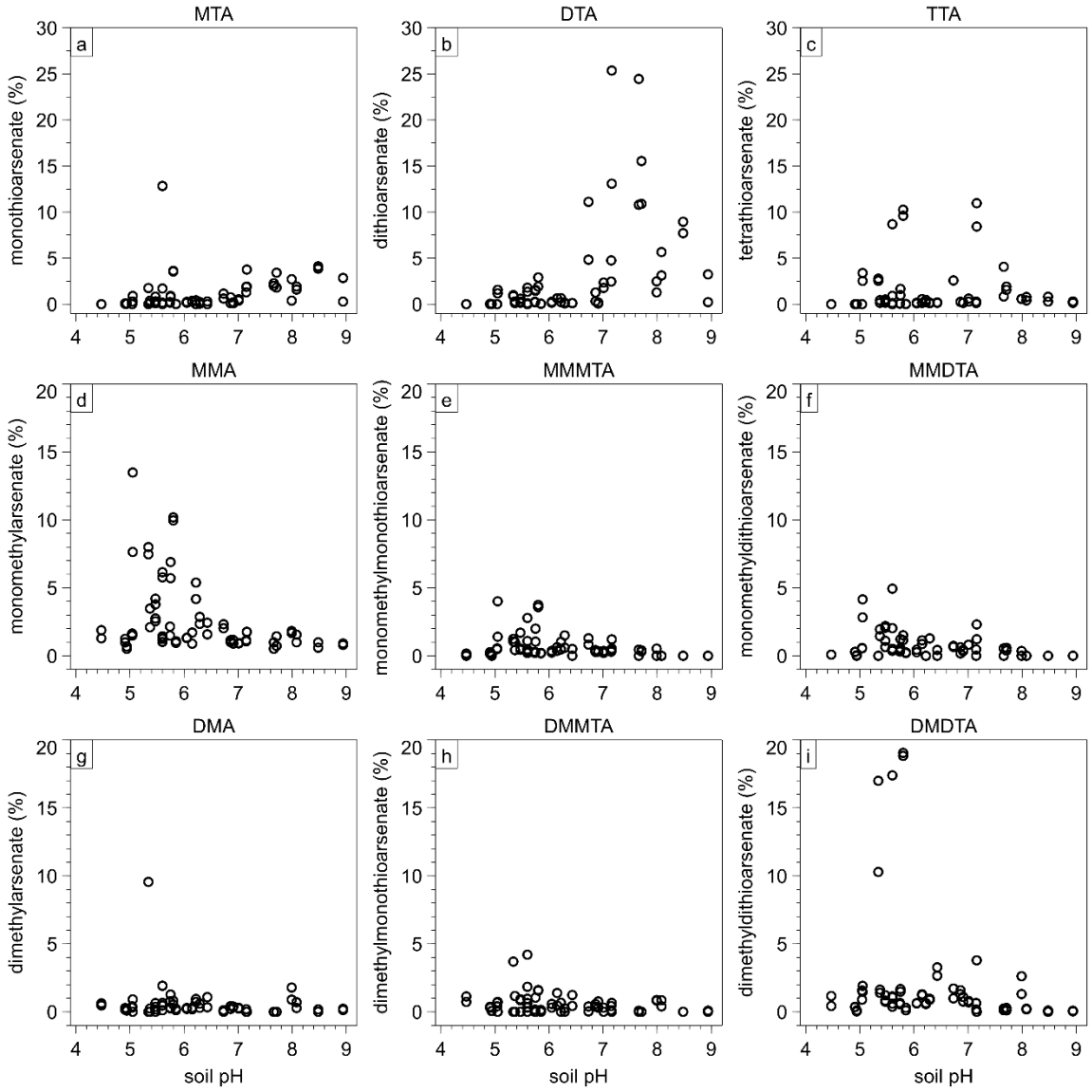

Figure S11 | Individual As speciation of inorganic thioarsenates in anaerobic soil incubations in relation to soil pH. a) MTA, b) DTA, c) TTA, d) monomethylated oxy- (MMA) and thioarsenates e) MMMTA, f) MMDTA) and g) dimethylated oxy- (DMA), and thioarsenates h) DMMTA, i) DMDTA .Data from control and sulfate addition for 31 paddy soils from China and 2 paddy soils from Italy were combined.

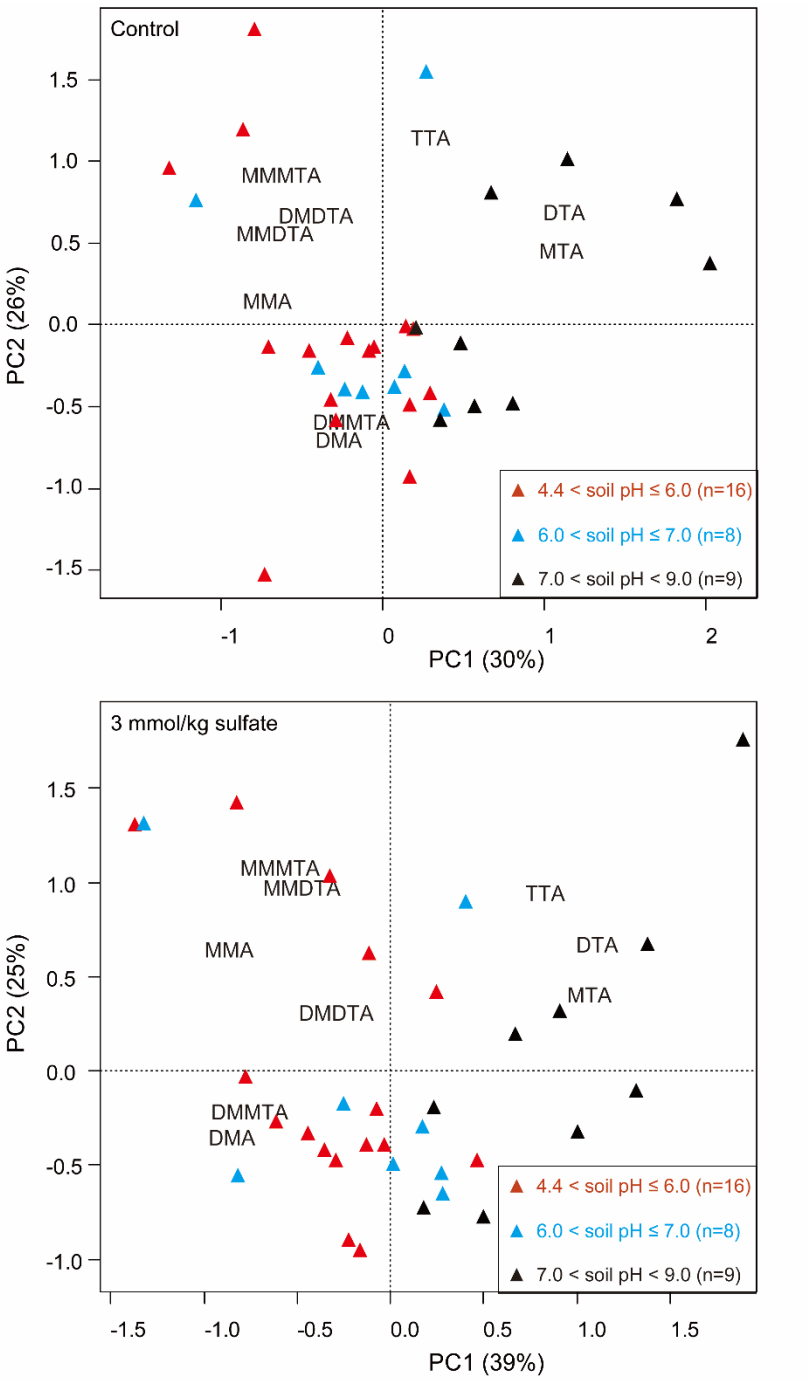

Figure S12 | Principal component analysis of As speciation in anaerobic soil incubations. Site distribution reveals clustering of methylated oxyarsenates (DMA and MMA) and methylated thioarsenates (DMDTA, MMDTA, MMMTA, DMMTA) with low pH soils and inorganic thioarsenates (MTA, DTA, and TTA) with high pH soils during anaerobic incubation of 31 paddy soils from China and 2 paddy soils from Italy; a) control treatment and b) sulfate addition.

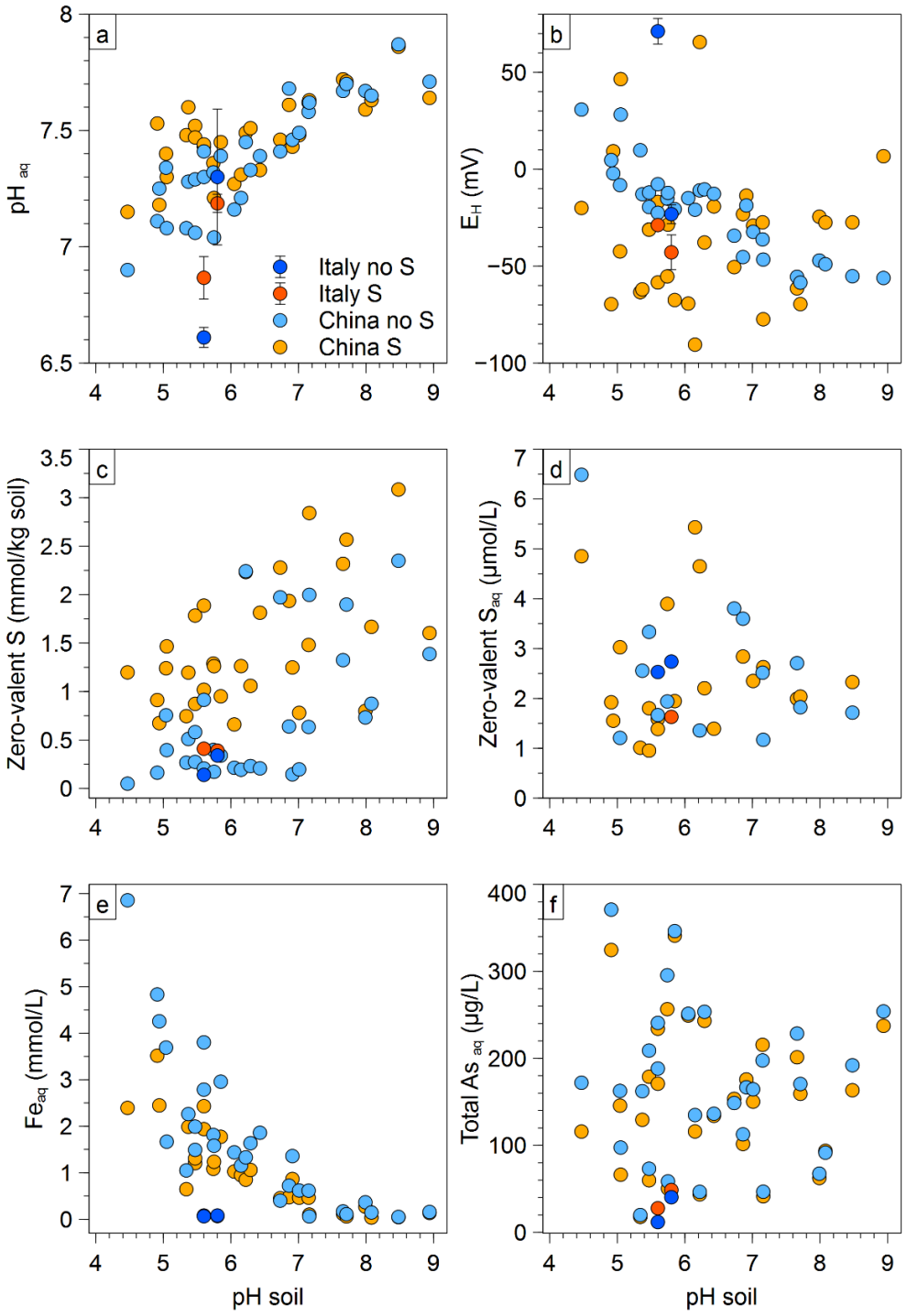

Figure S13 | Pore-water chemistry for anaerobic soil incubations as a function of soil pH. a) pore-water pH, b) E_H, c) solid phase zero-valent S, d) aqueous zero-valent S, e) total dissolved Fe, and f) total dissolved As for anaerobic soil incubations of 31 paddy soils from China and 2 paddy soils from Italy (experimental triplicates); blue colors refer to control treatments (no S), orange-red colors to sulfate addition (S).

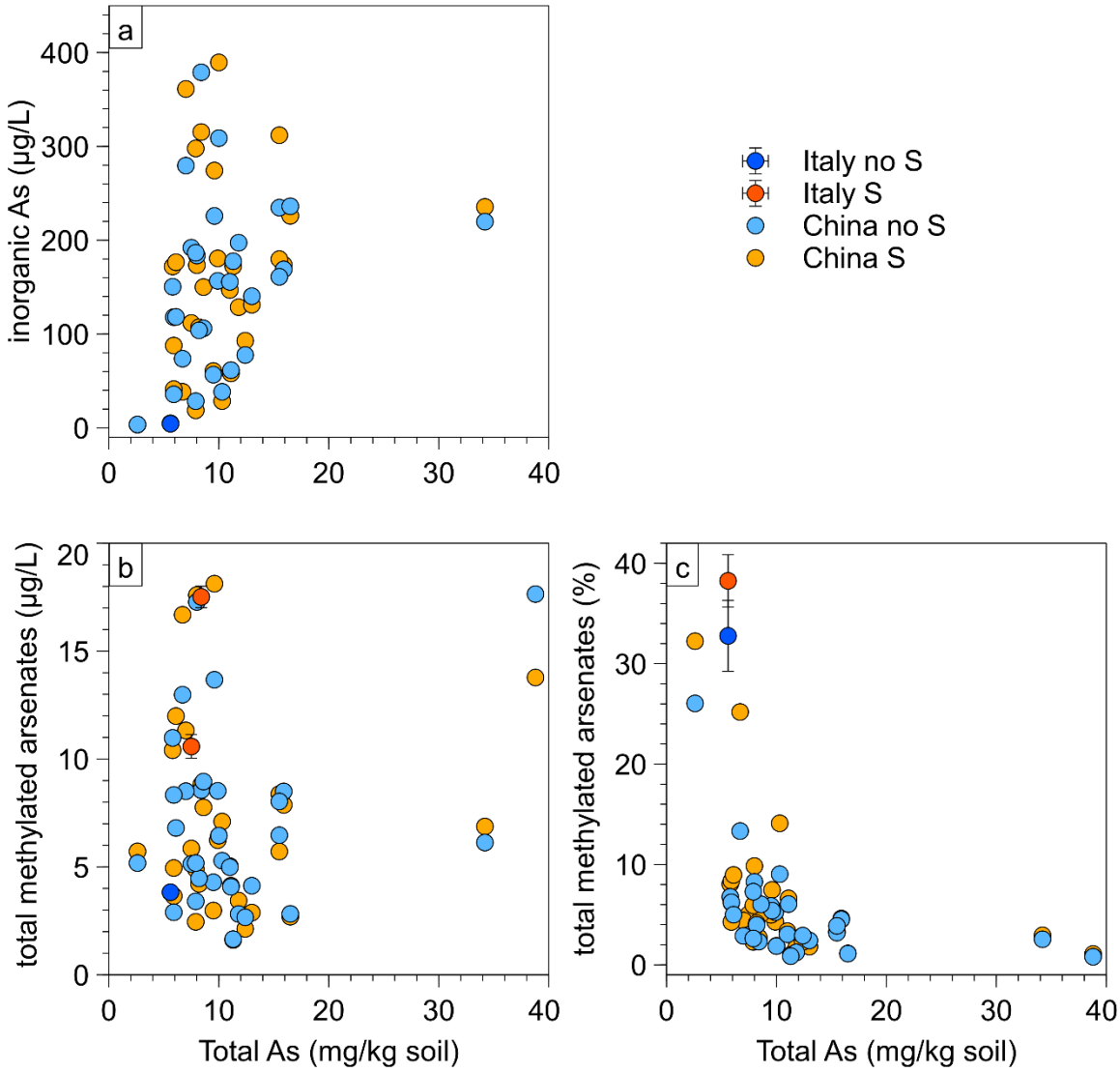

Figure S14 | Absolute and relative concentrations of inorganic As (a, b) and methylated oxyarsenates (c, d) in relation to total soil As. Anaerobic soil incubations were conducted with 31 paddy soils from China and 2 paddy soils from Italy (experimental triplicates); blue colors refer to control treatments (no S), orange-red colors to sulfate addition (S).

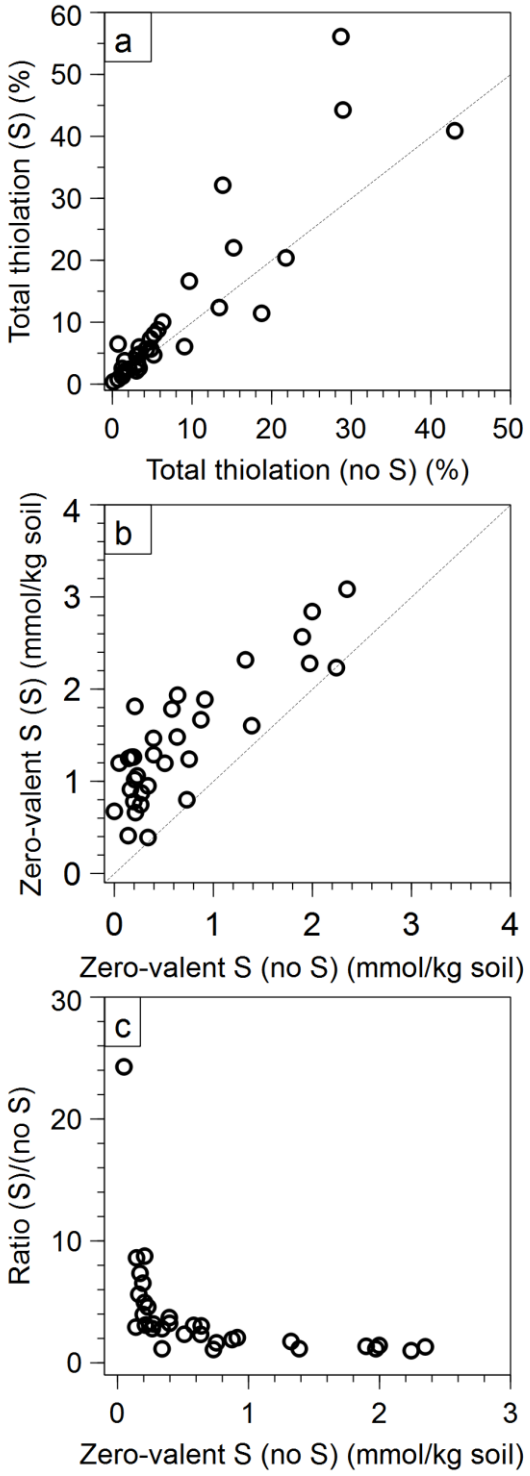

Figure S15 | Effects of sulfate addition on arsenic thiolation and zero-valent S formation. Comparison of a) total thiolation (%) and b) solid phase zero-valent S with and without sulfate addition; c) ratio of zero-valent S increase from control to sulfate-treatment versus original zero-valent S concentrations in control for anaerobic soil incubations of 31 paddy soils from China and 2 paddy soils from Italy.

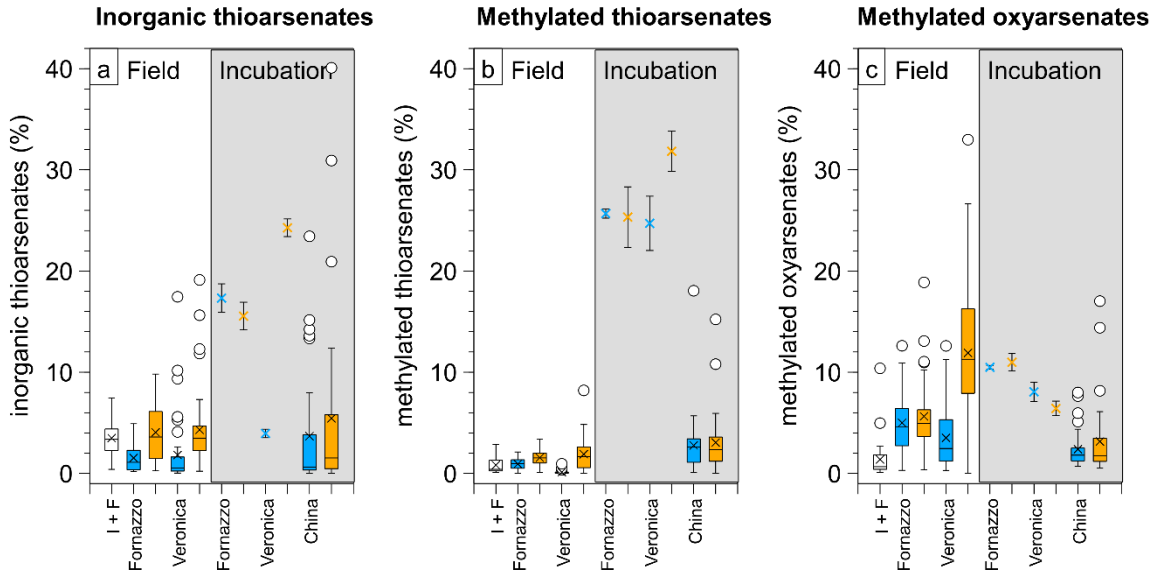

Figure S16 | Comparison of arsenic speciation in anaerobic soil incubations to mesocosm experiments and field survey. a) occurrence of inorganic thioarsenates, b) methylated thioarsenates, and c) methylated oxyarsenates in the field with plants (one-time survey Italy/France at late plant growth stage (I+F); n = 35; mesocosms with Veronica and Fornazzo soil integrated over whole rice cultivation period of 4 months; n = 42 each) and in anaerobic soil incubations (with Veronica and Fornazzo soils (n=3 each) and paddy soils over a pH-gradient in China (n=31 each); blue colors refer to control treatments (no S), orange colors to sulfate addition (S)

4. Methods

Table S8 | Quantitative Recovery of As species in a fresh model pore water sample spiked with 1 µg/L of DMA, DMMTA, arsenite, MMA, MMMTA, and arsenate, stabilized with 10 mM DTPA, and analyzed 1:5 and 1:10 diluted with deionized water using 2.4 % methanol in the eluent and either individual or averaged calibration

		DMA	DMMTA[a]	Arsenite	DMDTA[b]	MMA	MMMTA[c]	MMDTA[d]	Arsenate	MTA[e]	DTA[f]	Sum of As species
Counts per sencond	DMA 1 µg/L Standard	1001699										
	MMA 1 µg/L Standard					815446						
	Arsenate 1 µg/L Standard								876310			
	sample 1:10 dilution	151318	164191	37415	14160	114871	62514	87301	252602	19856	13196	
	sample 1:5 dilution	309624	400192	104872	36697	247134	153063	185058	415801	23790	13544	
Concentration (µg/L)	sample 1:10 dilution (measured concentration)	0.15	0.16	0.04	0.02	0.14	0.08	0.10	0.29	0.02	0.02	
	sample 1:5 dilution (measured concentration)	0.31	0.40	0.11	0.05	0.30	0.19	0.21	0.47	0.03	0.02	
	sample 1:10 dilution x 10 (final concentration by individual calibration)	1.51	1.64	0.37	0.17	1.41	0.77	1.00	2.88	0.23	0.15	10.85[g]

		DMA	DMMTA[a]	Arsenite	DMDTA[b]	MMA	MMMTA[c]	MMDTA[d]	Arsenate	MTA[e]	DTA[f]	Sum of As species
	sample 1:5 dilution x 5 (final concentration by individual calibration)	1.55	2.00	0.52	0.23	1.52	0.94	1.06	2.37	0.14	0.08	10.94[g]
	sample 1:10 dilution x 10 (final concentration by averaged calibration)	1.69	1.83	0.42	0.16	1.28	0.70	0.97	2.81	0.22	0.15	11.02[g]
	sample 1:5 dilution x 5 (final concentration by averaged calibration)	1.72	2.23	0.58	0.20	1.38	0.85	1.03	2.32	0.13	0.08	11.14[g]
(%) of total As	sample 1:10 dilution	13.9%	15.1%	3.4%	1.6%	13.0%	7.1%	9.2%	26.6%	2.1%	1.4%	>2.5% well detectable
	sample 1:5 dilution	14.1%	18.3%	4.8%	2.1%	13.9%	8.6%	9.7%	21.7%	1.2%	0.7%	>1% well detectable

Table S9 | Characteristics of irrigation water for mesocosm rice cultivation experiments.

Parameters	Irrigation water
pH	7.7
Conductivity (µS/cm)	508
TIC (mg/L)[a]	17.5
TOC (mg/L)[b]	1.2
Cl^- (mg/L)	11.4
NO_3^- (mg/L)	1.11
NO_2^- (mg/L)	1.05
PO_4^{3-} (mg/L)	1.01
SO_4^{2-} (mg/L)	11.1
Si (mg/L)	8.9
Mn (µg/L)	1.5
Cu (µg/L)	2.6
Zn (µg/L)	16.5
As (µg/L)	6.4

[a] Total inorganic carbon; [b] Total organic carbon

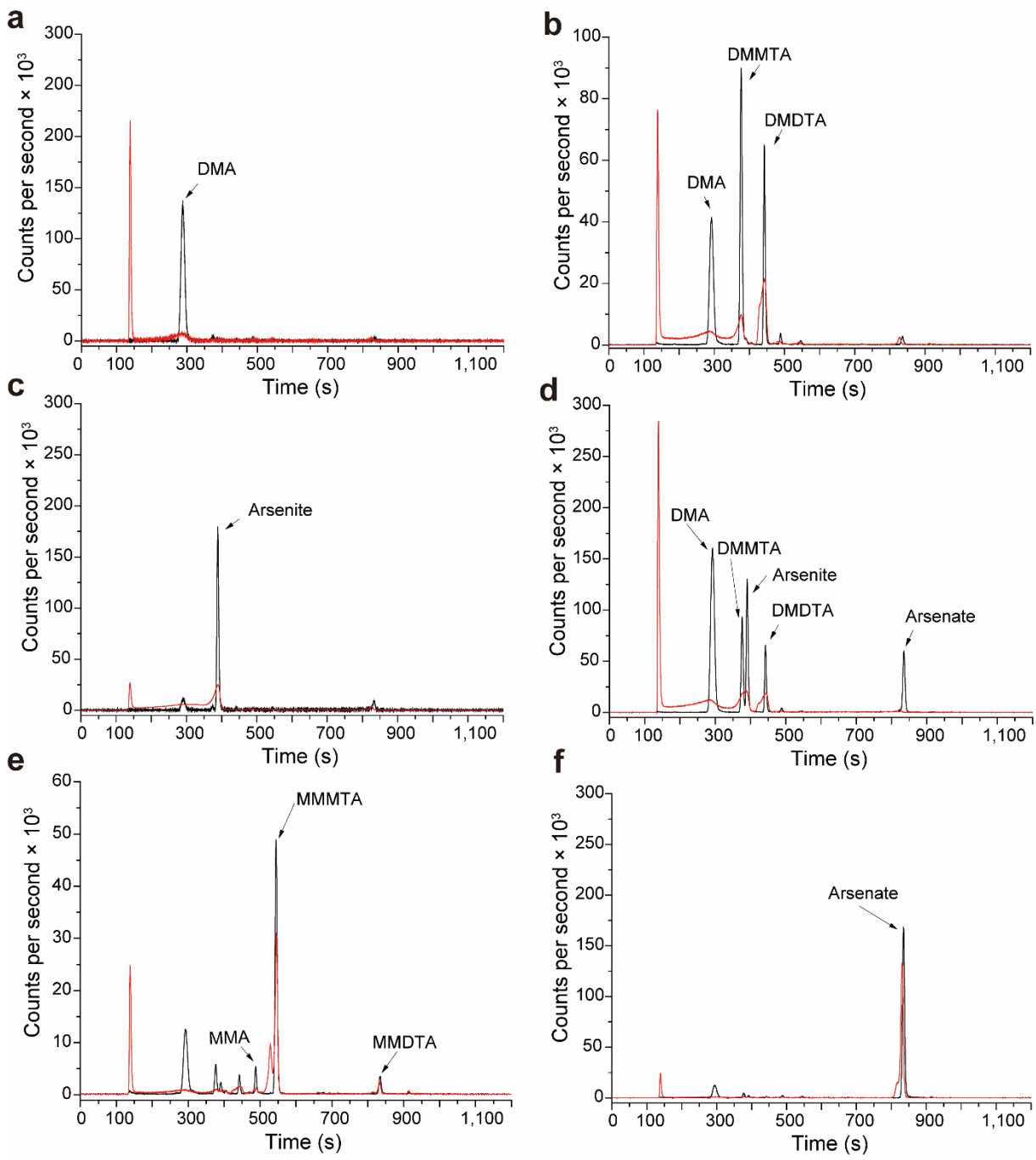

Figure S17 | Effect of 10 mM DTPA on retention time, peak shape, and resolution of As speciation. Tested model pore-water was spiked with 100 µg/L standards of a) DMA; b) DMMTA; c) arsenite; d) a mix of arsenite, DMA, DMMTA; e) MMMTA; f) arsenate; black lines = without DTPA, red lines = with 10 mM DTPA.

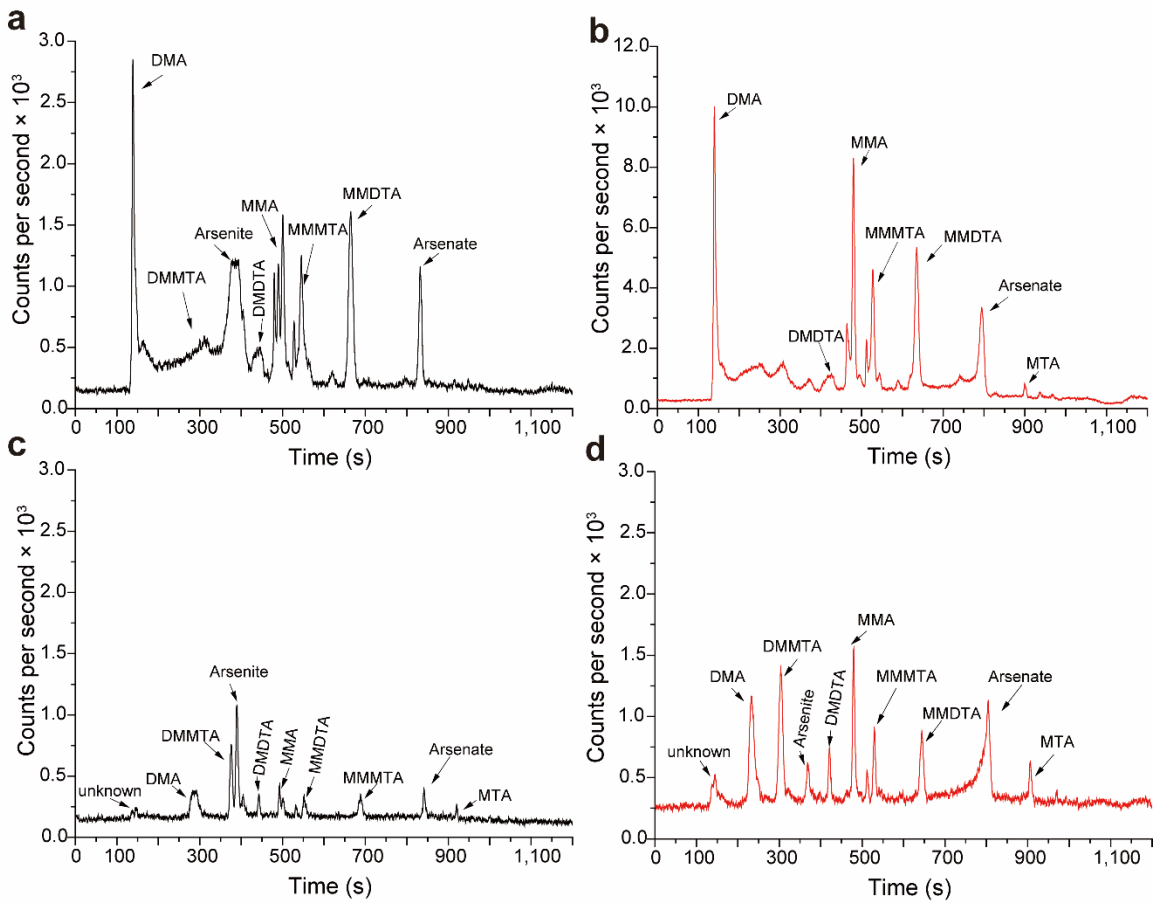

Figure S18 | Effect of sample dilution and use of methanol in the eluent on retention time, peak shape, and resolution of As speciation. Fresh, non-spiked model pore-water samples stabilized with 10 mM DTPAwere a) analyzed without dilution and without 2.4% methanol; b) analyzed without dilution with 2.4% methanol; c) diluted 1:10 with deionized water before analysis and analyzed without 2.4% methanol; d) diluted 1:10 with deionized water before analysis and analyzed with 2.4% methanol. All samples were analyzed with a 2.5-100 mM NaOH gradient eluent.

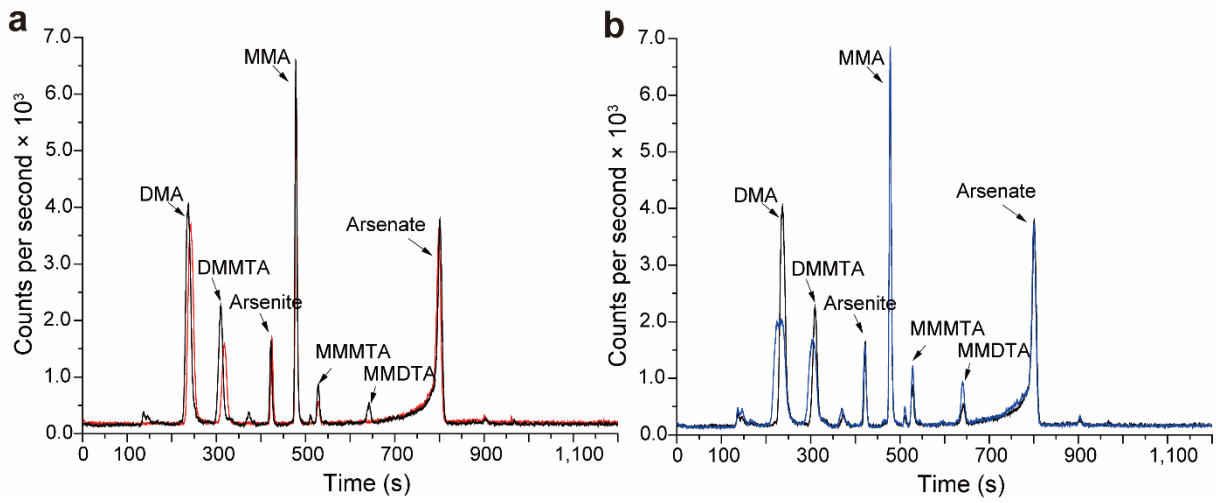

Figure S19 | Effect of pore-water matrix and different DTPA dilution on retention time, peak shape, and resolution of As speciation. Fresh model pore-water samples were spiked with 1 µg/L of DMA, DMMTA, arsenite, MMA, MMMTA, and arsenate,stabilized with 10 mM DTPA (all analyzed with 2.4% methanol in the eluent). a) comparison deionized water (red) and pore-water matrix 1:10 diluted (black), b) comparison 1:5 (blue) vs. 1:10 (black) dilution of pore-water matrix sample.

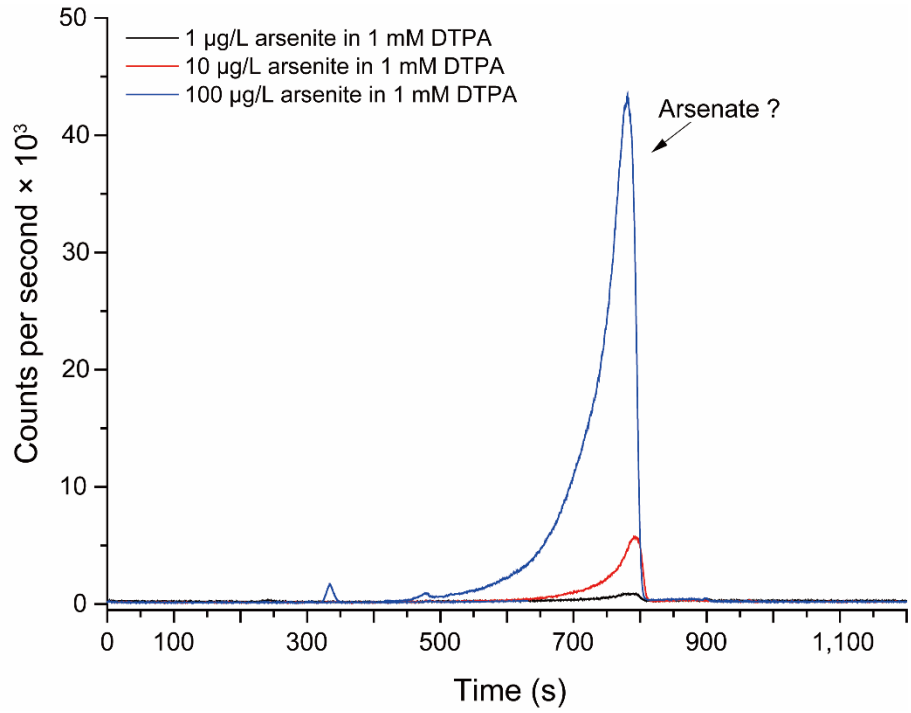

Figure S20 | Effect of 1 mM DTPA on arsenite standards in deionized water. The question mark behind the arsenate label indicates that the observed transformation product elutes at the retention time of arsenate but might also be an unidentified As-DTPA complex**.**

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Study 2: Redox Dependence of Thioarsenate Occurrence in Paddy Soils and the Rice Rhizosphere

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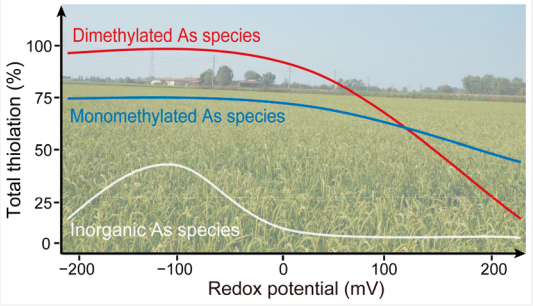
ABSTRACT: In flooded paddy soils, inorganic and methylated thioarsenates contribute substantially to arsenic speciation besides the much-better-investigated oxyarsenic species, and thioarsenate uptake into rice plants has recently been shown. To better understand their fate when soil redox conditions change, that is, 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.



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 nonthreshold 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 have been reported. Monomethylated arsenate (MMA) and dimethylated arsenate (DMA) are usually minor species in paddy soil pore waters, 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, for example, created by root radial oxygen loss, or changes from reducing to oxidizing conditions, for example, 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)-

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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; $As^VS(OH)_3$), di- (DTA; $As^VS_2(OH)_2^-$), and trithioarsenate (TTA; $As^VS_3(OH)^{2-}$), and (2) Methylated thioarsenates, which include mono- (MMMTA; $(CH_3)As^VS(OH)_2$) and dimethylated monothioarsenate (DMMTA; $(CH_3)_2As^VS(OH)$); mono- (MMDTA; $(CH_3)As^VS_2(OH)^-$) and dimethylated dithioarsenate (DMDTA; $(CH_3)_2As^VS_2^-$). Inorganic thioarsenates form by reaction of arsenite, sulfide, and zerovalent 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, that is, 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 toward oxidation for monothiolated than higher order thiolated species, for example, 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 thioarsenate 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 Supporting Information (SI) 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 days. 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 min), 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 sample processing were 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 days of soil reduction and 25 days 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 days, 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 sample processing were 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 (SI Figure S1a). For each microcosm, 400 g fresh soil (2 mm sieved) was 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.

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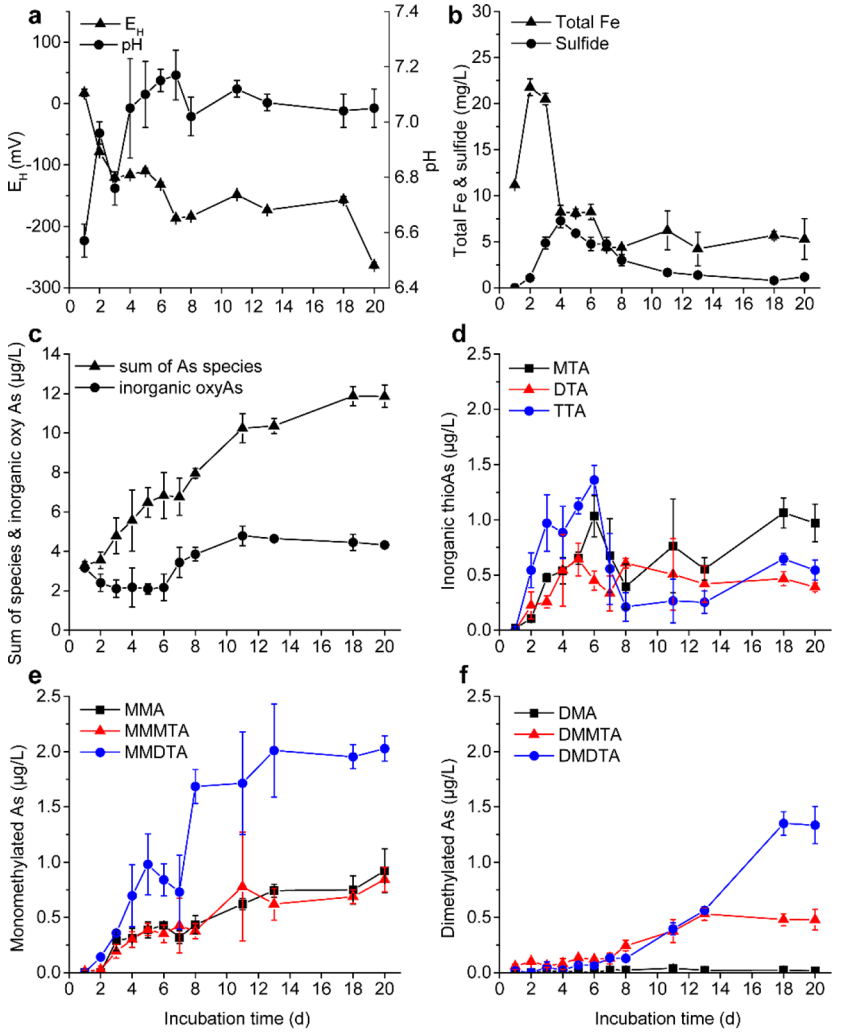

Figure 1. Pore-water chemistry (a, b) and As speciation (c, d, e, f) dynamics in reduction batch incubations using Veronica soil (spiked with 1 mmol L^{-1} sulfate). Bars represent standard errors (n = 3). For data of control experiment without sulfate spike see SI Figure S3.

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 days (without any spike), drained for 4 days, and subsequently reflooded for another 14 days 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 SI Figure 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 (SI Figure 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 six 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 g of air-dried paddy soil, equilibrated with 500 mL of tap water and developed 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 days, 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 and 20 °C, respectively. Fertilizers used were ammonium sulfate, potassium sulfate, and triple superphosphate. Besides an initial

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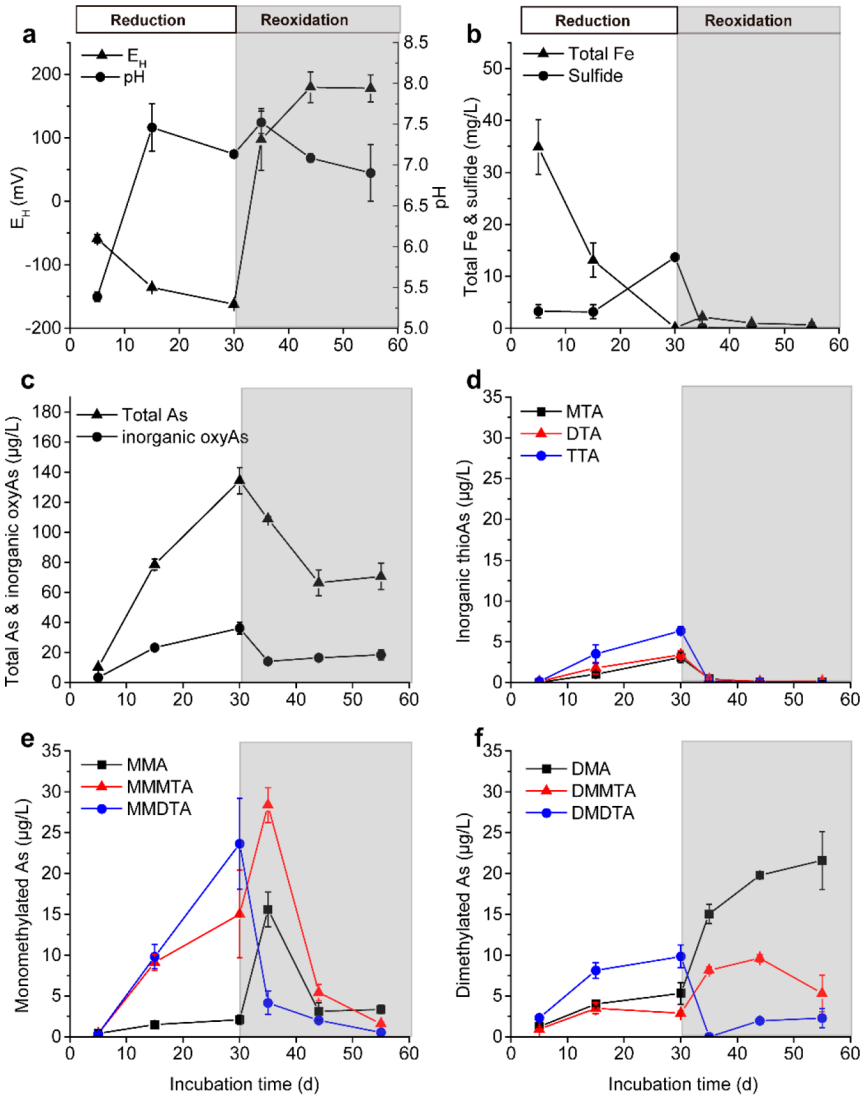

Figure 2. Pore-water chemistry (a, b) and As speciation (c, d, e, f) dynamics in reduction-reoxidation batch incubations using Veronica soil (spiked with 9 mmol L^{-1} sulfate, 100 μg L^{-1} arsenate). Bars represent standard errors (n = 3).

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 days (15 days preincubation and 75 days in the climate chamber), pore-water was extracted as described above.

Analytical Techniques. During sampling, unstable chemical parameters, that is, 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 diethylenetriaminepentaacetic 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 (Figure 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 (Figure 1b). In parallel, thiosulfate (maximum 0.5 mg L^{-1}; SI Figure S2b) and sulfide (Figure 1b) concentrations increased, along with decreasing sulfate concentrations (SI Figure S2b). Sulfide peaked at day 4 with a concentration of 8 mg L^{-1}, then decreased to <2 mg L^{-1} after 1 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} (Figure 1c). Besides inorganic oxyarsenic species (Figure 1c) and

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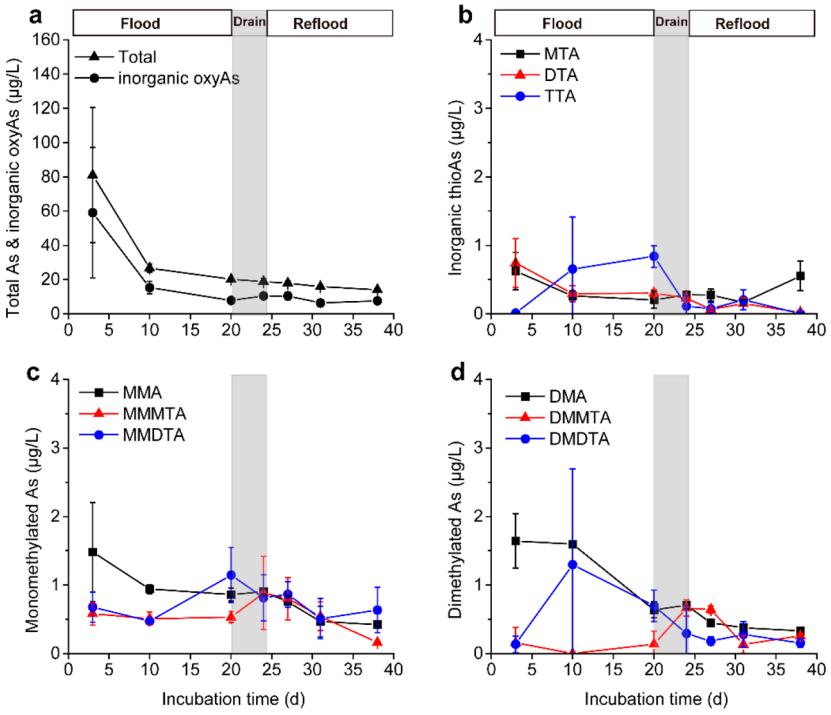

Figure 3. Arsenic speciation dynamics in flood-drain-reflood microcosm incubations using Fornazzo soil. Bars represent standard errors (n = 3). For arsenic speciation dynamics in microcosm incubations using Veronica soil see SI Figure S6.

methylated oxyarsenates (Figure 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 (Figure 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 days, before reaching concentrations of 0.5 and 1.3 μg L^{-1} for DMMTA and DMDTA, respectively (Figure 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 (Figure 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 (SI Figure 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 (Figure 2a) compared to −260 mV at day 20 with acetate addition as described before. Upon reoxidation, E_H increased up to 97 mV within 5 days, before stabilizing at ∼180 mV. The pH increased to neutral conditions during soil reduction, followed by a slight decrease of 0.5 units toward the end of the reoxidation period. Total dissolved Fe was 35 mg L^{-1} at day 5, followed by a fast decrease (Figure 2b). Sulfide increased substantially from 3 to 14 mg L^{-1} between day 15 and 30. Both total Fe and free sulfide concentrations were close to detection limit after reoxidation.

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

Upon reoxidation, total As decreased markedly from day 30 to day 44 before reaching ∼70 μg L^{-1}. Inorganic oxyarsenic species dropped from 36 μg L^{-1} to 14 μg L^{-1} within 5 days, and remained at that level thereafter (Figure 2c), whereas all inorganic thioarsenates decreased to negligible concentrations after reoxidation. Methylated dithiolated As (MMDTA and DMDTA) dropped steeply after 5 days of reoxidation and were constant at low levels with prolonged oxidation (Figure 2e,f). The decrease of MMDTA was accompanied by the 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 (Figure 2e). The decrease of DMDTA was accompanied by an increase of DMA and DMMTA (day 35). DMA increased slowly afterward, concurrent with a slight decrease of DMMTA toward the end of reoxidation (Figure 2f). The major As species that survived prolonged reoxidation of 25 days were, in the order DMA, inorganic oxyarsenic species, and DMMTA.

Dynamics of Thioarsenates in Flood-Drain-Reflood Microcosm Incubations. In the microcosm incubations

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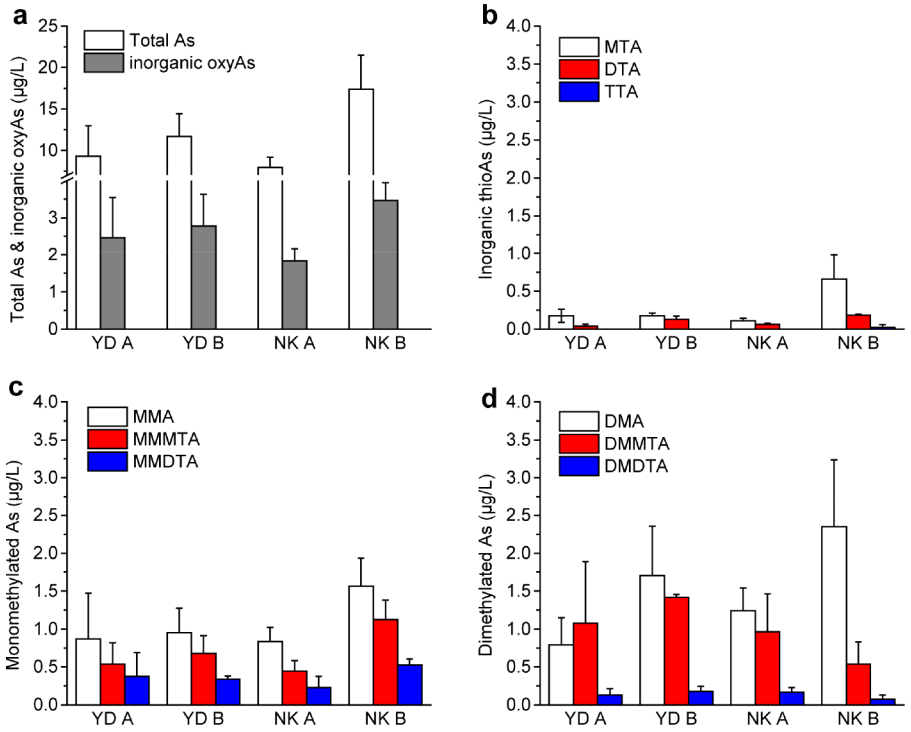

Figure 4. Arsenic speciation in paddy soil pore waters of planted rhizobox experiments using Fornazzo soil; YD A: rice variety Yangdao, sampled at depth A (9 cm below soil-water interface); YD B:Yangdao, sampled at depth B (16.5 cm below soil-water interface); NK A: rice variety Nongken, sampled at depth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

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 days and dropped again to around 10 mV after reflooding (SI Figure 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 (SI Figure S5b,d). Total Fe decreased slightly after drainage for 4 days, 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 (Figure 3a) and Veronica, respectively (SI Figure 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 toward 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 (Figure 3b–d) than in Veronica soil (SI Figure 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, that is, MMDTA and DMDTA, were detected as the main methylated thioarsenates (Figure 3c,d and SI Figure S6c,d, blue line), which was consistent with observations from the batch incubations.

After drainage and oxidation for 4 days and reflooding, DTA and TTA concentrations were close to detection limit. MTA was the only inorganic thioarsenate species that remained (Figure 3b and SI Figure S6b). Among the methylated thioarsenates, both MMDTA and DMDTA concentrations were substantially lower than before drainage. MMMTA and DMMTA showed an increase in concentrations with reflooding (Figure 3c,d and SI Figure 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 (SI Figure 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 (Figure 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}) (Figure 4b). MMMTA was the second-highest thiolated species (up to 1.5 μg L^{-1}), with concentrations slightly lower than its precursor MMA (Figure 4c). DMMTA was detected as the dominant thioarsenate species (up to 2.2 μg L^{-1}), with concentrations comparable to its precursor DMA (Figure 4d). MMDTA and DMDTA were detected but in much lower concentrations compared to their respective monothiolated As forms (Figure 4c,d).

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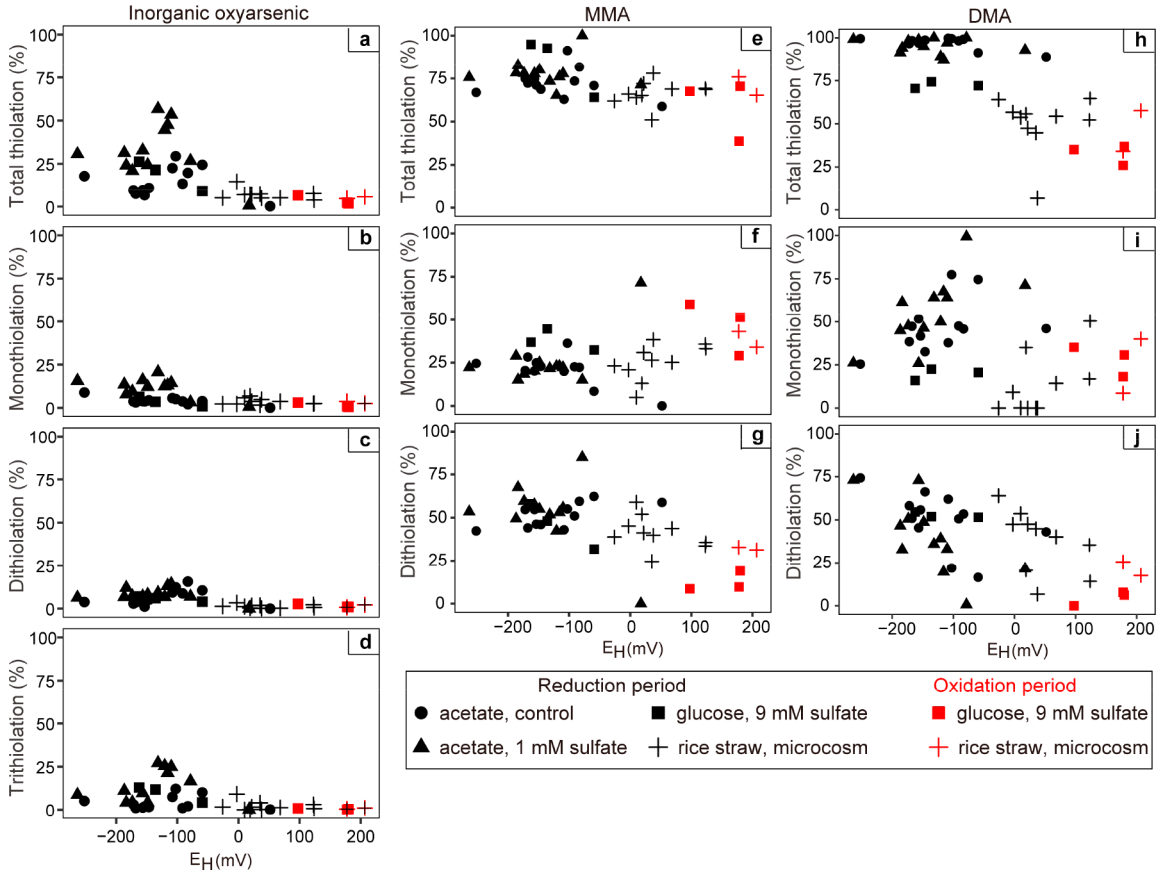

Figure 5. Percentage of total thiolation (a, e, h), mono- (b, f, (i), di- (c, g, j), and trithiolation (d) of inorganic oxyarsenic species (a–d), monomethylated (e–g), and dimethylated (h–j) arsenates as a function of pore-water redox potential (E_H); (calculation example: DMA dithiolation = DMDTA/(DMA+DMMTA+DMDTA));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

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) (Figure 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 (Figure 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 were 5.6 and 5.8, respectively). Especially in controls without sulfate spike, concentrations were very low (<0.5 $\mu g\ L^{-1}$) (SI Figure S3d). Sulfate spiking of anoxic soil lead to the formation of free sulfide and a rapid formation of inorganic thioarsenates (Figure 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 days, 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 toward 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 (Figure 1e,f and SI Figure 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 (Figure 5e,h). The high thiolation rate indicates spontaneous thiolation, which is not limited by sulfide supply, but

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intrinsically by the formation of the oxylated precursors MMA and DMA, which are usually detected only as minor As species in paddy soil pore waters.[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 (Figure 1b and SI Figure S3b), thereby greatly exceeding the sum of all As species (<0.15 μM, Figure 1c and SI Figure 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 from 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 (0.1−1 μM) by 1−2 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 (Figure 3c,d and SI Figure 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 (Figures 1e,f and 2e,f, and SI Figure S3e,f) and microcosm experiments (Figure 3c,d and SI Figure S6c,d) when E_H was <100 mV (Figure 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 ($\leq$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 Figures 1a, 2a, and SI Figures S3a and S5a,c). The reason is that pH upon flooding of acidic soils will increase due to proton-consuming reductions of, for example, 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 days after re-establishment of oxic conditions in both batch (Figure 2e,f) and microcosm experiments (Figure 3c,d and SI Figure S6c,d). The driving processes for dethiolation are likely depletion of sulfide (Figure 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 (Figure 2a) due to proton-producing oxidation processes, for example, 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 (Figure 5f) and DMA at E_H > −150 mV (Figure 5j), confirmed stepwise dethiolation. For DMA, dethiolation continued from DMMTA to DMA which increased in concentration under oxic conditions (Figure 2f) and became dominant at E_H > 100 mV (Figure 5i). For MMA, concentrations started to increase upon oxidation (day 35, Figure 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 (Figure 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 days under oxic soil conditions. In fact, concentrations during the reoxidation period were even higher than those observed during the initial reducing period due to dethiolation of DMDTA (Figure 2f).

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

In accordance with previous studies that indicate the potential contribution of DMMTA to rice grain As accumulation,[25,26] here we provide the first direct evidence that DMMTA occurs in the rhizosphere of paddy soils and is

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available for root uptake. DMMTA, which derives either from the thiolation of DMA or dethiolation of DMDTA, was identified as the dominant methylated thioarsenate species in the rhizosphere, irrespective of the root oxygen loss ability of rice cultivars (Figure 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 (Figure 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 need to be clarified in future studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b05639.

Summary of experimental designs (table and sketches), reduction batch incubations (control: pH, E_H, total Fe, sulfide, As speciation; control and 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 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Britta Planer-Friedrich − *Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), University of Bayreuth, 95440 Bayreuth, Germany;* orcid.org/0000-0002-0656-4283; Phone: +49-921-553999; Email: b.planer-friedrich@uni-bayreuth.de; Fax: +49-921-552334

Authors

Jiajia Wang − *Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), University of Bayreuth, 95440 Bayreuth, Germany*

Dipti Halder − *Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), University of Bayreuth, 95440 Bayreuth, Germany*

Laura Wegner − *Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), University of Bayreuth, 95440 Bayreuth, Germany*

Lena Brüggenwirth − *Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), University of Bayreuth, 95440 Bayreuth, Germany*

Jörg Schaller − *Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), University of Bayreuth, 95440 Bayreuth, Germany; Leibniz Center for Agricultural Landscape Research (ZALF), 15374 Müncheberg, Germany;* orcid.org/0000-0003-1996-0127

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Maria Martin − *Department of Agriculture, Forest and Food Sciences, University of Turin, 10124 Turin, Italy;* orcid.org/0000-0003-1075-7739
Daniel Said-Pullicino − *Department of Agriculture, Forest and Food Sciences, University of Turin, 10124 Turin, Italy*
Marco Romani − *Rice Research Centre, Ente Nazionale Risi, 27030 Castello d'Agogna, Pavia, Italy*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.9b05639

Notes

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Supporting Information

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

*Jiajia Wang[1], Dipti Halder[1], Laura Wegner[1], Lena Brüggenwirth[1], Jörg Schaller[1,4], Maria Martin[2], Daniel Said-Pullicino[2], Marco Romani[3] and Britta Planer-Friedrich[1]**

[1]Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), Bayreuth University, 95440 Bayreuth, Germany

[2]Department of Agriculture, Forest and Food Sciences, University of Turin, 10124 Turin, Italy

[3]Rice Research Centre, Ente Nazionale Risi, 27030 Castello d'Agogna, Pavia, Italy

[4]Leibniz Center for Agricultural Landscape Research (ZALF), 15374 Müncheberg, Germany

* Corresponding author phone: +49 921 55 3999,

E-mail: b.planer-friedrich@uni-bayreuth.de

(10 pages, 6 figures, 2 tables)

CONTENTS

Table S1. Summary of experiments discussed throughout this manuscript.

Experiments	Redox managements	Paddy soils	Setup	Exogenous carbon	Sulfate addition
Reduction batch incubations	20 days of soil reduction	Cascina Veronica (air-dried, 2 mm sieved)	120 mL crimp glass vials (20 g soil and 40 mL solution)	acetate	control and 1 mmol L^{-1} sulfate, respectively
Reduction-reoxidation batch incubations	30 days of soil reduction and 25 days of soil reoxidation			glucose	9 mmol L^{-1} sulfate
Flood-drain-reflood microcosm incubations	20 days of soil flood, 4 days of soil drain and 14 days of soil reflood	Cascina Fornazzo, Cascina Veronica (fresh, 2 mm sieved)	unplanted microcosm (see Fig S1a)	rice straw	no additional sulfate during flooding; reflooded with 1 mmol L^{-1} sulfate
Rhizobox rice cultivations	kept flooded during rice cultivation	Cascina Fornazzo (air-dried, 2 mm sieved)	Rhizobox (see Fig S1b)	／	sulfate introduced only in fertilizers

Table S2. Percentage of total thiolation, mono-, di-, and trithiolation of inorganic oxyarsenic species, MMA, and DMA in the rhizobox experiment using Fornazzo soil

Species	Rice cultivars & Depth	Total thiolation	Monothiolation	Dithiolation	Trithiolation
Inorganic oxyarsenic	YD A	8.0	6.6	1.4	0.0
	YD B	9.9	5.7	4.2	0.0
	NK A	8.5	5.4	3.1	0.0
	NK B	20.0	15.2	4.2	0.5
	Median	**9.2**	**6.1**	**3.6**	**0.0**
MMA	YD A	51.2	30.2	21.0	/
	YD B	51.7	34.4	17.3	/
	NK A	44.7	29.4	15.3	/
	NK B	51.3	35.0	16.3	/
	Median	**51.3**	**32.3**	**16.8**	/
DMA	YD A	60.4	53.8	6.7	/
	YD B	48.4	42.9	5.5	/
	NK A	47.8	40.7	7.0	/
	NK B	20.7	18.2	2.5	/
	Median	**48.1**	**41.8**	**6.1**	/

YD A: rice variety Yangdao, sampled at depth A (9 cm below soil-water interface); YD B: Yangdao, sampled at depth B (16.5 cm below soil-water interface); NK A: rice variety Nongken, sampled at depth A; NK B: Nongken, sampled at depth B. data represents mean values of each depth (n = 3); numbers in bold type indicate median for each As species

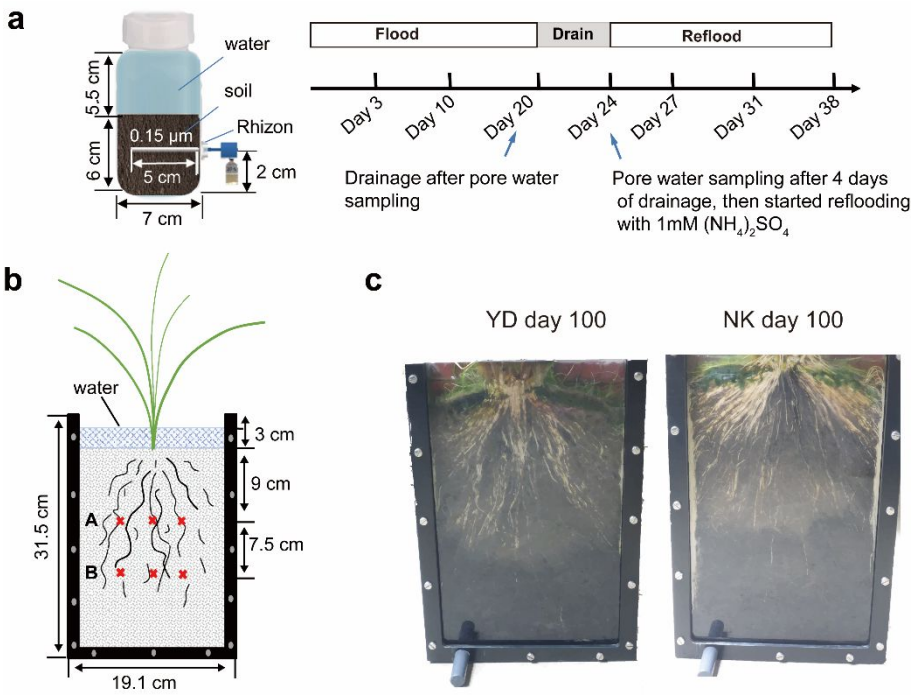
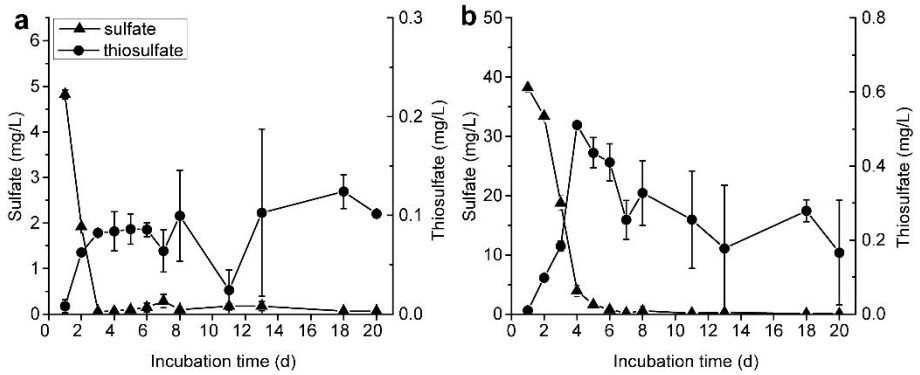


Figure S1. Unplanted microcosm experimental setup (a), rhizobox rice cultivation experimental setup (b), picture of rice roots development (c).



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58 **Figure S2.** Pore-water sulfate and thiosulfate dynamics in reduction batch incubations
59 using Veronica soil. (a) control, (b) spiked with 1 mmol L^{-1} sulfate.

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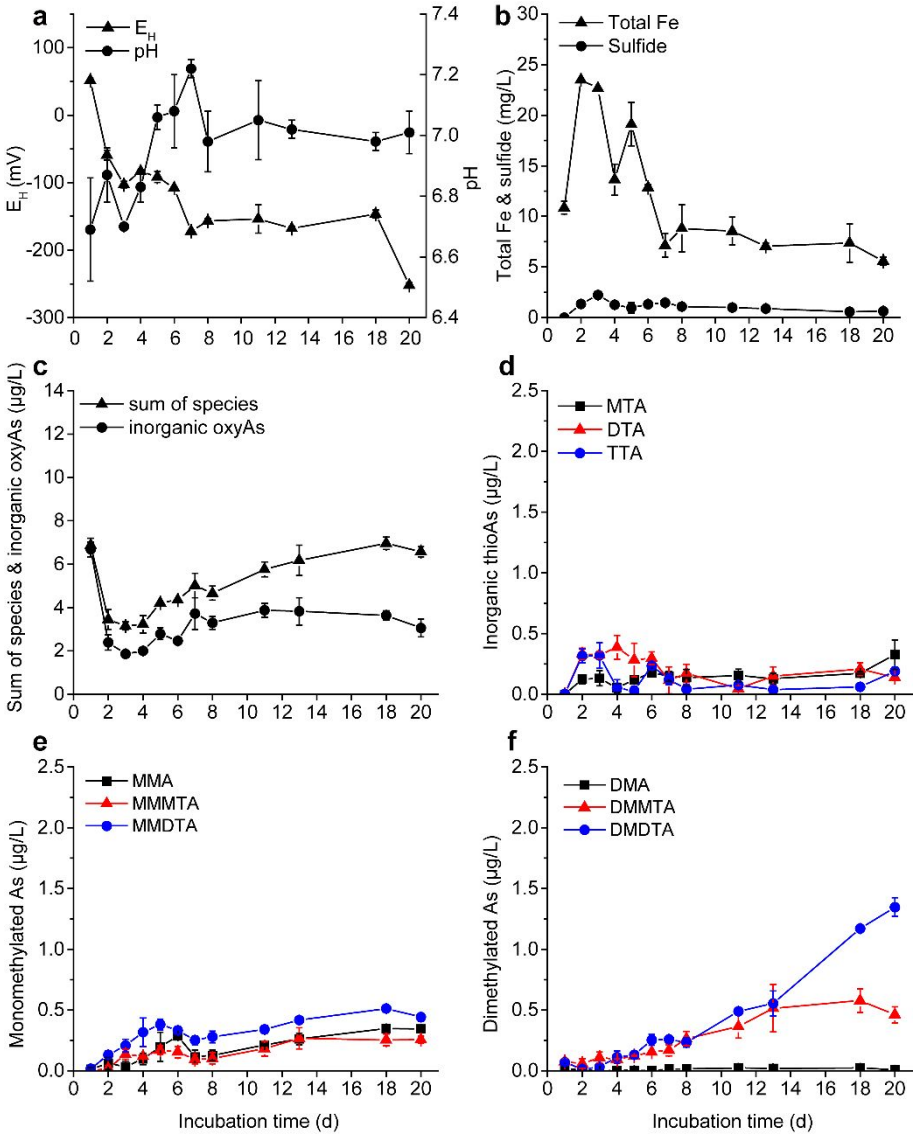

Figure S3. Pore-water chemistry (a, b) and As speciation (c, d, e, f) dynamics under reduction batch incubations using Veronica soil (control without sulfate spike).

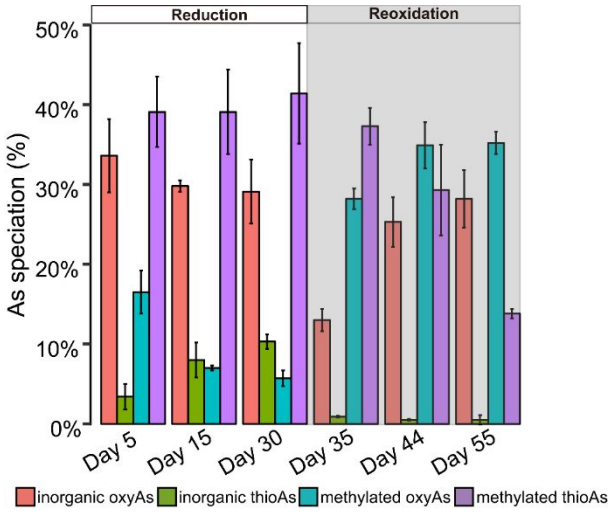

Figure S4. As speciation (% of total As) dynamics in reduction-reoxidation batch incubations using Veronica soil (spiked with 9 mmol L^{-1} sulfate, 100 µg L^{-1} arsenate).

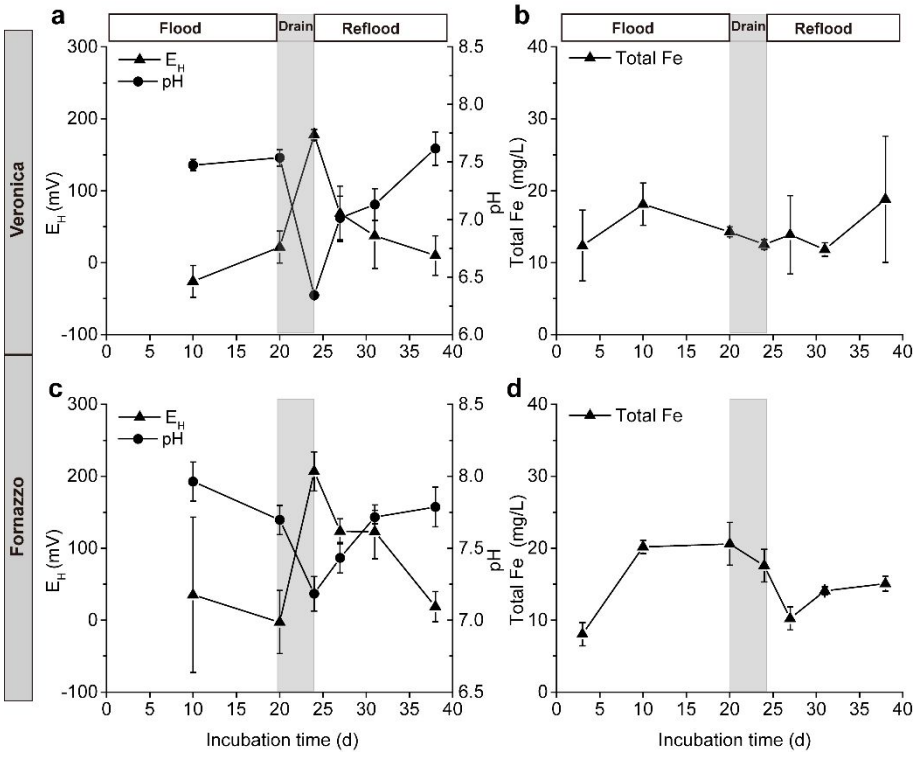

Figure S5. Pore-water chemistry dynamics in flood-drain-reflood microcosm incubations using Veronica (a, b) and Fornazzo (c, d) soil. Bars represent standard errors (n = 3).

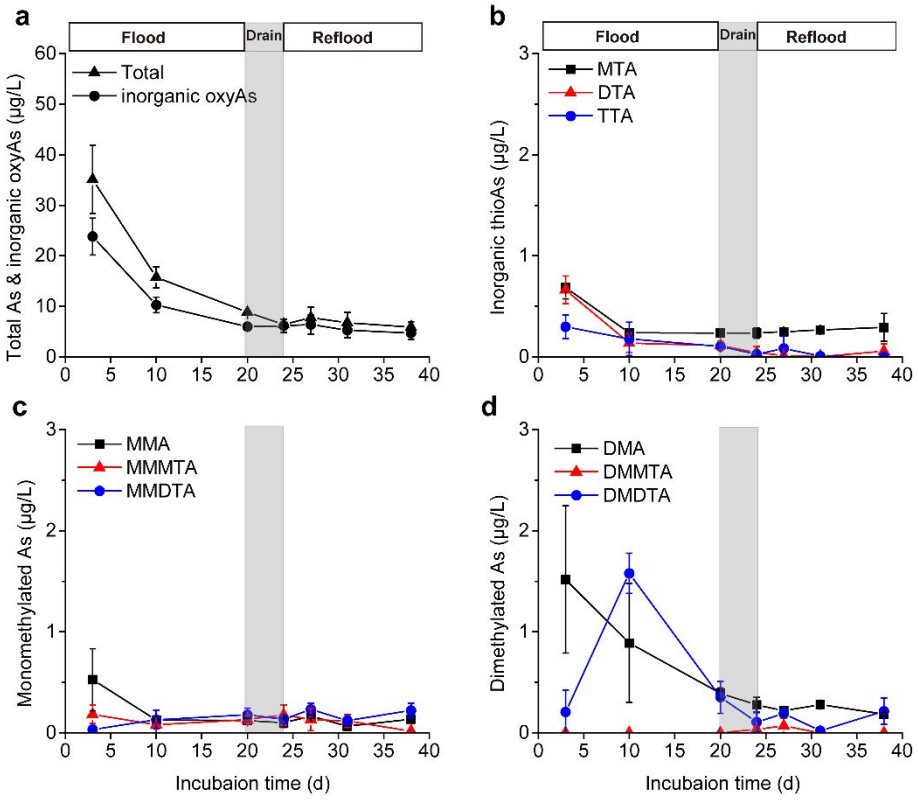

Figure S6. As speciation dynamics in flood-drain-reflood microcosm incubations using Veronica soil. Bars represent standard errors (n = 3).

Study 3: Sulfate fertilization influences the transfer of inorganic and methylated arsenic from paddy soil to rice grain

Jiajia Wang; Shuai Zhang, Carolin Kerl, Umberto Roll, Gianluca Beltarre, Maria Martin, Daniel Said-Pullicino, Marco Romani, Britta Planer-Friedrich

First draft

Sulfate fertilization influences the transfer of inorganic and methylated arsenic from paddy soil to rice grain

Jiajia Wang · Shuai Zhang · Carolin Kerl ·Umberto Roll · Gianluca Beltarre · Maria Martin · Daniel Said-Pullicino · Marco Romani · Britta Planer-Friedrich

Jiajia Wang · Carolin Kerl · Britta Planer-Friedrich (✉)

Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), University of Bayreuth, 95440 Bayreuth, Germany
email: b.planer-friedrich@uni-bayreuth.de

Shuai Zhang

Key Laboratory of Water Environment Evolution and Pollution Control in Three Gorges Reservoir, Chongqing Three Gorges University, 404100 Chongqing, China

Umberto Roll · Gianluca Beltarre · Marco Romani

Rice Research Centre, Ente Nazionale Risi, 27030 Castello d'Agogna, Pavia, Italy

Maria Martin · Daniel Said-Pullicino

Department of Agriculture, Forest and Food Sciences, University of Turin, 10124 Turin, Italy

Keywords

Arsenic, Inorganic arsenic, Methylated arsenic, Rice, Sulfate, Paddy soil

Abstract

Aims

Rice contains both inorganic arsenic, a class-1 carcinogen, and less toxic methylated arsenic. Sulfate fertilization has been suggested to mitigate arsenic accumulation, but reported results are often conflicting, and its effects on arsenic speciation in both soil and rice are unclear. We aim to determine how sulfate affects inorganic and methylated arsenic bioavailability in paddy soils, and their accumulation in grain, under different seeding practices and water managements.

Methods

Mesocosm rice cultivations were conducted open air with two paddy soils, amended with either sulfate or non-sulfate chemical fertilizers for two years. Pore-water and grain arsenic speciation during rice cultivation were analyzed.

Results

In both soils, sulfate fertilization significantly decreased pore-water inorganic arsenic concentration over rice growing season, mitigating its accumulation in white rice, irrespective of management practices. Pore-water methylated arsenic concentration was largely increased after two years of sulfate application. However, its ultimate accumulation in grain was strongly impacted by soil types and management practices.

Conclusions

Sulfate fertilization affects arsenic availability and speciation in flooded paddy soil, with opposite effects on inorganic and methylated arsenic, and could be an effective way to decrease the accumulation of cancerogenic inorganic arsenic in rice.

Introduction

Inorganic arsenic (As) is a chronic, non-threshold carcinogen. Rice is particularly effective in accumulating As from soil (Su et al. 2010), containing approximately 10-fold more As than other cereals (Meharg et al. 2009). Rice consumption represents a main source of human exposure to inorganic As (inorg-As) (Li et al. 2011; Meharg and Rahman 2003).

Besides inorg-As (usually referring to the sum of arsenite and arsenate), rice contains also varying amount of methylated As (methyl-As) (Zhao et al. 2013), mainly dimethylarsenate (DMA). Since rice plant cannot methylate As, grain methyl-As is of soil microbial origin (Lomax et al. 2012), greatly impacted by environmental factors and agronomic practices (Zhao et al. 2013). Health risks associated with DMA are less certain, but the level of ingestion from rice diet is believed to be much lower than that of concern (Zhao et al. 2013). Therefore, there is an urgent need to develop mitigation measures to reduce rice accumulation of As, especially inorg-As (Zhao and Wang 2020).

Due to its potential to decrease As bioavailability in flooded paddy soils (Hashimoto and Kanke 2018; Jia et al. 2015) and to decrease As translocation in rice plants (Dixit et al. 2016; Zhang et al. 2016; Zhang et al. 2011), sulfate fertilization has long been suggested to combat rice As problem. However, its ultimate effect on As in rice grain has only been evaluated in few studies to date (summarized in Table 1). Those studies often report contradictory findings of either decrease (Fan et al. 2013; Xu et al. 2019; Zhang et al. 2016) or no effect (Boye et al. 2017) on grain total As. To the best of our knowledge, only one relevant study from Zhang et al., (2016) determined As speciation in whole grain (with husk) (Table 1). The overlook of As speciation is rather surprising since food guideline thresholds only address cancerogenic inorg-As (Ministry of Health of the People's Republic of China 2017; The European Commission 2015) and inorg-As percentage in grain varies greatly from 10 to 100% (Meharg et al. 2009; Zhao et al. 2013). Sulfate fertilization may influence pore-water As speciation via impacting the activities of sulfate-reducing bacteria (SRB) (Xu et al. 2019) and methanogen (Schütz et al. 1989), which have very recently been showed to be involved in As methylation and demethylation in flooded paddy soils, respectively (Chen et al. 2019). Yet, it remains unknown whether/how sulfate availability impacts methyl-As in rice paddy pore waters.

In present study, we systematically evaluated how sulfate fertilization affected the bioavailability of inorg-As and methy-As in pore-water, and their ultimate accumulation in rice grains over two cropping years. Unlike above-mentioned studies, which based on pot experiments under greenhouse (Table 1), we conducted mesocosm rice cultivation (0.82 m^2, > 300 rice plants, ~20 cm depth of topsoil) open air thus enabled us to manage rice cultivation similar to field settings. We further examined the effectiveness of sulfate fertilization under different agronomic practices including seeding practices and water managements. Our study is the first to investigate the effect of sulfate on methy-As bioavailability during rice cultivation, and one of the very few evaluating the how sulfate fertilization influences grain As speciation.

Materials and methods

Paddy soil

Soil materials were collected from the plow layer of two Italian paddy fields: Cascina Veronica (E 8°53′48″, N 45°10′39) and Cascina Fornazzo (E 8°57′50″, N 45°13′54″). The two soils had the same total soil As contents (5.5 mg/kg) and were relatively similar in soil pH (5.6 and 5.8 for Veronica and Fornazzo, respectively). In compared with Veronica, Fornazzo had higher contents of 0.5 M HCl-extractable Fe (4.0 versus 2.9 g/kg), total C (4.7 versus 2.0%) and 0.5 M $NaHCO_3$ extractable sulfate (95 versus 29 mg/kg). Detailed information about soil characteristics has been reported previously (Wang et al. 2020).

Mesocosm rice cultivation

Twenty-four plastic containers (0.82 m^2) were settled open air at the property of Rice Research Centre Ente Nazionale Risi (ENR) in Castello d'Agogna (Pavia, Italy). Each container was first filled with ~30 cm of gravel (ø 2-5 cm) before overlaid a soil layer of ~20 cm. Twelve containers each were filled with soil from Veronica and Fornazzo, respectively. Sulfate fertilization was conducted with six containers of each soil were fertilized with sulfate-containing N-, or K-fertilizers (ammonium sulfate and potassium sulfate; S treatments), while the other six containers of each soil were fertilized with equivalent amounts of fertilizer without sulfate (urea and potassium chloride; no S treatments). P-fertilizers were applied in form of triple superphosphate. Rice cultivator used was *Oryza sativa* L. cv. Selenio. In 2017, two seeding practices were adopted under continuous flooding, namely water seeded with continuous flooding (W-CF) and dry seeded with continuous flooding (D-CF). Three containers of each soil and

fertilization treatment were managed via W-CF, the other three containers via D-CF. Since no significant interactions between fertilization treatment and seeding practice were found in 2017, only water seeded was adopted in 2018; with all previous D-CF managements been replaced by water seeded with alternate wet-dry irrigation (W-WD). Continuous flooding was conducted by maintaining a standing water of ~10 cm depth during whole growing season once flooded, while alternate wet-dry was conducted by inserting two soil drying periods into continuous flooding (7 and 8 days without daily irrigation at half stem elongation and before flowering, respectively). Details for management practices and experimental setups were summarized in Table 2 and Fig. S1-2, respectively.

Pore-water sampling and rice harvest

Pore-water was extracted by microrhizon samplers (Rhizon MOM, Rhizosphere Research Products, The Netherlands), connected to 100 mL evacuated glass bottles. The bottles were prepared prior to sampling by purging them with argon (purity > 99.9%) for 15 min, sealing them with a butyl rubber septum. Pore-water 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. Non-stable chemical parameters (pH, redox potential) were measured immediately on-site. A list of samples collected and their corresponding rice growing stages were summarized in Table 2 and Fig. S2. Information on pH, E_H for 2017 can be found in Wang et al., (2020), and for 2018 see Table S1.

At harvesting, aboveground biomass of each mesocosm were cut manually, dried in a Static Tray Dryer (Scolari S.r.l., Ospitaletto, Brescia) at 40°C for three days. Then, rice grain was separated from straw, weighted as grain production. The weight of straw was also recorded. Rice straw harvested in 2017 was returned back to corresponding mesocosm for next cropping season. Harvested rice grain was further dried to a humidity of approximately 10% (PM-650 - version 6501, Instant Moisture Meter, Kett, Villa Park, California). After machinery dehusking (Colombini & Co. Srl, Abbiategrasso, Milano) and polishing (TM-05 grain testing mill, Satake Engineering Co., Tokyo, Japan), white rice was ground to fine powder for future As extraction and speciation analysis.

Arsenic determination

Pore-water total As and grain total As (microwave digested in concentrated HNO_3 and 30% H_2O_2, 0.2 µm filtered) was quantified by inductively coupled plasma-mass spectrometry (ICP-MS, XSeries2, Thermo-Fisher). Arsenic was detected as AsO^+ at m/z 91 with an O_2/He mixture (10:90%) serving as reaction gas and signal drift was corrected using Rhodium (Rh^+ m/z 103) as an internal standard added manually to each sample. To determine grain As speciation, 1.0000±0.001 g rice flour samples were extracted with 10 mL of 0.28 M HNO_3 for 90 min at 95°C in a heating block (Digi PREP Jr, SCP Science, Canada) and filtered through 0.45 µm filter (SCP Science). Arsenic species in the extracts were separated by ion-chromatography (IC) (Dionex ICS-3000, Dionex Corp., USA, PRPX-100 column using a 10-40 mM $NH_4H_2PO_4$ pH 5.6 gradient) and coupled to ICP-MS (Muehe et al. 2019). Sum of all As species was in good agreement with total As measured in grain samples (Fig S3). Arsenic species in the pore-water were quantified by IC (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 as previously described (Wang et al. 2020).

Statistical analysis

All statistical analyses were performed via R statistical computing environment (R version 3.6.1). Bartlett test of homogeneity of variances was conducted ($p>0.05$) before being subjected to analysis of variance (ANOVA). A one-way ANOVA was performed on grain As accumulation (separated by cropping years and two studied soils), followed by Fishers Least Significant Difference (LSD) test. Two-way analysis of variance (two-way ANOVA) was used to assess the significance of the effects of sulfate fertilization and agronomic managements (seeding practice and water management), as well as of their interaction, separated by cropping years and two studied soils.

Table 1 A compilation of researches regarding the effect of sulfate fertilization on grain arsenic accumulation

Setup	Soil As content (mg/kg)	As spiked (mg/kg)	S additions	Rice plant per pot	Seeding & water managements	Effect on rice grain As [a]	Reference
Rhizobag (2 kg soil)	3.96	/	sodium sulfate (60, 120 mg S/kg)	2	W-CF [b]	brown rice (total As −)	Fan et al. 2013
		20		2	W-CF	brown rice (total As −)	
Pots (3.0 kg soil)	2.78	20	sodium thiosulfate (120 mg S/kg)	1	W-CF	whole grain (total As −, inorg. As −, methly. As =)	Zhang et al. 2016
Pots (1.3 kg soil)	15.40	/	gypsum (20 and 60 mg S/kg)	4	W-CF	brown rice (total As =)	Boye et al. 2017
	11.00	/		4	W-CF	brown rice (total As =)	
Pots (3.0 kg soil)	72.8	/	sodium sulfate (100 mg S/kg)	2	W-CF	brown rice (total As −)	Xu et al. 2019
	75.8	/		2	W-CF	brown rice (total As −)	

[a] signs "−""=" indicate decrease and no effect of sulfate additions on grain arsenic accumulations, respectively; [b] Water seeded with continuous flooding

Table 2 Summary of management practices and pore-water sampling in this study

Year	2017	2018
Cultivation areas	0.82 m^2	0.82 m^2
Seedlings per mesocosm	340	330
Seeding[a] & water managements	Water seeded with continuous flooding (W-CF)	Water seeded with continuous flooding (W-CF)
	Dry seeded with continuous flooding (D-CF)	Water seeded with alternate wet-dry (W-WD)
Fertilization [b]	180 kg N/ha, 150 kg K_2O/ha, 40 kg P_2O_5/ha	240 kg N/ha, 350 kg K_2O/ha, 40 kg P_2O_5/ha
Amount of S from sulfate fertilizer [c]	81 mg S/kg	124 mg S/kg
Rice straw addition per mesocosm [d]	200 g	785-1355 g
Pore-water sampling [e]	Tillering (June 14th), Stem elongation (July 4th), Booting (July 18th), Flowering (August 1th), Grain filling (August 8th), Dough (August 22th), Mature (September 13th)	Tillering (June 8th) Stem elongation (July 13th, start of first soil drying; July 18th, in the middle of soil drying) Booting (July 27th, starting of second soil drying; July 31th, in the middle of soil drying) Flowering (August 9th)

[a] In water seeded, soils were flooded from one day before seeding; in dry seeded, oxic soil conditions were maintained 20 days after seeding.

[b] N-, or K-fertilizers were applied of in form of ammonium sulfate and potassium sulfate (S treatments), urea and potassium chloride (no S treatments); P-fertilizers were applied in form of triple superphosphate. Due to the persistent rainy days after seeding in 2018, one drainage was conducted at May 19th, which could cause fertilizers loss. Thus, more N- and K-fertilizers were added in 2018 to meet the demands of rice.

[c] Estimation was based on the soil depth of 20 cm in the mesocosm and a bulk density of 1.55 g/cm^3.

[d] In 2018, soils were mixed with rice straw harvested in 2017 from the corresponding mesocosm.

[e] All dates in the bracket indicate pore-water sampling time.

Results

Pore-water arsenic during rice cultivation

Soil flooding caused large As mobilization into pore-water, especially in no S treatments (Fig. 1-2 and S4). In pore-water, four groups of As species were identified, i.e. inorganic oxyarsenic (arsenite and arsenate), methylated oxyarsenates (DMA and monomethylarsenate (MMA)), inorganic thioarsenates (mono-, di-, and trithioarsenate) and methylated thioarsenates (mono and dimethylated monothioarsenate; mono- and dimethylated dithioarsenate). Since thioarsenic species can be taken up by rice plants and dethiolated considerably during translocation (Kerl et al. 2018; Kerl et al. 2019), in present study, inorganic oxyarsenic and inorganic thioarsenates were summed as inorg-As, while methylated oxyarsenates and methylated thioarsenates as methyl-As. Pore-water As in 2017 has been reported previously to study the effects of sulfate fertilization on thioarsenates formation (Wang et al. 2020), and was reintegrated here to compare the difference in inorg-As and methyl-As between no S and S treatments (Fig. 2 and S4).

Sulfate fertilization decreased significantly the pore-water total As concentration over rice grow seasons in two cropping years and in both soils, irrespective of management practices (Fig. 1-2 and S4). The decrease of pore-water As was represented by the decrease of inorg-As (mainly arsenite and arsenate). Despite greater mobilization of As in Veronica in compared with Fornazzo in no S treatments, similar range of inorg-As (typically less than 30 µg/L after rice tillering) was found in two soils during rice cultivation in S treatments for both cropping years, irrespective of management practices (Fig. 1-2 and S4). Sulfate fertilization had no appreciable effects on pore-water methyl-As in 2017, and methyl-As in pore-water was typically less than 3 µg/L (Fig. 2a). However, sulfate fertilization resulted in a significant increase of methyl-As, especially in Veronica soil, over rice growing season in 2018 (Fig. 1 and 2b).

Comparison between the two years showed generally higher inorg-As and methyl-As in pore-water in 2018 than in 2017, likely due to the much higher rice straw addition in 2018 (Table 2). Both inorg-As and methyl-As was significantly decreased by short-term soil drying in 2018, but rebounded back to previous levels with prolonged reflooding (Fig. 1).

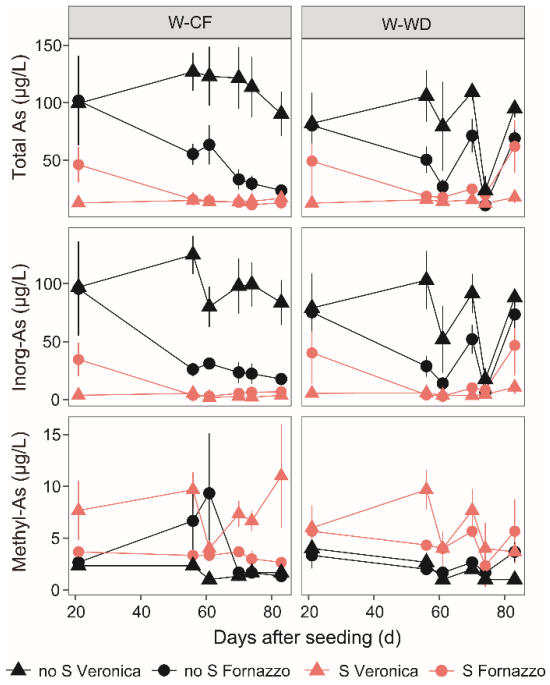

Fig. 1 Pore-water As concentrations over time in paddy soils from Veronica and Fornazzo in 2018. Bars represent standard deviations (n = 3). Abbreviations: W-CF = water seeded, continuous flooding; W-WD = water seeded, alternate wet-dry. For the year of 2017 see Fig. S4.

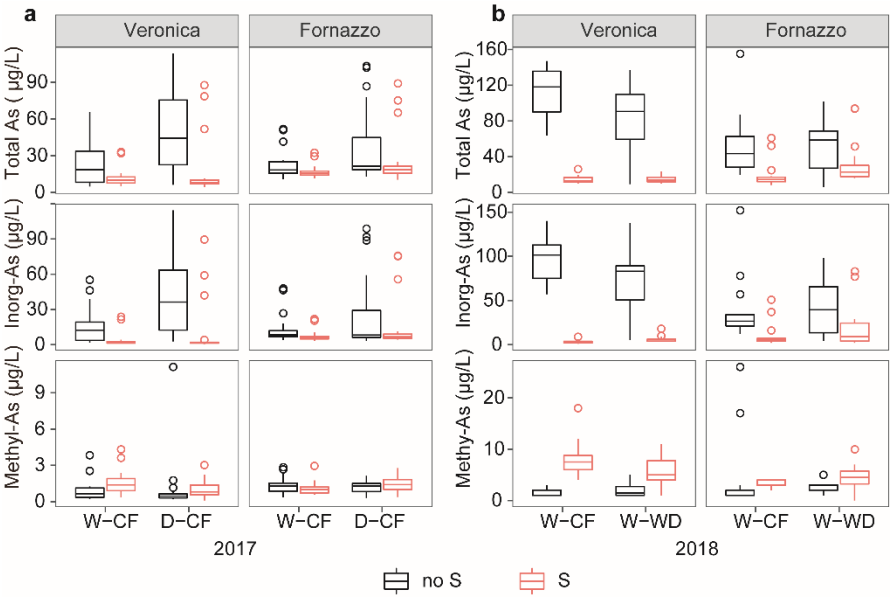

Fig. 2 Pore-water As concentrations integrated over time for 2017 (a) and 2018 (b). Boxplots: line: median; cross: mean; box: interquartile range; whiskers: 1.5 interquartile range; data from 3 mesocosms over 7 times (n=21) and 3 mesocosms over 6 times (n=18) for 2017 and 2018, respectively. Abbreviations: W-CF = water seeded, continuous flooding; W-WD = water seeded, alternate wet-dry.

Effect of sulfate on arsenic accumulations in rice

We analyzed As accumulation in white (polished) rice harvested from two cropping seasons. Four As species were identified (Table S2), i.e. arsenite, arsenate, DMA and MMA. Arsenite and arsenate were summed as inorg-As, while DMA and MMA as methyl-As in grains.

Sulfate fertilization decreased grain total As in Veronica soil for both seasons, especially for D-CF (2017, decrease by 34%) and W-CF (2018, decrease by 49%), while no appreciable effect was found for grain total As in Fornazzo soil (Fig. 3). Sulfate fertilization decreased grain inorg-As in two cropping years and in both soils, independent of management practices (Fig. 3). The magnitude of decrease in inorg-As ranged from 36% to 48% and from 23% to 30% for Veronica and Fornazzo, respectively. Unlike inorg-As, the effect of sulfate fertilization on grain methyl-As was inconsistent (Fig. 3). While sulfate fertilization slightly increased grain methyl-As in Fornazzo for both cropping years, significant decrease in grain methyl-As for D-CF (2017, decreased by 21%) and W-CF (2018, decreased by 49%) were found in Veronica. Despite variations in methyl-As, over two cropping years, inorg-As (%) (percentage of inorganic As relative to the sum of all As species in grain) was significantly decreased by sulfate fertilization in both soils and in all treatments (expect W-CF (2018) in Veronica) (Fig. 3). The decreased of inorg-As(%) ranged from 9% to 18% and from 11% to 17% for Veronica and Fornazzo, respectively. In summary, our grain speciation data showed that sulfate fertilization could produce white rice with lower inorg-As, but proportionally more methyl-As.

Effects of seeding practices and water managements

Seeding practices had no impact on grain inorg-As accumulation, despite a slight increase of methyl-As thus a decrease of inorganic As (%) in Veronica was found in dry seeded, in compared with water seeded (Fig.3a). Two-way ANOVA analysis showed no interaction between sulfate fertilization and seeding practices (expect for the methyl-As in Veronica soil, Table S3).

In comparison with continuous flooding, alternate wet-dry decreased significantly grain methyl-As, but had no effect on inorganic As, resulting in an increase in inorg-As (%) (Fig.3). The relationships between sulfate fertilization and grain methyl-As accumulation

depended strongly on water managements in Veronica ($P < 0.001$) but not in Fornazzo (Table S3). No interaction between sulfate fertilization and water managements was found for inorg-As accumulation in both soils (Table S3).

Effect of Sulfate fertilization on Yield

Sulfate fertilization had no significant effect on grain yield in both soils (Fig. S5a and Table S4). However, the production of straw showed a slight increase (values above 1:1 line in Fig. S5b), suggesting the stimulation of vegetative growth by sulfate fertilization.

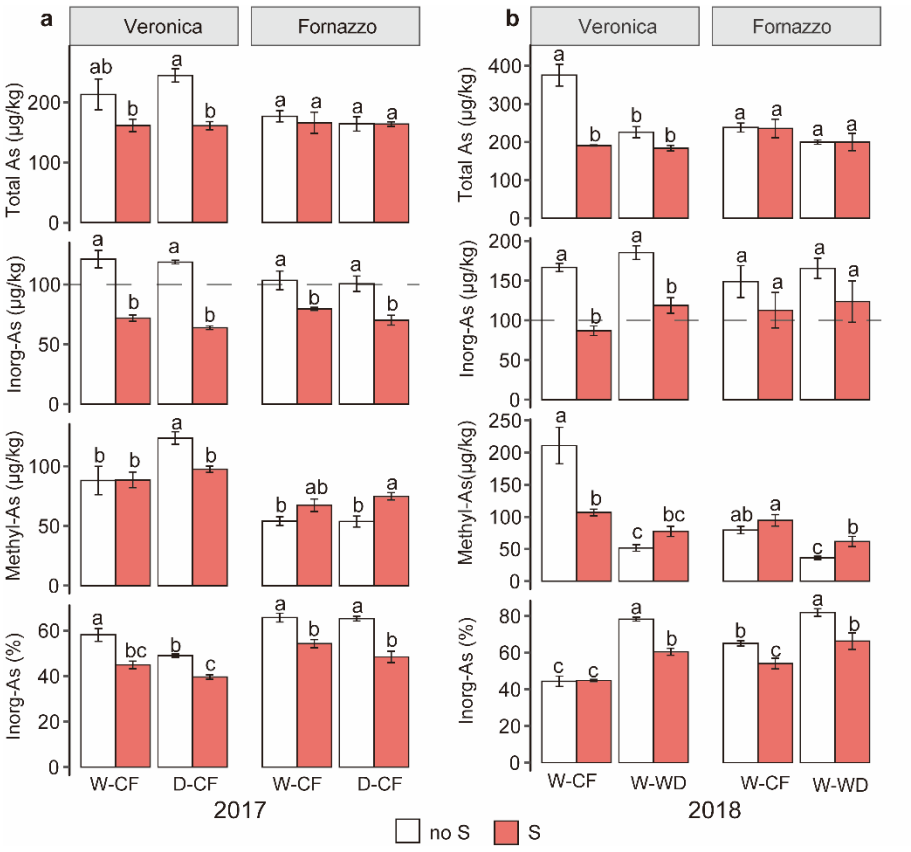

Fig. 3 Effects of sulfate fertilization on As accumulation in white rice harvested from Veronica and Fornazzo for 2017 (a) and 2018 (b). Error bars represent standard deviations (n = 3). Different lowercase letters indicate significant ($p < 0.05$) differences between treatments (separated by cropping years and two studied soils). Abbreviations: W-CF = water seeded, continuous flooding; D-CF = dry seeded, continuous flooding; W-WD = water seeded, alternate wet-dry. The horizontal dash line at 100 µg/kg indicates the limit of inorg-As concentration for European Union standard for rice based baby foods.

Discussion

During rice cropping, a large amount of sulfate is often introduced with concomitant applications of chemical fertilizers (Hashimoto and Kanke 2018). The application of sulfate-containing fertilizers is predicted to more prevailing due to the increasing sulfur deficiency in agriculture in recent decades since the reduction of sulfur dioxide emissions (Aula et al. 2019). Based on a typical Italian soil bulk density of 1.55 g/cm^3, the amounts of inorganic sulfur introduced were 81 mg S/kg in 2017, and 124 mg S/kg in 2018 (Table 2), comparable to sulfur added in previous plot experiments (Table 1). Our combined results indicate that change from non-sulfate chemical fertilizer (urea and potassium chloride) to sulfate-containing fertilizers (ammonium sulfate and potassium sulfate) can have considerable influences on the bioavailability and speciation of As in paddy soil pore waters and its transfer from soil to rice grains.

Sulfate fertilization effectively decreased inorg-As concentration in pore-water during rice cultivation (Fig. 1-2 and S4), resulting in a substantial decrease of inorg-As accumulation in white rice (Fig. 3), irrespective of management practices. Noticeably, sulfate fertilization decreased inorg-As concentration of white rice harvested from 2017 in both soils to < 100 μg/kg , thus met the European Union standard for rice based baby foods (The European Commission 2015). Inorg-As concentration of white rice harvested from 2018 was only slight higher than 100 μg/kg in S treatments, likely due to the large amount of rice straw amended (Table 2), which led enhanced As mobilization in pore-water in compared with in 2017(Fig. 2). The effect of sulfate in deceasing As bioavailability has been reported before in both unplanted soil incubations and pot experiments (Burton et al. 2014; Jia et al. 2015; Xu et al. 2019), which is explained by the enhanced precipitation of As with newly formed Fe-As-S minerals (Jia and Bao 2015; Saalfield and Bostick 2009; Xu et al. 2019). Since inorg-As availability was largely limited by sulfate fertilization, seeding practices and water managements were found to have no effects on the effect of sulfate fertilization (Fig. 3 and Table S3), indicating the effectiveness of sulfate fertilization in mitigating rice inorg-As accumulation under different agronomic practices.

Unlike inorg-As, methyl-As concentration in pore-water was increased by continuous sulfate fertilization (Fig. 1-2). A recent study by Chen et al. (2019) shows that SRB and methanogenic archaea are involved in As methylation and demethylation, respectively.

The large input of sulfate in 2018 together with potential sulfate legacy from 2017 (Table 2) could possibly stimulate the activity of SRB, but simultaneously suppress activity of methanogenic archaea as indicated by previous sulfate fertilization studies (Meharg and Rahman 2003; Schütz et al. 1989; Wörner et al. 2016; Xu et al. 2019), therefore explained the increased methyl-As concentration in pore-water during the second cropping year. Increase of methy-As in pore-water has been found in previous plot studies by rice straw addition (Jia et al. 2013; Yang et al. 2018), due to the stimulation of As methylating microbes, and by silicate gel addition (Liu et al. 2014), due to the inhibition of DMA adsorption on the soil solid phase. To the best of our knowledge, this is the first time that sulfate fertilization has been reported to increase methyl-As concentration in paddy soil pore waters. In consistent with the increased methyl-As in pore-water, sulfate fertilization slightly increased methyl-As contents in white rice harvested from Fornazzo soil in both years (Fig. 3). However, its effect on white rice methyl-As contents harvested from Veronica soil was inconsistent (Fig. 3), suggesting other plant-soil factors may also impact methyl-As accumulation. For example, sulfate fertilization may impact As accumulation indirectly via affecting root growth and exudation (Gauci et al. 2008; Kimura et al. 2004). Indeed, strong interaction between sulfate fertilization and water managements was found for grain methyl-As accumulation in Veronica but not Fornazzo (Table S3). Soil-dependent change of grain methyl-As accumulation upon sulfate fertilization warrants future investigation.

In our study, sulfate fertilization was found to have much greater impacts on the concentration of grain inorg-As than methyl-As (Fig. 3). This differs from other agronomic practices such as alternative wet-dry and straw incorporation, which are reported to have a much greater impact on the concentration of grain methyl-As, but much less effective in impacting grain inorg-As (Carrijo et al. 2019; Li et al. 2009; Ma et al. 2014; Yang et al. 2018) (also Fig. 3b). Greater effect on inorg-As found here is in line with the study of Zhang et al. (2016), which reports a significant decrease in grain inorg-As but no effect on DMA by thiosulfate application (Table 1). This observation is explained by the authors as the selective binding of arsenite, but not DMA, in rice vegetative parts via thio-rich compounds stimulated by sulfate addition (Dixit et al. 2016; Zhang et al. 2016). In addition to the substantial decrease of inorg-As availability in pore-water (Fig. 1-2 and S4), sulfate-fertilization-enhanced selective binding of arsenite may also play a role in our study, as suggested by the stimulated vegetative rice growth by sulfate fertilization

(Fig. S5b). Despite variations in inorg-As and methyl-As, over two cropping seasons, inorg-As (%) in white rice was significantly decreased (up to 18%) by sulfate fertilization (Fig. 3). This seems to be a general pattern, independent of studied soils and agronomic practices, strongly evidencing sulfate as an important but previously overlooked environmental factor in influence grain As speciation.

In comparison with Fornazzo soil, sulfate fertilization was found to have a stronger impact in Veronica with respect to inorg-As (decrease) and methyl-As (increase) in pore-water (Fig. 2). In our previous study, we found stronger impacts of sulfate on the formation of zero-valent sulfur and thioarsenates in Veronica compared to Fornazzo soil, indicating a higher sulfate turnover rate in Veronica soil (Wang et al. 2020). This is likely due to relative lower total C (2% versus 4.7%) and HCl-extractable Fe (52 mmol/kg versus 71 mmol/kg) contents in Veronica than in Fornazzo, which resulting in less inorganic sulfur sequestration in soil solid phase (Wan et al. 2014; Yu et al. 2015).

In previous sulfate fertilization studies, researchers often analyzed brown rice (only total As, see Table 1), despite the fact that they are unpopular in subsistence rice diets. One possible reason could be that grains produced in previous plot experiment, where limited numbers of rice seedlings (1-4 plants per plot), were not sufficient for machinery polishing therefore afterwards As analysis (Table 1). In order to make comparison with those studies, we analyzed brown rice harvested in 2018 (Table S5). Speciation data showed that the effects of sulfate fertilization on As accumulation in brown rice had similar patterns as in white rice, but with stronger decrease in total As and inorg-As (Table S6 and Fig. S6). As such, we suggested that the reported inconsistent effects of sulfate fertilization on total As could possibly be reconciled in the light of grain As speciation. Specially, the reported decrease in total As of brown rice by sulfate fertilization mostly likely caused by the decrease of inorg-As; while the reported no effect may either due to no sufficient sulfate input or sulfate-fertilization-induced increase of grain methyl-As, which somehow counteracts the decease of inorg-As. Variations in grain As speciation, rather than total As, need to be considered in future sulfate fertilization studies.

It should be noted that, within the groups of inorg-As and methyl-As, the formation of inorganic and methylated thioarsenates in pore-water were increased by sulfate fertilization (Wang et al. 2020). Thioarsenates can be taken up by rice plants (Kerl et al.

2018; Kerl et al. 2019), and dimethylated monothioarsenate has been detected in rice grains before (Ackerman et al. 2005; Mantha et al. 2017). Thioarsenates, if existed, would be determined together as their oxyarsenic counterparts due to the hot acid digestion of rice grains in our study (Kerl et al. 2019). Since there exists no solid method to extract and quantify thioarsenates in rice grain, it remains unclear about the role of thioarsenates in grain As accumulation.

In summary, the present study has shown that sulfate fertilization has opposite effects on the bioavailability of inorganic and methylated arsenic in flooded paddy soil, resulting in a significant decrease in former but increase in later. Since As root-to-shoot translocation in rice seedlings is greatly enhanced by sulfur deficiency (Dixit et al. 2016; Zhang et al. 2016; Zhang et al. 2011), sulfate fertilization can be of critical importance to both rice yield and the mitigation of rice As accumulation in paddy fields under sulfur deficiency (Islam and Ponnamperuma 1982). For paddy fields where DMA-related rice straighthead disease is prevalent (Zheng et al. 2013), however, sulfate fertilizer should be applied with care due to its potential to increase As methylation. Sulfate fertilization could be a feasible mitigation measure to reduce the accumulation of cancerogenic inorg-As in rice. The combinations of appropriate sulfate fertilization, limited amount of fresh organic carbon amendment and alternative wet-dry are highly recommended in rice farming to combat rice As problem. Future studies are needed to test the effectiveness of sulfate fertilization under field conditions and with different soil types.

Acknowledgments

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Supplementary material

Summary of experimental designs & agronomic managements, pore-water pH &E_H, As in rice grain (concentration of arsenite, arsenate, DMA and MMA, relationship between total As concentration and the sum of As species), two-way ANOVA tests of the effects sulfate fertilization/seeding practices or sulfate fertilization/water managements on white grain arsenic accumulations, effects of sulfate fertilization on the grain yield and straw production.

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Supporting information

Sulfate fertilization influences the transfer of inorganic and methylated arsenic from paddy soil to rice grain

Jiajia Wang · Shuai Zhang · Carolin Kerl · Umberto Roll · Gianluca Beltarre · Maria Martin · Daniel Said-Pullicino · Marco Romani · Britta Planer-Friedrich

Jiajia Wang · Carolin Kerl · Britta Planer-Friedrich (✉)

Department of Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BAYCEER), University of Bayreuth, 95440 Bayreuth,Germany
email: b.planer-friedrich@uni-bayreuth.de

Shuai Zhang

Key Laboratory of Water Environment Evolution and Pollution Control in Three Gorges Reservoir, Chongqing Three Gorges University, 404100 Chongqing, China

Umberto Roll · Gianluca Beltarre · Marco Romani

Rice Research Centre, Ente Nazionale Risi, 27030 Castello d'Agogna, Pavia, Italy

Maria Martin · Daniel Said-Pullicino

Department of Agriculture, Forest and Food Sciences, University of Turin, 10124 Turin, Italy

(17 pages, 6 Figures, 6 tables)

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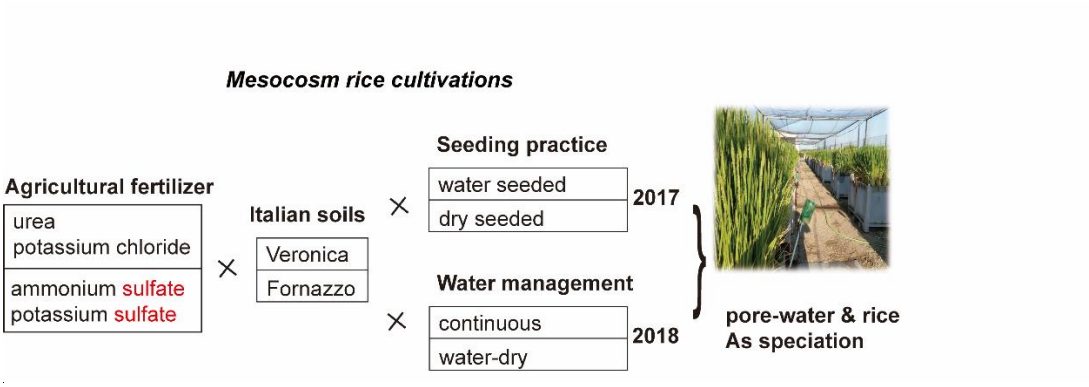

Fig. S1 Experimental setups for mesocosm rice cultivation at the Rice Research Centre ENR in Castello d'Agogna (Pavia, Italy) in 2017 & 2018.

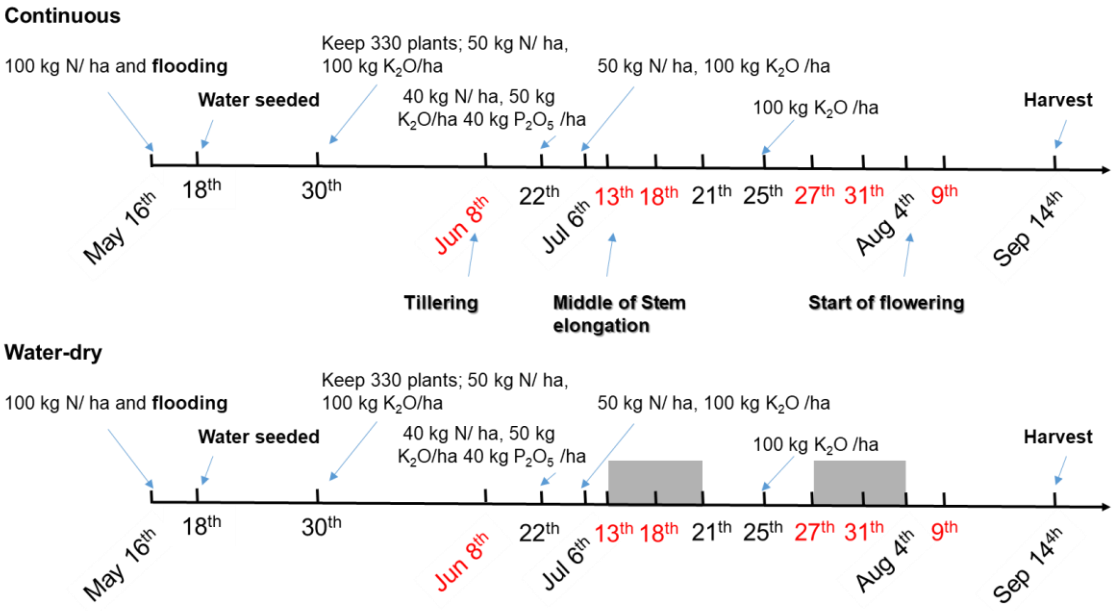

Fig. S2 Agronomic managements of continuous flooding and alternative water-dry mesocosm rice cultivation experiments at the Rice Research Centre ENR in Castello d'Agogna (Pavia, Italy) in 2018. For sulfate treatments, ammonium sulfate and potassium sulfate were applied, while urea and potassium chloride were used equivalent in N and K for control treatments. Dates in red indicate pore-water sampling dates. Shades on the axis indicate soil drying.

Table S1 Pore-water chemistry over rice growing seasons in the mesocosm experiments (2018) (Mean ± SE, n=3)

Pore-water parameters	Soil & irrigation	Fertilization	Jun 8th	Jul 13th	Jul 18th	Jul 27th	Jul 31th	Aug 9th
pH	**Veronica**							
	continuous	no S	6.71 ± 0.05	6.74 ± 0.10	6.8 ± 0.15	6.69 ± 0.16	6.75 ± 0.09	6.75 ± 0.10
		S	6.65 ± 0.13	6.75 ± 0.07	6.83 ± 0.11	6.93 ± 0.17	6.57 ± 0.04	6.93 ± 0.13
	wet-dry	no S	6.79 ± 0.03	6.66 ± 0.06	6.8 ± 0.03	6.65 ± 0.07	6.71 ± 0.00	6.8 ± 0.03
		S	6.66 ± 0.04	6.61 ± 0.04	6.85 ± 0.02	6.78 ± 0.12	6.81 ± 0.11	6.94 ± 0.10
	Fornazzo							
	continuous	no S	6.77 ± 0.10	6.79 ± 0.08	6.95 ± 0.04	7.09 ± 0.16	7.08 ± 0.19	6.87 ± 0.11
		S	6.71 ± 0.01	6.75 ± 0.19	7.13 ± 0.11	7.16 ± 0.03	7.00 ± 0.16	7.05 ± 0.09
	wet-dry	no S	6.87 ± 0.05	6.76 ± 0.11	6.93 ± 0.05	6.97 ± 0.04	6.95 ± 0.07	6.82 ± 0.06
		S	6.78 ± 0.18	6.85 ± 0.12	7.11 ± 0.10	6.86 ± 0.13	6.95 ± 0.09	6.75 ± 0.24
E_H (mV)	**Veronica**							
	continuous	no S	178 ± 13	157 ± 6	164 ± 11	163 ± 22	167 ± 24	189 ± 6
		S	151 ± 10	168 ± 66	142 ± 26	148 ± 7	140 ± 9	173 ± 3

	wet-dry	no S	199 ± 62	175 ± 15	167 ± 31	168 ± 6	184 ± 12	184 ± 3
		S	133 ± 21	166 ± 35	150 ± 7	149 ± 23	162 ± 29	171 ± 7
	Fornazzo							
	continuous	no S	137 ± 18	135 ± 11	124 ± 30	107 ± 18	169 ± 2	155 ± 5
		S	119 ± 15	85 ± 20	99 ± 3	90 ± 9	165 ± 33	159 ± 10
	wet-dry	no S	146 ± 26	127 ± 16	152 ± 7	112 ± 4	163 ± 2	158 ± 2
		S	112 ± 46	126 ± 10	110 ± 9	114 ± 19	127 ± 31	171 ± 19

Table S2 Arsenic species in white (polished) rice as determined by IC-ICP-MS in the mesocosm experiments (2017& 2018) (Mean ± SD, n=3)

Soil & Seeding & Year	Irrigation	Fertilization	Arsenite	DMA	MMA	Arsenate
			µg/kg	µg/kg	µg/kg	µg/kg
Veronica (2017)						
water seeded	continuous	no S	120.65 ± 7.94	88.08 ± 11.89	0.00 ± 0.00	0.58 ± 0.63
		S	71.95 ± 2.66	86.12 ± 6.28	2.41 ± 0.31	0.00 ± 0.00
dry seeded	continuous	no S	117.12 ± 2.01	123.61 ± 5.28	0.00 ± 0.00	1.58 ± 0.63
		S	63.87 ± 1.53	93.37 ± 2.09	4.14 ± 0.79	0.00 ± 0.00
Fornazzo (2017)						
water seeded	continuous	no S	100.65 ± 7.29	51.13 ± 2.5	2.33 ± 1.16	0.57 ± 0.55
		S	79.65 ± 1.32	64.97 ± 5.02	2.45 ± 0.31	0.00 ± 0.00
dry seeded	continuous	no S	100.33 ± 6.13	51.49 ± 4.69	2.24 ± 0.85	0.26 ± 0.37
		S	69.94 ± 3.95	71.74 ± 3.37	3.08 ± 0.57	0.26 ± 0.19
Veronica (2018)						

water seeded	continuous	no S	162.06 ± 5.84	208.7 ± 28.20	2.15 ± 0.40	4.54 ± 0.66
		S	85.01 ± 7.02	102.48 ± 4.41	4.32 ± 0.61	1.91 ± 1.20
water seeded	wet-dry	no S	180.52 ± 8.41	51.65 ± 5.16	0.00 ± 0.00	4.76 ± 1.36
		S	116.01 ± 9.94	75.01 ± 7.20	2.49 ± 0.74	2.61 ± 0.08
Fornazzo (2018)						
water seeded	continuous	no S	141.19 ± 15.88	77.91 ± 5.83	1.48 ± 0.47	7.59 ± 4.30
		S	100.57 ± 16.54	90.95 ± 8.71	3.60 ± 0.28	12.11 ± 5.80
water seeded	wet-dry	no S	156.82 ± 8.58	36.32 ± 2.51	0.00 ± 0.00	8.57 ± 4.83
		S	111.77 ± 20.21	59.3 ± 7.72	2.33 ± 0.36	11.86 ± 5.89

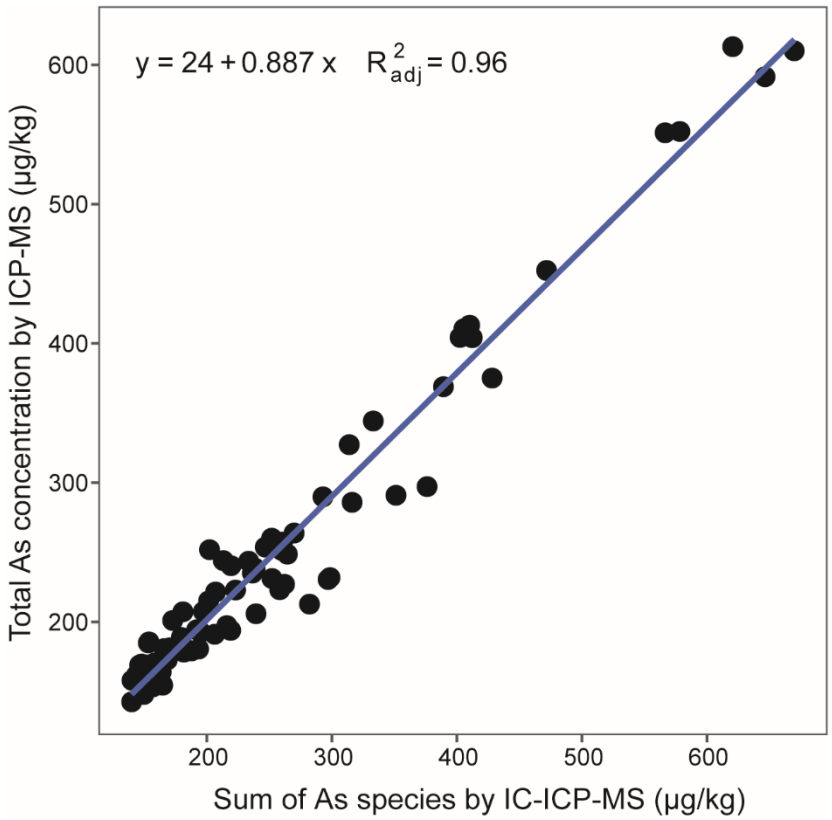

Fig. S3 Relationship between total As concentration in rice grain determined by ICP-MS and the sum of As species determined by IC-ICP-MS.

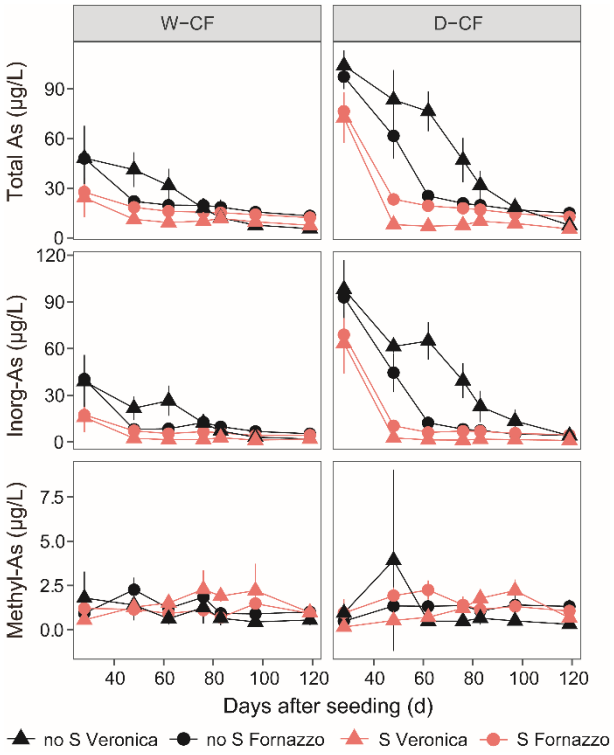

Fig. S4 Pore-water As concentrations over time in paddy soils from Veronica and Fornazzo in 2017. Bars represent standard deviations (n = 3). Abbreviations: W-CF = water seeded, continuous flooding; D-CF = dry seeded, continuous flooding.

Table S3 Two-way ANOVA tests of the effects sulfate fertilization/seeding practices or sulfate fertilization /water managements on white (polished) grain arsenic accumulations. Signs "*", "**", "***" indicates $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. The associated bar charts with error bars are shown in Fig. 3.

Response	Soil	2017				2018		
		no S/S	water seeded/ dry seeded	Interaction		no S/S	continuous/ water-dry	Interaction
Total As	Veronica	***				***	***	***
	Fornazzo							
Inorg-As	Veronica	***				***	**	
	Fornazzo	***				*		
Methyl-As	Veronica	*	**	*		**	***	***
	Fornazzo	***				**	***	
Inorg-As (%)	Veronica	***	***			***	***	***
	Fornazzo	***				***	***	

Table S4 The yield of whole grain (with husk) in the mesocosm experiments (2017& 2018) (Mean ± SD, n=3).

Soil & Seeding & Year	Irrigation	Fertilization	Whole grain	Straw
			g / mesocosm	g / mesocosm
Veronica (2017)				
water seeded	continuous	no S	967 ± 23	1076 ± 100
		S	863 ± 65	1072 ± 39
dry seeded	continuous	no S	765 ± 34	785 ± 43
		S	815 ± 17	1068 ± 12
Fornazzo (2017)				
water seeded	continuous	no S	908 ± 60	1355 ± 97
		S	919 ± 15	1233 ± 31
dry seeded	continuous	no S	939 ± 29	891 ± 39
		S	991 ± 64	1092 ± 73
Veronica (2018)				
water seeded	continuous	no S	930 ± 56	787 ± 60
		S	932 ± 53	1068 ± 79
water seeded	wet-dry	no S	895 ± 33	782 ± 42
		S	930 ± 51	993 ± 23
Fornazzo (2018)				
water seeded	continuous	no S	1107 ± 64	1027 ± 75
		S	1127 ± 45	1143 ± 70
water seeded	wet-dry	no S	1085 ± 22	1018 ± 13
		S	1023 ± 51	1213 ± 75

Table S5 Arsenic species in brown rice as determined by IC-ICP-MS in the mesocosm experiments (2018) (Mean ± SD, n=3)

Soil & Seeding & Year	Irrigation	Fertilization	Arsenite	DMA	MMA	Arsenate
			µg/kg	µg/kg	µg/kg	µg/kg
Veronica (2018)						
water seeded	continuous	no S	350.62 ± 14.26	212.53 ± 25.16	1.49 ± 0.43	21 ± 2.05
		S	149.91 ± 13.07	107.23 ± 4.38	2.62 ± 0.53	5.20 ± 3.79
water seeded	wet-dry	no S	449.52 ± 54.22	54.31 ± 3.77	1.38 ± 0.69	32.73 ± 8.96
		S	206.3 ± 20.49	78.57 ± 7.34	2.41 ± 0.86	6.37 ± 1.83
Fornazzo (2018)						
water seeded	continuous	no S	264.07 ± 33.07	78.33 ± 8.84	1.32 ± 0.3	15.1 ± 4.56
		S	135.67 ± 1.84	81.19 ± 5.95	3.14 ± 2.02	5.53 ± 1.74
water seeded	wet-dry	no S	310.52 ± 41.85	37.97 ± 8.85	0.41 ± 0.58	19.56 ± 4.63
		S	162.4 ± 1.69	54.08 ± 6.93	1.19 ± 0.44	4.38 ± 0.38

Table S6 Comparison of As accumulation between brown rice and white rice in the mesocosm experiments (2018) (Mean ± SD, n=3). Different lowercase letters indicate significant ($p < 0.05$) differences between treatments (separated by two studied soils).

Soil & Seeding & Year	Irrigation	Fertilization	Total As	Inorg-As	Methyl-As	Inorg-As (%)
			The ratio for brown rice to white rice			
Veronica (2018)						
water seeded	continuous	no S	1.64 ± 0.05 ab	2.23 ± 0.1 ab	1.02 ± 0.03 a	1.43 ± 0.02 a
		S	1.46 ± 0.15 c	1.81 ± 0.33 b	1.03 ± 0.04 a	1.30 ± 0.09 ab
water seeded	wet-dry	no S	2.56 ± 0.53 a	2.60 ± 0.29 a	1.08 ± 0.03 a	1.14 ± 0.02 b
		S	1.59 ± 0.05 ab	1.79 ± 0.07 b	1.05 ± 0.04 a	1.20 ± 0.02 b
Fornazzo (2018)						
water seeded	continuous	no S	1.70 ± 0.18 ab	1.94 ± 0.46 a	1.01 ± 0.17 a	1.20 ± 0.04 a
		S	1.22 ± 0.06 c	1.30 ± 0.23 a	0.90 ± 0.13 a	1.16 ± 0.03 a
water seeded	wet-dry	no S	1.95 ± 0.14 a	2.03 ± 0.41 a	1.04 ± 0.19 a	1.10 ± 0.02 a
		S	1.41 ± 0.11 bc	1.41 ± 0.27 a	0.91 ± 0.12 a	1.14 ± 0.04 a

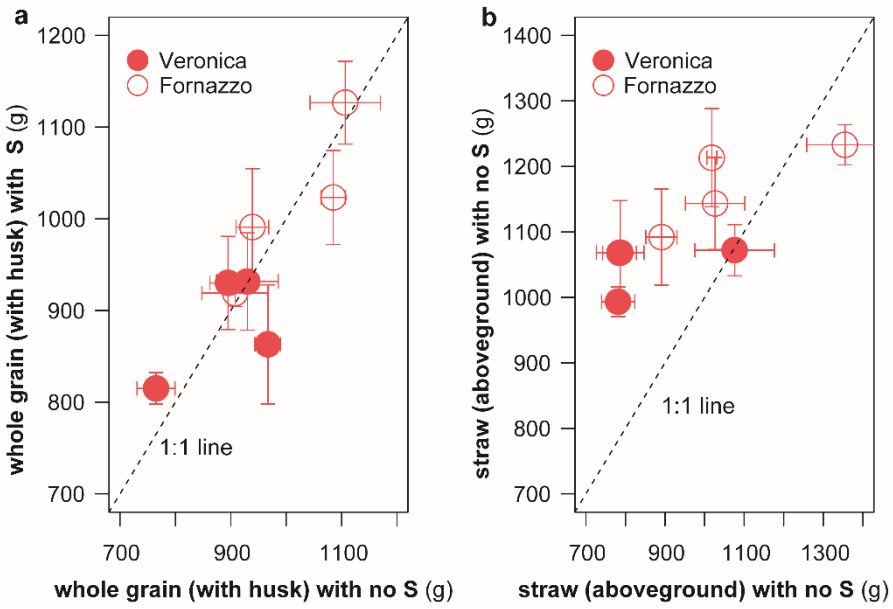

Fig. S5 Effects of sulfate fertilization on the yield of whole grain (with husk) (a) and straw (aboveground) (b) per mesocosm (0.82 m^2) from two-year rice cultivations using paddy soils from Veronica and Fornazzo. Values above 1:1 line (dash line) represent increased effect of sulfate, whereas values below 1:1 line represent decreased effect of sulfate. Bars represent standard deviations (n = 3).

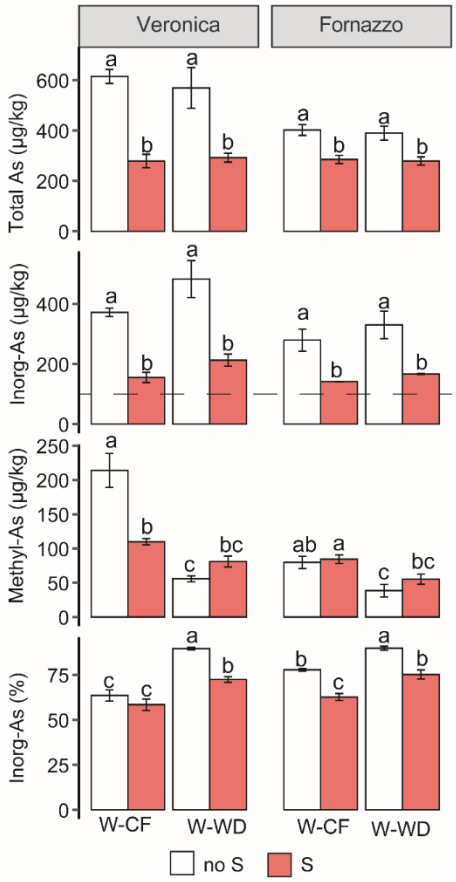

Fig. S6 Effects of sulfate fertilization on As accumulation in brown rice (2018) harvested from Veronica and Fornazzo. Error bars represent standard deviations (n = 3). Different lowercase letters indicate significant ($p < 0.05$) differences between treatments (separated by cropping years and two studied soils). Abbreviations: W-CF = water seeded, continuous flooded; W-WD = water seeded, alternate wet-dry. The horizontal dash line at 100 µg/kg indicates the limit of inorg-As concentration for European Union standard for rice based baby foods.

List of publications

The following publications/manuscripts have been published or are to be submitted during the work on this thesis:

Wang, Jiajia .; Kerl, C. F.; Hu, P.; Martin, M.; Mu, T.; Brüggenwirth, L.; Wu, G.; Said-Pullicino, D.; Romani, M.; Wu, L.; Planer-Friedrich, B., Thiolated arsenic species observed in rice paddy pore waters. Nature Geoscience 2020, 13, (4), 282-287.

Wang, Jiajia.; Halder, D.; Wegner, L.; Brüggenwirth, L.; Schaller, J.; Martin, M.; Said-Pullicino, D.; Romani, M.; Planer-Friedrich, B., Redox Dependence of Thioarsenate Occurrence in Paddy Soils and the Rice Rhizosphere. Environmental Science & Technology 2020, 54, (7), 3940-3950.

Wang, Jiajia.; Kerl, C.; Zhang, S.; Roll, U.; Beltarre, G.; Martin, M.; Said-Pullicino, D.; Romani, M.; Planer-Friedrich, B., Sulfate fertilization influences the transfer of inorganic and methylated arsenic from paddy soil to rice grain. (first draft) 2020.

Other publications:

Schaller, J.; **Wang, Jiajia**.; Islam, M. R.; Planer-Friedrich, B., Black carbon yields highest nutrient and lowest arsenic release when using rice residuals in paddy soils. Scientific Reports 2018, 8, (1), 17004.

Supervised theses

The following theses have been co-supervised during the work on this thesis:

1. Laura Wegner (2018): Effect of redox changes and sulfate application mode on arsenic thiolation in paddy soil incubations, Bachelor Thesis, University of Bayreuth, Environmental Geochemistry.

Results of this project are included in study 2 of this thesis.

2. Lena Brüggenwirth (2019): Effect of root radial oxygen loss on arsenic speciation in the rice rhizosphere, Master Thesis, University of Bayreuth, Environmental Geochemistry.

Results of this project are included in study 2 of this thesis.

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