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Thioarsenates in rice plants and grains: implications for food safety

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Abstract

Rice is typically grown under flooded conditions which mobilizes naturally occurring arsenic (As) from paddy soils. Besides the well-known oxyAs species, inorganic As (iAs: arsenite and arsenate), monomethylarsenate (MMA^V), and dimethylarsenate (DMA^V), inorganic and methylated thioarsenates have recently been found in paddy soil pore waters. Moreover, the uptake of thioarsenates by rice plants has been confirmed. Amongst methylated thioarsenates, dimethylmonothioarsenate (DMMTA) is particularly relevant because of its high toxicity in mammalian cells. Despite mounting evidence of the ubiquitous presence of thioarsenates in soil pore waters little is known about their interaction with plants and accumulation in grains.

This thesis aimed to investigate the relevance of thioarsenates, particularly DMMTA, for food safety and rice plants. The occurrence of thioarsenates in rice grains and products was examined. Additionally, the toxic effects of DMMTA on plants were assessed and the path of DMMTA to the grains, specifically (trans)formation, accumulation, transport, and translocation, was studied in rice plants.

The first two studies investigated the occurrence of thioarsenates in rice grains and products. Since routine acid-based extractions co-determined DMMTA as DMA^V, a method to detect thioarsenates was developed. The method consists of a two-step enzymatic extraction followed by chromatographic separation with 2.5-100 mmol L^{-1} NaOH as eluent. The extraction efficiency of the method was confirmed by using a rice-certified reference material. Contents of DMA^V, iAs, and sum of As species were all in agreement with the certified values. Formation of DMMTA during the enzymatic extraction was ruled out by the complete recovery of a DMA^V spike added before extraction. Analysis of commercial samples showed that thioarsenates, namely DMMTA, dimethyldithioarsenate (DMDTA), and monothioarsenate (MTA) accumulated in the grains. Notably, puffed rice cakes had particularly higher contents of thioarsenates in comparison to the rice samples. Screening of a larger dataset of commercial puffed rice cakes (n = 80) revealed that DMMTA and DMDTA were widely present in the samples, accounting for up to 38 and 46% of total As, respectively. Additionally, MTA, dithioarsenate (DTA), monomethylmonothioarsenate (MMMTA), and monomethyldithioarsenate (MMDTA) were also widely present as minor species. A comparison between the As speciation of rice grains and their respective puffed rice cake revealed that the high content of thioarsenates detected in the puffed rice is a consequence of the high temperatures employed during the puffing treatment. Reduced sulfur originating from the thermal degradation of sulfur-containing compounds in rice was speculated to cause thiolation.

The following two studies focused on the behavior of DMMTA in plants. Hydroponic experiments with *Arabidopsis thaliana* demonstrated DMMTA caused a strong root growth inhibition, more than arsenite and by far more than DMA^V. Unlike arsenite, DMMTA exposure did not lead to the accumulation of reactive oxygen species but caused deformation of root epidermal cells revealing different toxicity mechanisms between the As species. Furthermore, like DMA^V, the phytochelatin pathway did not contribute to DMMTA detoxification, hence speciation in roots and shoots revealed efficient translocation of DMMTA within plants. Shoot growth and development were also severely affected by the translocated DMMTA. Alterations such as curling of the leaves due to dehydration, decrease in chlorophyll *a* and *b* and carotenoids, and accumulation of anthocyanins were observed. Hydroponic experiments with rice plants during grain filling revealed that both DMMTA- and DMA^V-exposed plants accumulated similar shares of DMMTA in the leaves, grains, and husks and DMDTA in the leaves and grains, unveiling *in planta* (de)thiolation processes. Stem-girdling experiments indicated preferential transport of DMMTA in the phloem. Phloem transport was further confirmed by the detection of DMMTA in grains after flag leaf-feeding experiments. For the first time, arsenite and MMA^V *in planta* thiolation was observed in rice seedlings. *In planta* thiolation of DMA^V was also detected in *A. thaliana* and the kinetics of DMA^V thiolation showed this process is not purely abiotic. Significantly lower DMA^V thiolation was observed in glutathione (GSH) deficient mutants compared to wild-type *A. thaliana* plants, thus suggesting GSH concentration as an important parameter influencing *in planta* thiolation of As species.

The fifth study merged our findings and other evidence available on thioarsenates occurrence in rice and thioarsenates detection challenges and put it in context with cytotoxicity data and regulatory limits for As in rice worldwide. The study highlighted that overlooking thioarsenates poses a potential food safety threat, particularly when it comes to the misidentification of highly cytotoxic DMMTA as unregulated DMA^V.

Overall, our findings revealed the importance of studies on the interaction of thioarsenates, specially DMMTA, with plants with respect to food safety. DMMTA was shown to be highly mobile and toxic for plants. Even in the absence of thioarsenates in soil pore water, *in planta* thiolation of DMA^V can lead to DMMTA and DMDTA accumulation in grains. The occurrence of thioarsenates in commercial rice grains and puffed rice cakes indicates that further monitoring and risk assessment characterization of ingesting thioarsenate-containing rice is urgently needed.

Zusammenfassung

Reis wird in der Regel unter gefluteten Bedingungen angebaut, wodurch natürlich vorkommendes Arsen (As) aus den Reisböden mobilisiert wird. Neben den bekannten Oxy-As-Spezies, anorganischem As (iAs: Arsenit und Arsenat), Monomethylarsenat (MMA^V) und Dimethylarsenat (DMA^V) wurden vor Kurzem auch anorganische und methylierte Thioarsenate im Porenwasser von Reisböden gefunden. Außerdem wurde die Aufnahme von Thioarsenaten durch Reispflanzen bestätigt. Unter methylierten Thioarsenaten ist Dimethylmonothioarsenat (DMMTA) aufgrund von nachweislich hoher Toxizität in Säugetierzellen, besonders relevant. Trotz zunehmender Nachweise von Thioarsenaten im Reisboden Porenwasser ist derzeit nur wenig über die Interaktion mit Pflanzen und deren Akkumulation im Reiskorn bekannt.

Ziel dieser Arbeit war es, die Bedeutung von Thioarsenaten, insbesondere von DMMTA, für die Lebensmittelsicherheit und Reispflanzen zu erforschen. Das Vorkommen von Thioarsenaten in Reiskörnern und Reisprodukten wurde untersucht. Darüber hinaus wurden die toxischen Effekte von DMMTA auf Pflanzen beurteilt und der Weg von DMMTA zu den Reiskörnern, mit besonderem Fokus auf Bildung, Umwandlung, Akkumulation, Transport und Translokation, in Reispflanzen untersucht.

In den ersten beiden Studien wurde das Vorkommen von Thioarsenaten in Reiskörnern und Reisprodukten untersucht. Da etablierte säurebasierte Extraktionen DMMTA als DMA^V erfassen, wurde eine Methode zum Nachweis von Thioarsenaten entwickelt. Diese umfasst eine zweistufige enzymatische Extraktion und eine anschließende chromatografische Trennung mit 2,5-100 mmol L^{-1} NaOH als Elutionsmittel. Die Extraktionseffizienz der Methode wurde mit zertifizierten Reis Referenzmaterial bestätigt. Gehalte von DMA^V, iAs und Summe der As Spezies stimmten mit den zertifizierten Werten überein. Bildung von DMMTA während der Extraktion konnte durch vollständige Wiederfindung, von vor der Extraktion zugesetzten DMA^V, ausgeschlossen werden. Die Analyse von handelsüblichen Waren zeigte, dass sich Thioarsenate, besonders DMMTA, Dimethyldithioarsenat (DMDTA) und Monothioarsenat (MTA), in den Reiskörnern anreichern. Reiswaffeln wiesen im Vergleich zu Reisproben einen besonders hohen Gehalt an Thioarsenaten auf. Screening eines umfangreichen Datensatzes kommerziell erwerblicher Reiswaffeln (n = 80) zeigte eine weite Verbreitung von DMMTA und DMDTA, die jeweils bis zu 38 und 46 % des gesamt-As Gehaltes ausmachten. Darüber hinaus waren MTA, Dithioarsenat (DTA), Monomethylmonothioarsenat (MMMTA) und Monomethyldithioarsenat (MMDTA) in geringeren Konzentrationen ebenfalls weit verbreitet. Ein Vergleich der As-Speziierung von Reiskörnern und jeweils zugehöriger Reiswaffeln zeigte, dass der hohe Gehalt an Thioarsenaten in Reiswaffeln eine Folge der hohen Temperaturen ist, die bei dem Reispuffen angewendet werden. Es wurde vermutet,

dass reduzierter Schwefel, der durch die thermische Zersetzung schwefelhaltiger Verbindungen im Reis entsteht, die Thiolierung verursacht.

Die beiden folgenden Studien befassten sich mit dem Verhalten von DMMTA in Pflanzen. Hydroponische Experimente mit *Arabidopsis thaliana* zeigten eine starke Hemmung des Wurzelwachstums durch DMMTA, stärker als durch Arsenit und bei weitem stärker als durch DMA^V. Im Gegensatz zu Arsenit führte die Exposition mit DMMTA nicht zur Akkumulation reaktiver Sauerstoffspezies, sondern verursachte eine Deformation der epidermalen Wurzelzellen, was auf unterschiedliche Toxizitätsmechanismen der beiden As-Spezies hinweist. Außerdem, ähnlich wie bei DMA^V, trugen Phytochelatine nicht zur Entgiftung von DMMTA bei, da die As-Speziierung in den Wurzeln und Sprossen eine effiziente Verlagerung von DMMTA innerhalb der Pflanzen ergab. Auch das Wachstum und die Entwicklung der Sprossen wurden durch das Verlagern von DMMTA stark beeinträchtigt. Veränderungen, wie das Kräuseln der Blätter aufgrund von Austrocknung, eine Abnahme von Chlorophyll *a* und *b* und Carotinoiden sowie eine Anhäufung von Anthocyanen wurde beobachtet. Hydroponische Experimente mit Reispflanzen während der Reiskornfüllungsphase zeigten, dass sowohl DMMTA- als auch DMA^V-exponierte Pflanzen ähnliche Anteile an DMMTA in den Blättern, Körnern und Schalen, und DMDTA in den Blättern und Körnern akkumulierten, was *in planta* Dethio- bzw. Thiolierungsprozesse zeigt. Stamm-girdling Experimente zeigten, dass DMMTA bevorzugt über das Phloem transportiert wird. Zusätzlich konnte der Transport im Phloem durch den Nachweis von DMMTA in den Reiskörnern über das Fahnenblatt exponierter Pflanzen bestätigt werden. Darüber hinaus wurde *in planta* Thiolierung von Arsenit und MMA^V zum ersten Mal in Reissetzlingen beobachtet. *In planta* Thiolierung von DMA^V konnte auch in *A. thaliana* nachgewiesen werden und die Kinetik der DMA^V-Thiolierung zeigte, dass die Thiolierung kein rein abiotischer Prozess ist. Eine deutlich geringere DMA^V-Thiolierung wurde in Mutanten mit Glutathion (GSH)-Mangel im Vergleich zu Wildtyp-Pflanzen von *A. thaliana* beobachtet, was darauf hindeutet, dass die GSH-Konzentration ein wichtiger Parameter ist, der die Thiolierung von As-Spezies *in planta* beeinflusst.

In der fünften Studie wurden unsere Ergebnisse mit anderen verfügbaren Ergebnissen zum Vorkommen von Thioarsenaten in Reis und Problemen beim Nachweis von Thioarsenaten zusammengeführt und in Zusammenhang mit den Zytotoxizitätsdaten und den weltweit geltenden Grenzwerten für As in Reis gebracht. Diese Studie verdeutlichte, dass das Übersehen von Thioarsenaten eine potenzielle Gefahr für die Lebensmittelsicherheit darstellt, insbesondere wenn es um die falsche Identifizierung von hoch zytotoxisches DMMTA als unreguliertem DMA^V geht.

Insgesamt zeigen unsere Ergebnisse, wie wichtig Studien über die Wechselwirkung von Thioarsenaten, insbesondere von DMMTA, mit Pflanzen im Hinblick auf die Lebensmittelsicherheit

sind. DMMTA erwies sich als sehr mobil und toxisch für Pflanzen. Selbst bei Abwesenheit von Thioarsenaten im Porenwasser des Bodens kann die Thiolierung von DMA^V in Pflanzen zu einer Anreicherung von DMMTA und DMDTA in den Reiskörnern führen. Das Vorkommen von Thioarsenaten in handelsüblichen Reiskörnern und Reiswaffeln unterstreicht, dass ein weiteres Monitoring und eine Risikobewertung zum Verzehr von thioarsenathaltigem Reis dringend erforderlich sind.

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List of Abbreviations

As	Arsenic
CONTAM	Panel on Contaminants in the Food Chain
DMA^{III}	Dimethylarsenite
DMA^{V}	Dimethylarsenate
DMDTA	Dimethyldithioarsenate
DMMTA	Dimethylmonothioarsenate
DTA	Dithioarsenate
EFSA	European Food Safety Authority
GSH	Glutathione
HG-AAS	Hydride generation atomic absorption spectrometry
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
iAs	Inorganic arsenic
IC	Ion chromatography
IC_{50}	Half maximal inhibitory concentrations
ICP-MS	Inductively coupled plasma mass spectrometry
MMA^{III}	Monomethylarsenite
MMA^{V}	Monomethylarsenate
MMDTA	Monomethyldithioarsenate
MMMTA	Monomethylmonothioarsenate
MTA	Monothioarsenate
NanoSIMS	High-resolution secondary ion mass spectrometry
OVT	Ovular vascular trace
PBS	Phosphate buffer solution
PCs	Phytochelatins
S-XRF	Synchrotron X-ray fluorescence
TTA	Trithioarsenate
XANES	X-ray absorption near edge structure
XAS	X-ray absorption spectroscopy

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Extended summary

1. Introduction

1.1. Rice grains and arsenic - speciation beyond oxyarsenic species

Rice is a dietary staple for half of the world's population.[1] It is currently grown in over a hundred countries that, in 2021, produced more than 787 million tons of paddy rice.[2] Caribbean and South East Asian populations are amongst the highest rice consumers with countries like Cuba and Myanmar consuming over 200 g and 600 g per capita daily, respectively.[3] Furthermore, rice products such as puffed rice cakes, mueslis, crackers, noodles, cereal bars, rice milk, and rice syrup are significantly consumed in Western diets as these products cater to emerging restricted diets such as gluten-free and vegan diets.[3,4] Additionally, due to its mild flavor, nutritional properties, and low allergen potential, rice products such as rice porridge are typically used for weaning infants.[5]

Previous studies have identified rice as one of the major sources of arsenic (As) exposure in the human diet.[6–9] In fact, rice is nearly tenfold elevated in As content compared to other grain staples such as wheat and barley.[10] The main As species found in rice, inorganic As (iAs: arsenite and arsenate), are classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC), meaning there is sufficient evidence of carcinogenicity in humans, namely lung, urinary, bladder, and skin cancer.[11] The remaining As species typically encountered in rice, monomethylarsenate (MMA^V), and especially, dimethylarsenate (DMA^V), are classified by the IARC as possibly carcinogenic to humans (Group 2B), meaning there is some evidence that they can cause cancer in humans but currently, the evidence is far from conclusive.[11]

Rice is typically grown in flooded fields. Management of paddy soils involves tillage of the flooded soil and rice transplantation and growth in submerged conditions up to one or two weeks before harvest.[3,12] Upon flooding, microorganisms utilize organic substances as electron donors during respiration and, as long as it is available, molecular oxygen is the preferred electron acceptor. After oxygen is depleted, microorganisms utilize alternative electron acceptors such as NO_3^-, Mn^{4+}, Fe^{3+}, and SO_4^{2-}, decreasing the soil redox potential.[12] The reduction of Fe-(oxyhydr)oxides indicates the development of anoxic conditions.[12] Here, arsenate is released from As-bearing Fe-(oxyhydr)oxides into the pore water and is quickly reduced to arsenite. Thereafter, microbe-mediated As methylation leads to the formation of MMA^V and DMA^V.[13,14] Ultimately, speciation in pore waters is dominated by arsenite and, to a lesser extent, arsenate, DMA^V, and MMA^V.[3,15] The availability of these oxyAs species in paddy soil pore waters is one of the main reasons why most research on rice grain As

speciation has strongly focused only on iAs and DMA^V.[3,4,16–21] However, the recent detection of another group of As species, known as thioarsenates (pentavalent analogues of oxyAs species in which oxygen is replaced by sulfur, Figure 1), in rice paddy pore waters[22] has prompted the question of their relevance in rice plant and grain studies.

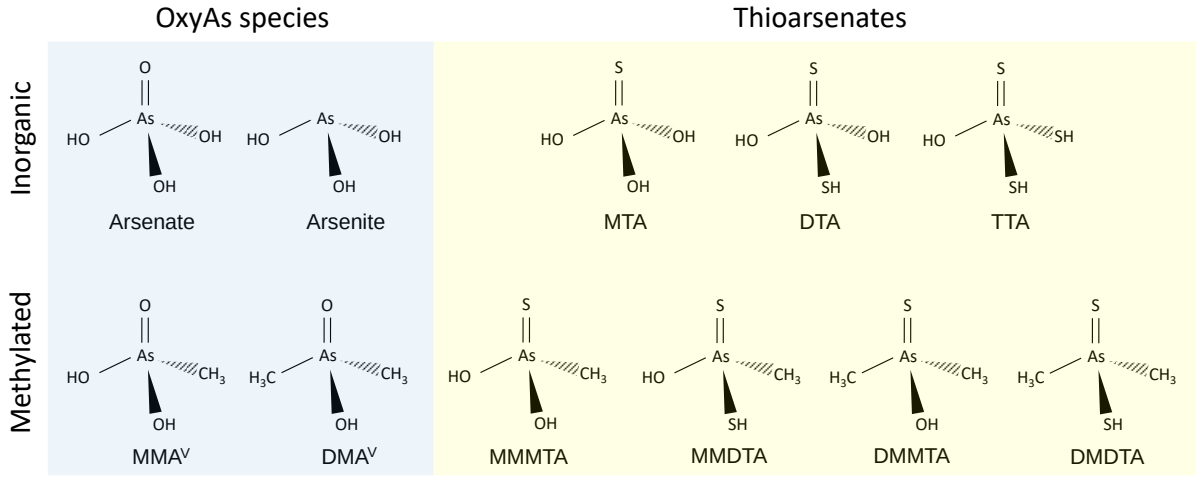

Figure 1. Overview of oxyAs species and thioarsenates discussed in this thesis. For simplicity, all species are depicted fully protonated. Abbreviations: MTA: monothioarsenate, DTA: dithioarsenate, TTA: trithioarsenate, MMA^V: monomethylarsenate, DMA^V: dimethylarsenate, MMMTA: monomethylmonothioarsenate, MMDTA: monomethyldithioarsenate, DMMTA: dimethylmonothioarsenate, and DMDTA: dimethyldithioarsenate.

1.2. Arsenic in rice plants: root-to-grain journey

1.2.1. Root uptake

Arsenic species in paddy soil pore water enter the rice root cells through transporters of nutrients with similar chemical properties.[23] Arsenate (pK_{a1} = 2.2 and pK_{a2} = 6.9) uptake happens via phosphate (pK_{a1} = 2.1 and pK_{a2} = 7.1) membrane transporters (e.g., OsPht1;1,[24] OsPht1;4,[25] and OsPht1;8[26]) against an electrochemical gradient (active transport).[27] Arsenite (pK_{a1} = 9.2) uptake is driven by a concentration gradient (passive transport) and occurs via nodulin 26-like intrinsic proteins, aquaglyceroporins in charge of silicic acid (pK_{a1} = 9.5) uptake, such as Lsi1 (OsNIP2;1).[28] In rice plants, the transporter Lsi1 has also been shown to take up, at a much slower rate, undissociated MMA^V (pK_{a1} = 4.2 and pK_{a2} = 8.8) and DMA^V (pK_a = 6.1).[29,30] Root uptake follows the order DMA^V < MMA^V < iAs.[29–31]

Regarding thioarsenates, direct uptake into rice roots has only been demonstrated in seedling experiments where MTA was externally applied and extracted with a species-preserving method. In

the same study, toxicity tests with different initial phosphate concentration revealed higher MTA toxicity with lower phosphate availability, implying MTA uptake in rice roots might occur via phosphate transporters.[32] Analysis of total As from MMMTA and DMMTA-exposed rice seedlings showed both species can enter the root cells and when comparing their total As accumulation in roots to that of methylated oxyAs species, the following root uptake trend was observed DMA^V < DMMTA < MMA^V < MMMTA.[33] However, there is still no direct analytical evidence of the presence of methylated thioarsenates in rice roots.[33] The transporters mediating inorganic and methylated thioarsenates uptake have not been identified yet.

1.2.2. *In planta* transformations: root detoxification mechanisms

The As species that are taken up into the roots can have three different fates: (i) efflux out of the cell, (ii) sequestration in the root cells, or (iii) long-distance transport to the aboveground tissues. Arsenate is rapidly reduced to arsenite inside the cells by arsenate reductases such as HAC1.[27,34,35] Arsenite can be effluxed back to the rhizosphere also via the Lsi1 transporter (which possess bidirectional permeability)[36] and other unknown efflux transporters.[36] Arsenite can also be complexed with thiol ligands such as glutathione (GSH) or phytochelatins (PCs),[31,37–39] which bind the trivalent As through their cysteine residues.[40] The As-thiol complexes are then stored in the vacuoles, where they remain stable due to the acidic environment (pH ~ 5.8) of this organelle.[41,42] In rice, the ABC-type transporter OsABCC1 is responsible for the vacuolar sequestration of the As-thiol complexes.[43,44] Like arsenate, MMA^V is first reduced in the roots and then complexed by GSH or PCs as well.[31,45] So far, DMA-thiol complexes have not been identified in rice or other plant roots.[31,45]

No prior studies have specifically investigated thiol complexation of thioarsenates in rice roots, however, a study conducted on *Arabidopsis thaliana* demonstrated that PCs play a relevant role in the detoxification of MTA.[46] In rice, efflux experiments with seedlings exposed to MTA showed rapid reduction of MTA to arsenite, hinting to a similar detoxification mechanism as arenate.[32]

1.2.3. Long-distance transport: xylem transport

The As species that are not effluxed or trapped in the roots can be further transported to the shoots. The xylem is the vascular tissue in charge of conducting water and dissolved minerals from the roots to the shoots. The main driving force for xylem transport is transpiration, thus, this process leads to the accumulation of elements in transpiring tissues (e.g., leaves and husks).[47,48] Efflux towards the xylem also happens via transporters of nutrients with similar chemical properties. Experiments with hydroponically grown rice showed that arsenate can be loaded into the xylem via the phosphate transporter OsPht1;8, however, it is only detected in traces in the xylem sap due to significant

intracellular reduction to arsenite.[26] Arsenite loading into the xylem is mediated by the silicon transporter Lsi2.[28] How MMA^V and DMA^V reach the xylem has not yet been elucidated, however, despite their lower root uptake, methylated As species have a higher root-to-shoot mobility compared to iAs,[31] especially DMA^V, which is not complexed by thiols and is therefore readily loaded into the xylem.[29]

Hydroponic experiments with rice seedlings have demonstrated the presence of MTA,[32] MMMTA,[33] and DMMTA[33] in the xylem sap, nevertheless, the transporters involved have not yet been identified. Analysis of total As from rice seedlings exposed to methylated oxyAs species and methylated thioarsenates revealed that DMMTA-exposed seedlings had particularly higher As contents in the shoots, suggesting DMMTA has a high potential to contribute to total As accumulation in grains.[33] It is, however, noteworthy that there is no direct analytical evidence of the presence of methylated thioarsenates in rice shoots. So far, only DMMTA and DMMTA-GS have been reported in the shoots of cabbage plants exposed hydroponically to DMA^V, suggesting *in planta* thiolation as a process involved in the metabolism of As species in plants.[49] Figure 2 summarizes the information available and knowledge gaps of the transport of As species from the roots to the xylem.

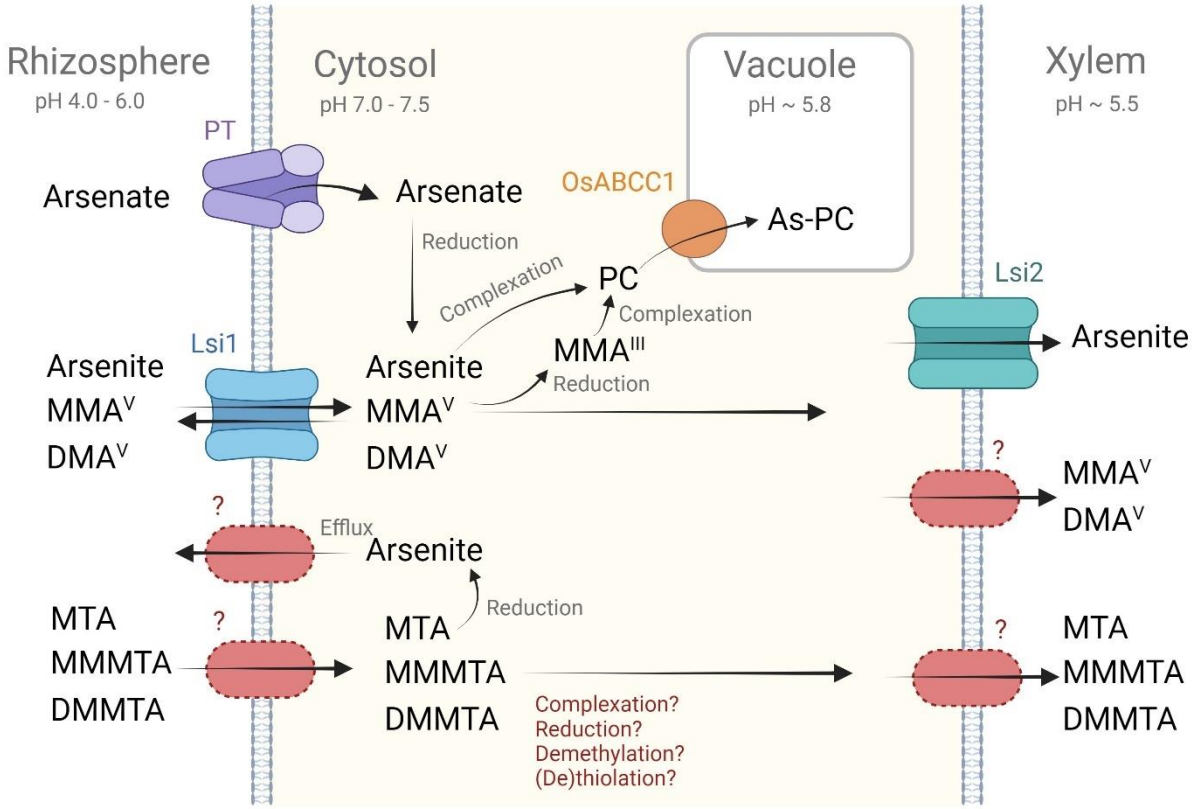

Figure 2. Simplified schematic representation of the movement of As species from the roots to the xylem in rice plants (adapted from Clemens 2019)[23]. PT: phosphate transporter. Red dotted outlines indicate unknown transporters. Created with BioRender.com

1.2.4. Long-distance transport: phloem transport

Once in the xylem, As species are transported mainly to the leaves, where they are either stored or further loaded into the phloem. The phloem is the vascular tissue in charge of transporting sugar and amino acids from source tissues like the leaves to sink tissues like the grains.[3] After phloem flow interruption, feeding experiments with excised rice plants revealed that phloem transport accounted for 90% of arsenite and 55% of DMA^V transport to the grains.[20] Despite the importance of phloem transport to food safety, very little is known about phloem loading and unloading.[3,23,50]

Presumably, the phloem is loaded via xylem-to-phloem transfer or via remobilization from the leaves.[23] In rice, it has been shown that phloem loading by intervascular transfer in the node I (Figure 3) plays a major role in the distribution of As to the leaves and grains.[51,52] The nodes are junctional regions where the leaves join the rice stem. Rice cultivars typically have between 13 to 18 nodes on the main stem, each connected to the lower and upper nodes through a complex yet organized vascular tissue system. Here, many unknown transporters are involved in the movement of elements to the grains.[53]

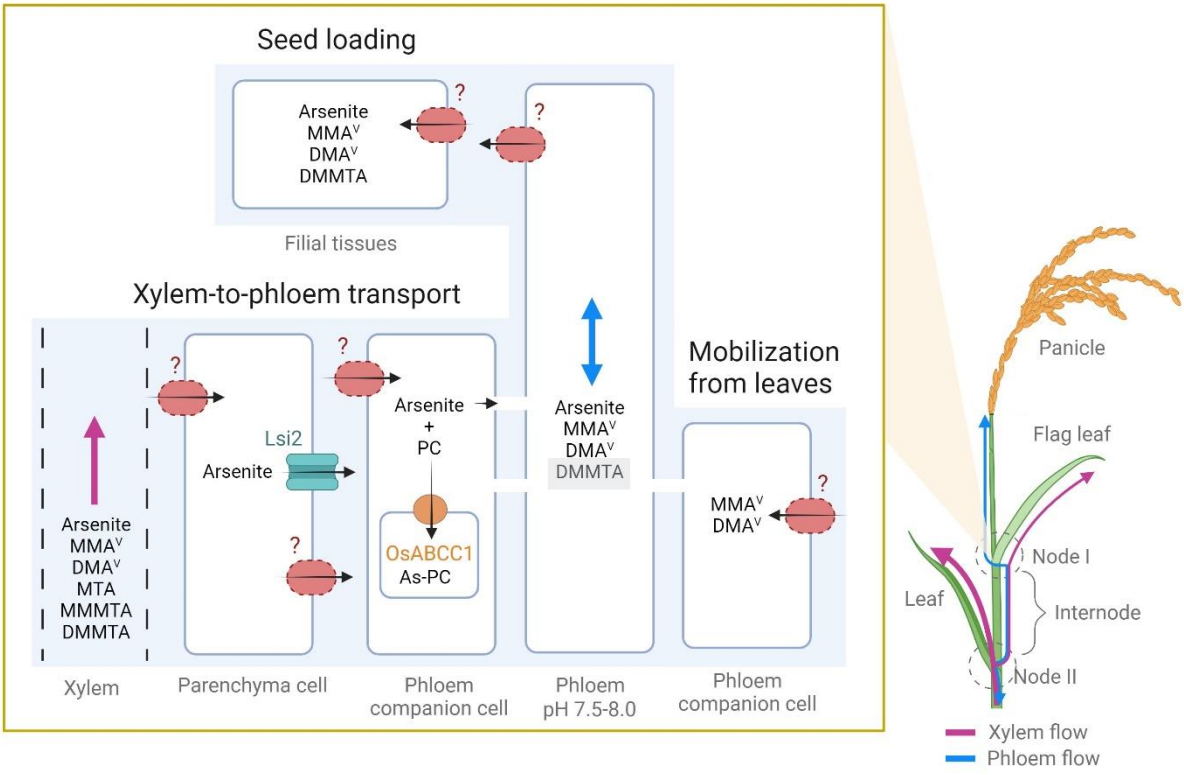

Figure 3. Simplified schematic representation of As species transport in the phloem of rice plants (adapted from Clemens & Ma 2016 and Clemens 2019)[23,50]. Red dotted outlines indicate unknown transporters. Parenchyma cell is not assigned to xylem or phloem. DMMTA in the phloem is shaded in grey to indicate indirect evidence. Nodes are numbered from top to bottom at the reproductive growth stage. Created with BioRender.com

Synchrotron X-ray fluorescence (S-XRF) and high-resolution secondary ion mass spectrometry (NanoSIMS) mapping in the node I, internode, and leaf sheath of rice plants exposed to arsenite, revealed a strong co-localization of As and S in the vacuoles of phloem companion cells in all the vascular tissues. Since the nodes contain proportionally more vascular tissues than the internodes and leaves, they can accumulate significantly higher As contents and restrict its distribution to the grains.[52] The transporter OsABCC1 has also been found expressed in the nodes of rice plants, and in the upper nodes, it was found localized to the phloem region of the vascular tissues. Arsenite feeding experiments with rice plants excised below node II showed a decrease in As tolerance in OsABCC1 knockout mutants compared to wild-type rice.[43] Hence, confirming As-thiol vacuolar sequestration as a detoxification strategy in the nodes. Additionally, only the efflux carrier Lsi2, located in the node parenchyma cells, has been identified in the process of phloem loading. Arsenite feeding experiments with rice plants excised below the node I showed higher As accumulation in the flag leaf and lower As accumulation in grains in *lsi2* mutant plants compared to wild-type rice.[51]

Even less information is known on DMA^V and MMA^V transport through the phloem. Flag leaf feeding experiments in rice demonstrated that in contrast to iAs, DMA^V and MMA^V were efficiently retranslocated from the leaves to the grains.[21] Despite the high mobility of the methylated oxyAs species, the transporters involved in their distribution remain unidentified. The involvement of Lsi2 in the transport of DMA^V was ruled out since DMA^V-exposed plants from *lsi2* mutants accumulated as much As as the wild-type plant.[51] There has been no reports on thioarsenate transport in the phloem, however, the tentative identification of DMMTA in two rice grain samples[54] (see section 1.3) provides indirect evidence of DMMTA transport in the phloem.

Finally, phloem unloading and transport into the grains are molecularly not understood. Since there is no symplastic continuity between the maternal and filial tissues, at least two additional transport steps across membranes are required.[55] Figure 3 summarizes the information available and knowledge gaps on As transport in the phloem.

1.2.5. Arsenic grain loading and distribution

Like nutrients, As species are loaded into the grains through the phloem and xylem of the ovular vascular trace (OVT).[20,48,55,56] During the early stage of grain development, nutrients are transported from the OVT to the pigment strand, into the nucellar projection, and finally into the aleurone and endosperm (Figure 4, left). Later, during the grain filling stage, circumferential transport predominates, with nutrients travelling from the OVT to the nucellar epidermis around the grains and then move centripetally towards the aleurone and endosperm.[3,57] Studies addressing the distribution of

As species in rice grains (see section 1.3 for As speciation techniques) have revealed that arsenite and As^{III}-thiol complexes are strongly localized in the bran aleurone layers (which are thickest near the OVT) while DMA^V readily permeates the endosperm (Figure 4, right).[20,48,57,58]

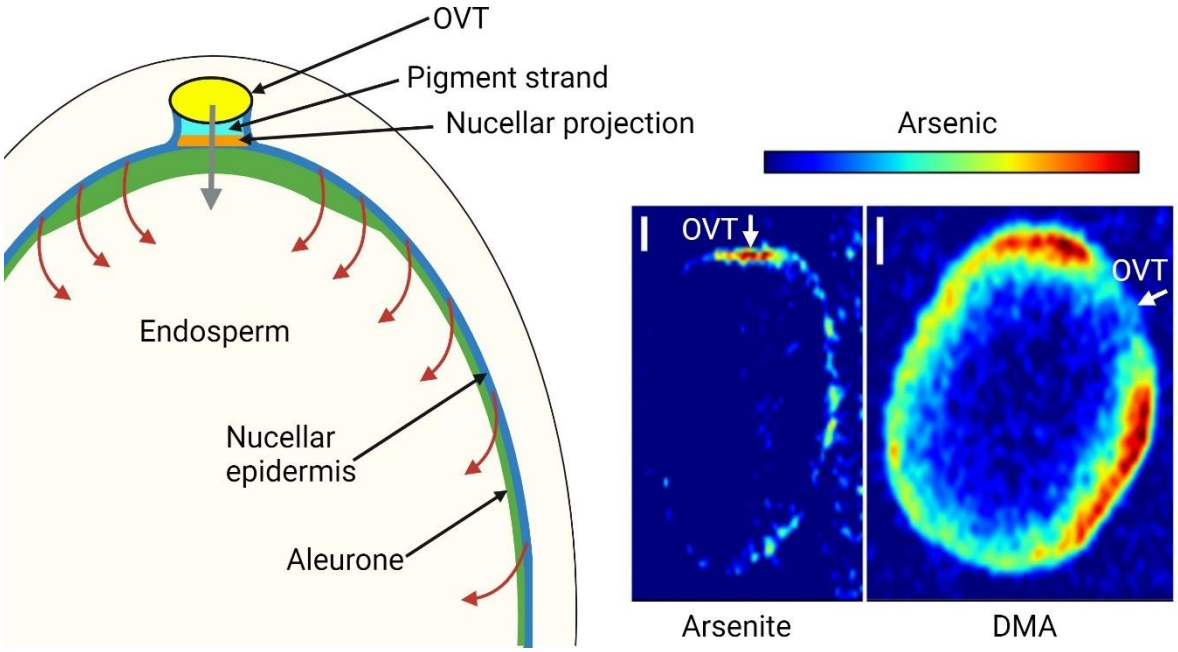

Figure 4. Left: simplified schematic representation of nutrient loading into developing rice grains. The grey arrow represents the loading pathway at the early stages of grain development and red arrows represent the circumferential pathway occurring mainly during grain filling. Right: high-resolution S-µXRF images showing As distribution in rice grains hydroponically exposed to arsenite (8 µmol L^{-1}) and DMA^V (5 µmol L^{-1}). Warmer colors are proportional to higher As concentrations (adapted from Limmer & Seyfferth 2022)[57]. Created with BioRender.com

1.3. Arsenic speciation in rice grains – challenges of detecting thioarsenates

Arsenic speciation in rice grains is dominated by iAs and DMA^V.[1,17,56,59] Both their contents (ranging up to hundreds µg kg^{-1}) and relative percentages (iAs: 10-100% and DMA^V: 0-90 %) vary widely.[59] *In situ* synchrotron X-ray absorption spectroscopy (XAS) approaches[20,48,57,60,61] and chemical extractions followed by chromatographic separation[1,5,7,17–19,54,61] are used to speciate As in grains. While these techniques have been successfully employed to differentiate between iAs and DMA^V, they have limitations and challenges when it comes to thioarsenates.

In situ speciation analysis using synchrotron-based X-ray absorption near edge structure (XANES) can distinguish arsenite, As^{III}-thiol complexes, and DMA^V in rice grains.[48,57] Regarding thioarsenates, a study reported that after rice panicles were pulsed with high DMA^V concentrations, most of the added DMA^V had transformed in the grain (As XANES absorption edge shifted to lower energies than

the DMA^V standard). Based on the observed absorption edge, formation of DMDTA or a DMA^{III}-DMA^V dimer thiol, both sharing a similar spectra to arsenite, was speculated but not confirmed.[20] Detection of thioarsenates in rice grain by XANES will likely be difficult, especially when multiple spectroscopically-similar species, with overlapping white lines, are present at low concentrations.[57,62,63]

For food safety monitoring, As speciation in rice grains is usually done by chemical extraction with dilute acids such as 2 mol L^{-1} trifluoroacetic acid[64] or 0.28 mol L^{-1} nitric acid.[19] When using a strong anion exchange column (e.g. Hamilton PRP X-100) with a mobile phase of either ammonium phosphate, nitrate, sulfate, carbonate or a combination of these (at pH 6-9), arsenite, arsenate, DMA^V , and MMA^V can be separated.[65] Thereafter detection is typically done via hydride generation atomic absorption spectrometry (HG-AAS) or inductively coupled plasma mass spectrometry (ICP-MS).[66]

Little is known about the presence of thioarsenates in rice grains. More than 15 years ago, DMMTA was tentatively identified in rice using a two-step enzymatic extraction that mimics cooked food entering the human digestive tract.[54] Notably, DMMTA was only detected in two samples at relatively high contents, 40 and 46 µg kg^{-1}, accounting for 13 and 20% of total As in the grains, respectively. The fact that DMMTA was only detected in two samples might be attributable to the chromatographic conditions employed (Hamilton PRP X-100 and 25 mmol L^{-1} $(NH_4)_2CO_3$ with pH adjusted to 8.6) which produced a relatively broad and flat DMMTA peak (Figure 5) and consequently, an insufficient detection limit. The study also demonstrated DMMTA was not an extraction artefact. Speciation discrepancies between the enzymatic extraction and conventional trifluoroacetic acid extraction revealed DMMTA transformed to DMA^V in the acidic extracts.[54]

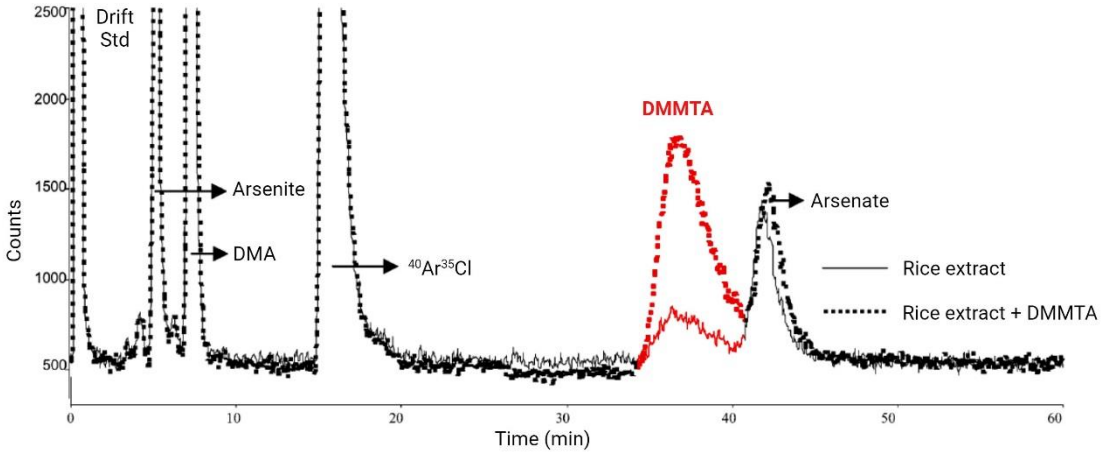

Figure 5. First analytical evidence of thioarsenates in rice grains. Ion chromatography (IC)-ICP-MS chromatogram of the enzymatic rice extract with the unknown As species characterized as DMMTA (solid line) and rice extract spiked with DMMTA standard (dotted line) (adapted from Ackerman et al., 2005)[54]. Modified with BioRender.com

In a later rice grain survey using an enzymatic extraction followed by IC-ICP-MS (Hamilton PRPX-100 and 10 mmol L^{-1} $NH_4NO_3/NH_4H_2PO_4$ with pH adjusted to 8.2), DMMTA was also detected.[7] The observed contents were lower (5-19 µg kg^{-1}), but since DMMTA recovery was not tested, the extent of transformation during the enzymatic extraction remained unclear (the authors reported "at least a partial preservation" for DMMTA throughout the procedure). The authors also reported dilute nitric acid converted DMMTA to DMA^V.[7] Despite these findings, no follow-up studies on the presence of DMMTA or other thioarsenates in rice grains have been conducted, hence their natural occurrence in rice grains remain unknown.

Detection of thioarsenates in rice grains would only be possible if their stability during extraction is accounted for since they tend to convert into their corresponding oxyAs species upon acidification, heating, or dilution.[7,67–69] Furthermore, adaptations from routine chromatographic conditions employed in rice As speciation studies would also be needed (e.g., to separate and quantitatively elute thioarsenates by IC-ICP-MS, highly alkaline conditions are required).[68,69]

1.4. Toxicity of thioarsenates – a threat for rice plants and humans

Even though thioarsenates have been detected in paddy soil pore waters and their uptake by rice plants has been shown before, very little is known about their toxicity in plants. Seedling growth experiments using the model plant *A. thaliana* revealed that MTA is less toxic than arsenite, but more toxic than arsenate, which was reflected in a stronger growth inhibition.[46] Similar growth inhibition results were also observed in experiments using rice seedlings, were MTA toxicity was similar to that of arsenate but lower than that of arsenite.[32] No information exists on toxicity effects of methylated thioarsenates in plants, despite their observed higher root uptake in rice seedlings compared to that of their corresponding oxyAs species (MMA^V < MMMTA and DMA^V < DMMTA).[33] Thioarsenates need to be investigated to better understand possible negative impacts on plant growth and grain yield.

The fact that DMMTA was previously reported to occur in rice grains is particularly concerning for food safety for three reasons, namely, (i) detection after extraction utilizing a procedure that mimics the human digestive tract implies that DMMTA is bioaccessible and released at least partially intact upon rice digestion, (ii) its known toxicity to human and other mammalian cells, and (iii) misidentification of DMMTA as DMA^V using routine acid extractions.

Evidence from studies with human cells indicate that DMMTA is among the most toxic As compounds, even though it is a pentavalent species which are generally regarded less toxic due to their lower uptake and cellular accumulation.[70] Experiments with human hepatocarcinoma HepG2 cells showed DMMTA was about 10-fold more cytotoxic than DMA^V [71,72] and over 100-fold more

cytotoxic than DMDTA.[72] In experiments with human epidermoid carcinoma A431 cells, DMMTA showed cytotoxic effects in the same concentration range as arsenite and DMA^{III}, whereas arsenate and DMA^{V} showed cytotoxic effects at concentrations 2 orders of magnitude higher.[70] Cytotoxicity of different As species in human bladder cancer EJ-1 cells was shown to follow the order $DMA^{V} \approx MMA^{V} <$ MMMTA < Arsenate << Arsenite < DMMTA $\approx DMA^{III}$, with calculated half maximal inhibitory concentrations (IC_{50}) for arsenite, DMMTA and DMA^{III} of 75, 17, and 13 µmol L^{-1}, respectively.[73] Similarly, on the basis of 24-h IC_{50} values, the relative cytotoxicity of As species in human lung (A549) and bladder (T24) cells followed the order $MMA^{V} < DMA^{V} <$ DMDTA < Arsenate << Arsenite ≤ DMMTA $\approx DMA^{III}$, with IC_{50} values for DMMTA that were 3.7-fold lower than for arsenite and 300-fold lower than for DMA^{V}.[74] Furthermore, apart from cytotoxicity, DMMTA has shown to cause oxidative stress and DNA damage in EJ-1 cells.[73] In UROtsa human urinary bladder cells, DMMTA caused cell cycle distribution, genotoxicity, and DNA strand break induction in a similar and even lower concentration range than arsenite (e.g., DMMTA inhibition of DNA-damage induced poly(ADP-ribosyl)ation was caused at 35,000-fold lower incubation concentrations than arsenite).[75] In the case of inorganic thioarsenates, a study in human HepG2 and UROtsa cells found that MTA and TTA were both more cytotoxic than arsenate but less cytotoxic than arsenite.[76]

Taken together, these results show that DMMTA is nearly as cytotoxic as arsenite and is by far more cytotoxic than DMA^{V}. Food guidelines in Europe only regulate the content of iAs (Group 1 carcinogen) in rice grains and rice products. The limits are set to a maximum of 300 µg kg^{-1} iAs for rice waffles, wafers, crackers, cakes, flakes, and popped breakfast, 250 µg kg^{-1} for parboiled and husked rice and rice flour, 150 µg kg^{-1} for white rice, and 100 µg kg^{-1} for rice used in the production of food for infants and young children.[77] Therefore, misidentification of highly cytotoxic DMMTA as unregulated DMA^{V} in routine acid-based extractions poses a potential health risk.

Considering that (i) thioarsenates have been reported in paddy soil pore waters, (ii) their uptake by rice plants has been shown before, (iii) speciation studies have shown, at least for partially preserved DMMTA, accumulation in a few rice grain samples, and (iv) the elevated cytotoxicity of DMMTA, concerns about its toxic effects and behaviour in plants and its occurrence in rice grains and products were addressed on this thesis. While other thioarsenates were studied, the focus of this thesis remained on DMMTA based on its high cytotoxicity and relevance for food safety.

1.5. Objectives

The overall aim of this thesis was to investigate the metabolic fate of thioarsenates in rice plants and to address open questions regarding their occurrence in rice and relevance for food safety, with a

particular emphasis on DMMTA. The first study aimed to develop a method to detect thioarsenates in rice grains and products and subsequently, assess their occurrence in commercial samples. The latter revealed a particularly high accumulation of DMMTA in puffed rice cakes, therefore, the second study addressed the effect of puffing on the As speciation, focusing on the transformations between DMA^V, DMDTA, and DMMTA. Additionally, it aimed to evaluate the occurrence of DMMTA in generic and infant-labeled commercial puffed rice cakes. To obtain a better mechanistic understanding of DMMTA's path into the grains, studies 3 and 4 focused on the effect and behavior of DMMTA in plants. The third study investigated the toxicity of DMMTA for plants using the model plant *A. thaliana*. The fourth study investigated the interaction of DMMTA with rice seedlings and rice plants during the grain filling stage, focusing on DMMTA dethiolation and DMA^V thiolation processes occurring *in planta*. Finally, the widespread occurrence of DMMTA observed in the screening of commercial samples from study 1 and study 2, the observed toxicity and efficient translocation of DMMTA within plants from study 3, and the observed *in planta* thiolation of DMA^V from study 4, led to study 5. This study aimed to raise awareness of the potential risks associated with ingesting DMMTA-containing rice and addressed the need for a revision of existing regulatory limits for As.

The specific objectives of this thesis were to:

1. develop and validate an extraction and chromatographic separation method to quantify thioarsenates, especially DMMTA and MTA, in rice grains and products and determine their contribution to total As accumulation in commercial samples (study 1 and study 2).

2. study the modes of toxicity of DMMTA in plants using *A. thaliana* as a model system and assess the behavior (uptake, accumulation, transport, translocation, and transformation) of DMMTA in rice plants (study 3 and study 4).

3. explain how current As regulatory limits and analytical methods overlook the potential food safety threat of ingesting thioarsenate-containing rice (study 5).

2. Methods

2.1. General practices and analytical methods

Due to the focus of studies 1-4, either rice grains, puffed rice cakes, or plant tissues were analyzed for total As content and As speciation, measuring commonly investigated As species and thioarsenates. The most relevant methods will be described in the following. Further analytical details can be found in the materials and methods sections of the studies included in the appendix.

2.1.1. Sample preparation

Rice grain samples were homogenized by milling using an ultra-centrifugal mill (Retsch MM 2000) at 30 Hz for 2 minutes, pausing every 30 s, to prevent them from heating. Puffed rice cakes were homogenized by blending using a Ninja 2-in-1 Mixer (stainless steel blades and 1200-watt motor) for 1 minute, also pausing every 30 s. For As speciation analysis, all the samples were freshly homogenized before extraction to exclude changes due to storage. Thereafter, the milled rice and rice cake samples were stored at -20 °C.

After *A. thaliana* or rice seedling exposure treatments, root tissues were harvested and washed for 10 min in 1 mmol L^{-1} KH_2PO_4, 5 mmol L^{-1} $Ca(NO_3)_2$, and 5 mmol L^{-1} 2-(*N*-morpholino)ethanesulfonic acid buffer. Then, both the clean roots and shoots were immediately frozen and ground in liquid nitrogen, and stored at -80 °C. In the case of rice plants in the grain filling stage, after exposure, the flag leaves were also collected, immediately frozen, ground in liquid nitrogen, and stored at -80 °C until analysis. The rice panicles were harvested and oven-dried at 60 °C for 7 days. Afterwards, grains from the top and mid sections were dehusked using a palm husker (Mercer Corporation). The grains were treated as previously described, and the husks were frozen with liquid nitrogen, homogenized with pestle and mortar, and stored at -20 °C.

2.1.2. Arsenic measurements

To determine the total As content, the samples were digested in H_2O_2 and HNO_3 (5.6 mol L^{-1} H_2O_2 and 6.4 mol L^{-1} HNO_3 for grains, husks, and puffed rice cakes and 1.9 mol L^{-1} H_2O_2 and 4.5 mol L^{-1} HNO_3 for leaves, shoots, and roots) using a CEM Mars5 microwave digestion system (CEM Corp., Matthews, NC). The digests were filtered using 0.2 μm cellulose acetate filters before analysis. Total As content was analyzed by ICP-MS, with an 8900 ICP – QQQ (Agilent) or XSeries2 (Thermo-Fisher), using oxygen as reaction cell gas (AsO^+, *m/z* 75 → 91). Signal drift was corrected using rhodium (Rh^+, *m/z* 103) as internal standard. The extraction of As species from the different

samples is detailed in the sections of each study. However, after extraction, the As speciation was analyzed by IC-ICP-MS using a Dionex ICS-3000 (Thermo-Fisher) or a 940 Professional IC Vario (Metrohm). Unless stated otherwise, an AG/AS16 IonPac column (Dionex; 2.5 to 100 mmol L^{-1} NaOH, flow rate of 1.2 mL min^{-1}, and 50 µL injection volume) was used. Quantification of As was done as previously described. Peak identification was done by using commercial and synthesized standards or by comparison with retention times previously reported in studies using the same chromatographic conditions.[67,68] Reference materials, sample triplicates, and standard spikes were included in every analytical run to ensure data accuracy.

2.2. Thioarsenates in rice and puffed rice cakes (study 1 and study 2)

Study 1 aimed to develop an analytical method to detect thioarsenates, particularly DMMTA and MTA, in rice grains and products. The approach of the study was to test the stability of standard spikes in various extractants, on each step of the extraction procedures, and test their elution in different chromatographic conditions. Table 1 summarizes the different methods evaluated, which involved a routine acid extraction[78] and two adaptations of a pepsin-pancreatin enzymatic extraction using a saline solution or a phosphate buffer solution (PBS).[79] The development of the method was a central aspect of this study, therefore, the details concerning this particular methodology will be addressed in the result and discussion section (section 3.1).

Table 1. Extraction and chromatographic conditions evaluated for the detection of thioarsenates in rice grains and products.

	Method A	**Method B**	**Method C**
		Extraction conditions	
Extractant	0.28 mol L^{-1} HNO_3	Saline solution: 120 mmol L^{-1} NaCl + 5 mmol L^{-1} KCl	PBS: 2 mmol L^{-1} of NaH_2PO_4 + 0.2 mmol L^{-1} of Na_2-EDTA
Extraction conditions	90 min at 95 °C	1 h at 80 °C in MQ 1 h at 37 °C in pepsin, pH 2 2 h at 37 °C in pancreatin, pH 6	1 h at 80 °C in MQ 1 h at 37 °C in pepsin, pH 2 2 h at 37 °C in pancreatin, pH 6
		Chromatographic conditions	
Column	PRP-X100 (Hamilton)	PRP-X100 (Hamilton)	AG/AS16 IonPac (Dionex)
Eluent	10-40 mmol L^{-1} $NH_4H_2PO_4$ (gradient), pH 5.6	10-40 mmol L^{-1} $NH_4H_2PO_4$ + NH_4NO_3 (gradient), pH 8.2	2.5-100 mmol L^{-1} NaOH (gradient) + 2.4% methanol, pH 12-13
Flow rate	1.0 mL min^{-1}	1.0 mL min^{-1}	1.2 mL min^{-1}
Sample volume	50 µL	50 µL	50 µL

The outcome of this study was the development and validation of Method C. The applicability of this method was assessed using commercial rice samples and products. For this, 0.2 g of powdered sample was soaked in 3.2 mL deionized water and heated for 1 h at 80 °C (this cooking step was omitted for

products that were already cooked). Then, the samples were incubated at 37 °C for 1 h in 10 mL PBS (adjusted to pH 2 with 5 mol L^{-1} HCl) and 0.5 mL pepsin solution (125 g per L of 0.1 mol L^{-1} HCl; ≥ 250 units mg $solid^{-1}$). After that, 1 mol L^{-1} $NaHCO_3$ was added to raise the pH to 6, and 2.5 mL pancreatin solution (1.9 g per L of 0.1 mol L^{-1} $NaHCO_3$; 4 x USP specifications) was added. The samples were incubated at 37 °C for 2 h. The final mass of the solution was adjusted to 15 g with PBS (pH 6), the samples were filtered (0.45 µm hydrophilic Teflon filters), and the speciation was immediately analyzed. Finally, to show that acid-based extractions codetermine DMMTA as DMA^V, the 10 commercial samples with the highest DMMTA content were also extracted using Method A for speciation comparison.

After analysis of the commercial samples from study 1, it became clear that puffed rice cakes contained higher amounts of thioarsenates compared to the rice grain samples. Therefore, study 2 investigated the effect of puffing on the rice As speciation, focusing on the (trans)formations of DMA^V, DMMTA, and DMDTA. For this, the As speciation of rice samples before and after puffing was analyzed. The puffed rice cakes were prepared in collaboration with a private company following their method to produce commercial rice cakes. The manufacturing details are confidential, however, the method followed standard procedures to produce puffed rice cakes.[80,81] Upon arrival from the company, a representative subsample of each rice and puffed rice cake was homogenized, and the As speciation was analyzed. The enzymatic extraction procedure followed the methodology outlined in study 1 (Method C). After 3 months of storage in closed bags at room temperature, another subsample of puffed rice cakes was collected, homogenized, and As speciation was analyzed to study the stability of DMMTA and DMDTA over time. To assess the occurrence of thioarsenates in generic and infant-labeled puffed rice cakes, 80 commercial samples (52 generic and 28 infant-labeled) from the German market were analyzed using Method C. Again, to emphasize the limitation of acid extractions to detect DMMTA, these samples were also extracted using Method A for speciation comparison.

2.3. Thioarsenates in *A. thaliana* and rice tissues (study 3 and study 4)

2.3.1. Extraction of As species from plant tissues

For studies 3 and 4, an As species-preserving extraction method for plant tissues that preserves thioarsenates and does not cause the thiolation of oxyarsenic species (arsenite, MMA^V, and DMA^V) was developed. For this, the stability of DMMTA, arsenite, MMA^V, and DMA^V in the extracts from *A.thaliana* and rice roots and shoots was tested. For the extraction, ground plant material (0.01 to 0.08 g), glass beads (0.4 g), and 1.5 mL of PBS (pH 6.0) spiked with 0.67 µmol L^{-1} DMMTA, arsenite, MMA^V, or DMA^V were boiled for 5 min to denature enzymes and other proteins, cooled in an ice bath for 2 min, and then vortexed for 53 min under anaerobic conditions. The samples were filtered, and

As speciation was immediately analyzed as described in section 2.1.2. After confirming species stability, this method was used in studies 3 and 4 to extract As species from *A. thaliana* and rice tissues after exposure treatments.

2.3.2. Toxic effects of DMMTA in plants (study 3)

Study 3 focused on investigating the toxic effects of DMMTA using seedlings of the model plant *A. thaliana*. The seedlings were grown in hydroponic culture for 3-4 weeks using short day conditions (8 h light/16 h dark). For all the tests, exposure to the different As species was done hydroponically via the roots. The root growth inhibitory effect of DMMTA (0, 10, 25, and 50 μmol L^{-1}) was compared to that of arsenite (0, 10, 25, and 50 μmol L^{-1}) and DMA^V (0, 50, 200, 750, and 1000 μmol L^{-1}) using the wild-type Col-0, the phytochelatin synthases-deficient mutant (*cad1-3*), the *abcc1/2* mutant (lacking the two most important As-PC transporters), and the arsenate reductase-deficient *hac1* mutant exposed for 24 h. Furthermore, toxic effects were also assessed by comparing the cell integrity in root tips of Col-0 seedlings exposed for 4 h to 10 and 25 μmol L^{-1} DMMTA, arsenite, or DMA^V. Root-to-shoot translocation factors were studied by exposing Col-0 seedlings to 10 and 50 μmol L^{-1} DMMTA, arsenite, or DMA^V for 5 days. Additionally, the thiol profiles of the plants, GSH and PCs (PC2 and PC3), were measured after extraction with high-performance liquid chromatography (HPLC).[82] Finally, the impact of DMMTA on shoot development was evaluated after photometric analysis by comparing pigment concentrations (chlorophyll a and b, carotenoids, and anthocyanin) in Col-0 seedlings exposed for 24 h to 50 μmol L^{-1} DMMTA, arsenite, or DMA^V.

2.3.3. Behavior of DMMTA in plants (study 4)

Study 4 aimed to investigate the behavior of thioarsenates in rice plants. For this, Kitaake rice plants (ssp. *japonica*, rapid life cycle model cultivar) were grown in pots with soil under tropical greenhouse conditions. At 10 days post-anthesis (beginning of the grain filling stage), the plants were harvested and exposed via the stems, which were cut below the flag leaf node, for 48 h to 13.3 μmol L^{-1} DMMTA or DMA^V. After exposure, As speciation and total content were measured in the flag leaves, grains, and husks to assess species uptake, accumulation, and transformation ((de)thiolation). Furthermore, to study species transport, the same exposure experiment was done using stems that were previously jet-steamed to interrupt phloem flow. To assess DMMTA translocation from the flag leaf into the filling grains, intact plants were exposed for 7 days to 66.6 μmol L^{-1} DMMTA or DMA^V through the flag leaves. Arsenic speciation and total content were measured in the grains. For the transport and translocation tests, strontium (Sr) and rubidium (Rb) were added with a final concentration of 1 mmol L^{-1} to the nutrient solution as markers of xylem and phloem transport, respectively. Strontium (*m/z* 88, standard mode) and Rb (*m/z* 85, standard mode) contents were

determined by ICP-MS after microwave digestion as described in section 2.1.2. To further study *in planta* As thiolation, Kitaake rice seedlings were exposed to 13.3 μmol L^{-1} arsenite, MMA^V, or DMA^V for 24 h. Additionally, DMA^V thiolation was also tested in different rice seedling varieties (Baldo, Carnaroli, S.Andrea, and Arborio). For all rice seedlings tests, the plants were grown in hydroponic culture for 20 days using long day conditions (16 h light/ 8 h dark), As exposure was done hydroponically via the roots, and As speciation was measured in roots and shoots. To study the mechanism underlying DMA^V thiolation, *A. thaliana* exposure tests with DMMTA and DMA^V were conducted. First, to assess uptake and *in planta* transformations, wild-type Col-0 seedlings were exposed via the roots for 5 days to 10 μmol L^{-1} DMMTA or DMA^V. Furthermore, kinetics of DMA^V uptake and thiolation were studied in roots of Col-0 seedlings exposed to 100 μmol L^{-1} DMA^V for 0.5, 1, 2, 3, 6, 24, and 48 h. Finally, DMA^V uptake kinetics (100 μmol L^{-1} DMA^V for 3, 6, and 24 h) were also measured in roots of the GSH-deficient mutants *cad2-1* and *pad2-1*. All exposure treatments included control plants without As addition and As speciation of the nutrient solutions was always analyzed before and after exposure.

3. Results and Discussion

3.1. Thioarsenates in rice and puffed rice cakes (study 1 and study 2)

Inorganic and methylated thioarsenates have been detected in paddy soil pore waters[22,83,84] and their uptake by rice plants has been confirmed.[32,33] Interestingly, DMMTA had been tentatively identified in two rice grain samples at relatively high concentrations more than 15 years ago,[79] however, no follow-up studies were conducted. Therefore, the extent of DMMTA preservation during extraction procedures and its natural occurrence in rice remained unknown. To address this, we developed an analytical method for the speciation of As, that enables the determination of thioarsenates in rice grains and products, focusing on DMMTA and MTA (study 1).

Initially, the elution and stability of DMMTA and MTA standard spikes were tested in different methods (Table 1). The chromatographic conditions of Method A were not able to elute DMMTA or MTA. Thus, to test their stability in all the steps of the acid extraction of Method A (study 1, Figure 1), the chromatographic conditions of Method B were used instead. Both thioarsenates were completely transformed into their respective oxyarsenic species using the acid extraction method. Furthermore, the chromatographic conditions of Method B were also discarded after evaluating the detection limit and peak shape of different concentrations (0.25, 0.5, 0.75, and 1 µg L^{-1}) of DMMTA spikes (study 1, Figure 2). Thereafter, the combination of the extraction of Method B and chromatographic conditions of Method C were also discarded due to the poor peak separation obtained after evaluating the elution of 10 µg L^{-1} arsenite, arsenate, DMA^V, MMA^V, and DMMTA spiked into the saline solution (study 1, Figure S1).

For Method C, the stability of DMMTA and MTA spikes was also evaluated in every step of the extraction (study 1, Figure 1). With Method C, 11% of the spiked DMMTA and 55% of the spiked MTA transformed back to DMA^V and arsenite, respectively. Since MTA is less cytotoxic than arsenite,[76] misidentification of MTA as arsenite likely poses no additional health risks since iAs is regulated in most food guidelines (study 5, Table 1). However, misidentification of DMMTA as unregulated DMA^V could be critical. Therefore, the focus of this study and thesis was centered on DMMTA. Method C, a two-step enzymatic extraction that mimics the conditions in the human digestive system followed by chromatographic separation with 2.5-100 mmol L^{-1} of NaOH as eluent, was selected for further tests since it allowed for the differentiation between DMMTA and DMA^V.

DMMTA artifact formation during extraction was ruled out by the complete recovery of a 10 µg L^{-1} DMA^V spike added before the extraction (study 1, Figure 3). Furthermore, the contents of extracted

DMA^V, iAs, and sum of As species were all in agreement with the values reported for the rice-certified reference material ERM-BC211. The recovery of sum of As species of Method C was 100 ± 1%. Analysis of commercial samples (n = 54), which included rice grains and puffed rice cakes, showed accumulation of DMMTA, DMDTA, and MTA. Additionally, DTA was detected but only in the puffed rice cakes (study 1, Figure 4). Regarding occurrence, DMMTA was the most frequently encountered thioarsenate in the samples (n = 50) and had the highest contents (up to 26 μg kg^{-1}). Finally, the As speciation comparison between Method A and Method C clearly showed that acid-based extractions codetermine DMMTA as DMA^V (study 1, Figure 5), allowing highly cytotoxic DMMTA to escape regulation.

The commercial sample screening from study 1 revealed that puffed rice cakes had a particularly higher content of thioarsenates in comparison to the rice samples analyzed. Lacking the original rice used to produce the puffed rice cakes, it was unclear if the rice processing treatment was responsible for the observed increase of thioarsenates in the puffed rice cakes. To address this, we investigated As speciation changes caused by rice puffing and As speciation changes during the storage of puffed rice cakes (study 2).

When comparing the As speciation of rice samples and their respective puffed rice cakes (study 2, Figure 1A), it was evident that the puffing treatment considerably increased the thioarsenate content in the rice cakes. Most of the DMA^V present in the rice samples was transformed to DMMTA and DMDTA. Small amounts of MTA and DTA, products of iAs thiolation, were also detected in the rice cakes (study 2, Table S1). Rice grains are known to have high sulfur concentrations[85], and many of its sulfur-containing compounds (e.g., cysteine, methionine, and thiamine)[86] produce hydrogen sulfide when exposed to thermal treatments above 200 °C.[87–89] Treatments between 200 °C and 300 °C are typically encountered in the production of puffed rice.[80,81] When opening the bags containing the freshly produced rice cakes, a strong sulfuric odor was perceived. The latter can be associated with volatile compounds such as hydrogen sulfide or dimethyl sulfide, commonly found in the aroma of cooked rice.[90,91] Since reduced sulfur must be present to form thioarsenates,[67,92] thiolation of iAs, DMA^V, and DMMTA (further thiolated to DMDTA) can be explained by the presence of hydrogen sulfide originating from the thermal degradation of sulfur-containing compounds during rice puffing. Furthermore, the high positive correlation between the DMA^V content in rice and the DMMTA (r^2 = 0.88) and DMDTA (r^2 = 0.85) contents in the puffed rice cakes (study 2, Figure 1B) confirmed that there is a potential hidden risk when rice grains with high DMA^V contents (not considered by food guidelines) are used to manufacture puffed rice.

Arsenic speciation of the puffed rice cakes was re-analyzed after 3 months of storage because the sulfuric smell was no longer perceived. After storage, most of the DMDTA was dethiolated and

converted mainly to DMMTA and some DMA^V (study 2, Figure 2), likely because of the loss of excess hydrogen sulfide. These results suggest that, despite DMDTA having a lower cytotoxicity than DMMTA,[74] it is also relevant from a food safety perspective as it can be a precursor of DMMTA. Our results highlight the importance of further studying the manufacturing (e.g., type of rice, rice moisture, puffing temperature) and storage (e.g., temperature, duration, ventilation, type of packing) conditions of puffed rice cakes to better understand the thiolation mechanism, determine which parameters play a key role in the extent of thiolation, and potentially how to mitigate it.

The occurrence of thioarsenates in infant (n = 28) and generic (n = 52) puffed rice cakes was analyzed. Of the 80 samples, DMMTA was detected in 79 samples, ranging between 2 to 256 μg kg^{-1} and DMDTA was found in 77 samples ranging between 1 to 343 μg kg^{-1} (study 2, Figure 3). Other thioarsenates such as MTA, DTA, MMMTA, and MMDTA were also widely detected as minor species. All the infant-labeled samples contained DMMTA and DMDTA. Furthermore, the screening of commercial samples revealed that infants are potentially at greater risk if they are fed generic puffed rice cakes, not only on an iAs body weight dose basis but also the additional threat posed by the elevated contents of DMMTA (again, not regulated despite its higher cytotoxicity than arsenite[74]) and DMDTA (precursor of DMMTA upon dethiolation) found in these samples. Overall, the results from studies 1 and 2 helped to confirm the widespread occurrence of thioarsenates in rice and puffed rice cakes and raised the concern whether these species are bioaccessible as they were extracted employing an enzymatic procedure that mimics the gastric and intestinal digestion of humans.[93]

3.2. Toxic effects and behavior of DMMTA in plants (study 3 and study 4)

The ubiquitous presence of DMMTA in rice grains indicated plant uptake, translocation, and grain loading of this methylated thioarsenate. Therefore, we were interested in assessing how DMMTA interacts with plants. The approach of study 3 was to compare the effects caused by DMMTA in *A. thaliana* seedlings to those caused by arsenite and DMA^V, both of which have been previously shown to be toxic for plants.[94,95] First, a comparison of the root lengths of seedlings exposed to different concentrations of DMMTA (0 to 50 μmol L^{-1}) revealed a concentration-dependent growth inhibition, with nearly no growth observed at 50 μmol L^{-1} (study 3, Figure 1). Furthermore, when equivalent arsenite concentrations were used, less inhibitory effects were observed. For instance, at 10 μmol L^{-1}, arsenite caused a root growth inhibition lower than 50%, whereas DMMTA inhibition was about 80%. In the case of DMA^V, even at 1000 μmol L^{-1} root growth was observed. Root cell integrity tests revealed that arsenite and DMMTA caused different negative effects on the root cells. Arsenite markedly increased reactive oxygen species accumulation in the root cortex cells (study 3, Figure 2) while DMMTA induced root epidermal cell deformation (study 3, Figure 3). Again, even at high concentrations, DMA^V had a negligible effect on *A. thaliana* roots. These results suggested that,

DMMTA is more toxic than arsenite and far more toxic than DMA^V, not only for human cells[70,71,74] but also for plant cells.

Root-to-shoot translocation tests showed that total As accumulation in roots followed the order DMA^V < DMMTA << arsenite (study 3, Figure 5A). This accumulation trend could, to some extent, explain the rather low toxicity effects observed for the DMA^V-treated seedlings. Furthermore, a comparison of the thiol profile of the plants (study 3, Figure 5C) revealed that, as expected,[45] the arsenite-treated roots accumulated PCs, whereas no significant PC accumulation occurred in the roots exposed to DMA^V. The DMMTA-treated roots also exhibited no PC accumulation above background levels, however, they showed a slight increase in GSH concentrations, suggesting complexation of DMMTA by GSH as previously demonstrated in *Brassica oleracea*.[49] Total As accumulation in shoots followed the order $DMA^V \approx$ arsenite < DMMTA. Translocation factors, at both tested concentrations, were near 0.4 for DMMTA and DMA^V, while for arsenite, they were below 0.009. Our results indicated that both methylated As species are efficiently translocated. Interestingly, similar trends for DMMTA and DMA^V root uptake and shoot accumulation have been reported for rice seedlings.[33] Alongside our observations, this provides evidence that monocots (rice) and dicots (*A. thaliana*) have similar transport and translocation capacities for DMMTA and DMA^V.

The leaves of the DMMTA-treated plants presented severe alterations. After 24 hours of exposure to DMMTA, curling of the leaves was observed likely due to dehydration.[96] Leaf pigment measurements revealed that the contents of carotenoids and chlorophyll *a* and *b* decreased only in the DMMTA-treated plants, negatively impacting the photosynthetic efficiency of the plants.[97] Pigment concentrations in the arsenite- and DMA^V-treated plants did not differ from the control conditions (study 3, Figure 6). Furthermore, darkening of the leaves in the DMMTA-treated plants suggested the accumulation of anthocyanins, a common indicator of abiotic stress in plants.[97] Our results revealed higher mobility and stronger toxic effects of DMMTA in plant tissues compared to arsenite and DMA^V.

The observed toxicity and efficient translocation of DMMTA in *A. thaliana* from study 3 and the data confirming DMMTA accumulation in rice grains from study 1 raised serious concerns regarding food safety. Therefore, study 4 aimed to investigate the behavior of DMMTA in rice plants and its path to the grains.

First, we examined the accumulation of As in different tissues of rice panicles exposed to DMMTA or DMA^V during grain filling (study 4, Figure 1). The plants exposed to DMMTA exhibited higher total As content in both leaves and grains compared to the plants exposed to DMA^V. This accumulation trend is in line with previous findings in roots and shoots of rice seedlings,[33] suggesting that for rice

plants, DMMTA has a higher mobility than DMA^V regardless of the growth stage of the plant. Furthermore, As speciation of the DMMTA-exposed panicles revealed DMMTA accumulation in the flag leaf (8 ± 1% of the ΣAs species), grains (9.1 ± 0.6% of the ΣAs species), and husks (1.4 ± 0.2% of the ΣAs species), however, significant dethiolation to DMA^V was observed in all the tissues. DMMTA transport in the xylem was indirectly confirmed due to its presence in the husk, which is primarily fed via this vascular tissue.[47,48] DMMTA transport in the phloem was also confirmed due to its translocation from the leaves into the filling grains after exposing the plants to DMMTA via the flag leaf [21] (study 4, Figure S8). Disruption of phloem flow led to a decrease of over 70% in DMMTA grain content (study 4, Figure S9), indicating a higher contribution of phloem transport compared to xylem transport.

Surprisingly, the As speciation results of the panicles exposed to DMA^V also showed DMMTA accumulation in the flag leaf (6 ± 1% of the ΣAs species), grains (8.3 ± 0.5% of the ΣAs species), and husks (1.3 ± 0.2% of the ΣAs species) (study 4, Figure 1). Speciation changes in the exposure solution were ruled out (study 4, Figure S7), thus our results are the first analytical evidence for DMA^V *in planta* thiolation. Notably, for both the DMMTA- and DMA^V-exposed panicles, the percentage of DMMTA in the flag leaf, grains, and husks was not significantly different ($p > 0.06$ for the 3 comparisons). Additionally, further thiolation of DMMTA to DMDTA in the leaves and grains was observed in both treatments (DMMTA- and DMA^V-exposed panicles). The parameters determining the extent of (de)thiolation in the different plant tissues are yet to be identified.

Using 20-day-old seedlings of different rice varieties (study 4, Figure 2), we confirmed DMA^V *in planta* thiolation is a common process occurring in rice, even during the early stages of growth. DMMTA and DMDTA were detected in both the roots and shoots of all the varieties. Moreover, detection of MTA, DTA, and TTA in arsenite-exposed seedlings and detection of MMMTA in MMA^V-exposed seedlings (study 4, Figure 3) confirmed *in planta* thiolation of other relevant As species.

Finally, after confirming that DMA^V thiolation also occurs in the model plant *A. thaliana* (up to 35 ± 7% (DMMTA and DMDTA) in roots and shoots; study 4, Figure S12), kinetic studies in seedling roots (study 4, Figure 4) revealed that DMA^V thiolation occurs rapidly (thiolation was already detected at 0.5 h after exposure). By comparing our kinetic results with calculated values using rate constants for abiotic thiolation[98] (study 4, Table S5), we were able to show that the observed rapid *in planta* thiolation is not a purely abiotic process but also enzymatically catalyzed. Finally, since GSH is the most abundant free thiol compound in plant cells[99], we compared DMA^V thiolation percentages of wild-type (Col-0) *A. thaliana* seedlings to that of two GSH-deficient mutants (*cad2-1* and *pad2-1*). The observed DMA^V thiolation percentages had the same trend as the GSH content of the seedlings

(*pad2-1* < *cad2-1* < Col-0),[99,100] suggesting GSH concentration is a key parameter contributing to *in planta* As thiolation.

Studies 3 and 4 shed light on the risks associated with the presence of DMMTA for both, plants and humans. In the case of rice plants, the observed heightened toxicity of DMMTA compared to DMA^V, coupled with the confirmation of DMA^V *in planta* thiolation, raises the question whether DMA^V is indeed triggering straighthead disease, a well-known problem occurring in rice[95], or if the formation of DMMTA in plant tissues could also be involved. Furthermore, our results confirm that besides the formation of DMMTA in paddy soil pore waters, plants can also thiolate DMA^V after uptake and accumulate DMMTA in the grains, hence increasing the food safety concerns related to As exposure and rice.

3.3. Thioarsenate-containing rice: a potential food safety threat (study 5)

The prior studies of this thesis provided evidence that the presence of thioarsenates, especially DMMTA, in rice grains warrants attention. Study 5 aimed to discuss the challenges and relevance surrounding thioarsenates in food and summarize the current state of As regulations in rice worldwide.

This study addressed three primary challenges concerning thioarsenates in rice. First, the detection of thioarsenates require more effort than the detection of oxyAs species. Studies have shown that the detection of thioarsenates in rice is only possible when considering their stability during extraction (e.g. matrix, pH, temperature) and detection (e.g., eluent and column selection).[7,79,101,102] The required methods for detecting and quantifying thioarsenates, such as our Method C from study 1 (two-step enzymatic extraction followed by chromatographic separation with 2.5-100 mmol L^{-1} of NaOH), are more costly and time-consuming than commonly applied acid-based extractions[78] and speciation on strong anion exchange columns (e.g., Hamilton PRP X-100) with slightly acidic phosphate eluents.[38] Hence, using standard procedures, thioarsenates are transformed and misidentified as their corresponding oxyAs counterparts or are not quantitatively eluted, which ultimately leads to a lack of data on their occurrence in food.

Second, there is also a lack of information and therefore understanding of the toxic effects thioarsenates can cause in humans. Currently, no in vivo toxicity data exists for any of the thioarsenates. Lacking information on absorption, metabolism, excretion, modes of toxicity, occurrence in human, epidemiology, amongst other, hinders food safety authorities to assess the risk or provide recommendations regarding the ingestion of thioarsenate-containing rice. It is, however, noteworthy that cell line studies[70,73,74,103,104], have shown that DMMTA is far more cytotoxic than

DMA^V and even arsenite (study 5, Figure 1), the most harmful As species for human health considering exposure from food.[105] Therefore, addressing the relevance of DMMTA in rice is urgently needed.

Finally, the lack of information regarding the toxicity of thioarsenates, leads to the third main challenge, which is establishing regulation criteria for thioarsenates, mainly DMMTA, in rice. Worldwide, countries handle As regulations in rice differently (study 5, Table 1), however, most of them limit only iAs. The lack of regulation of DMA^V (directly as a species or indirectly as total As) creates a blind spot in food guidelines (study 5, Figure 2), allowing highly cytotoxic DMMTA to bypass regulations. One potential solution might involve revising food guidelines to limit total As content in rice, rather than specific species. In this case, over or underestimation of an As species exposure risk by misidentification would no longer be a problem, and in the end, this is a safer solution since all As species are cancer promoters.[106]

4. General conclusions and outlook

The aim of this thesis was to investigate the relevance of thioarsenates for rice plants and food safety. The interactions of thioarsenates with plants were studied, particularly addressing DMMTA toxicity using the model system *A. thaliana* and DMMTA accumulation and (trans)formation in different rice tissues. Occurrence of thioarsenates was investigated in commercial rice grains and products, and the results were put in context with cytotoxicity data and regulatory limits for As. The main outcomes from studies 1-5 are summarized as a conceptual model in Figure 6.

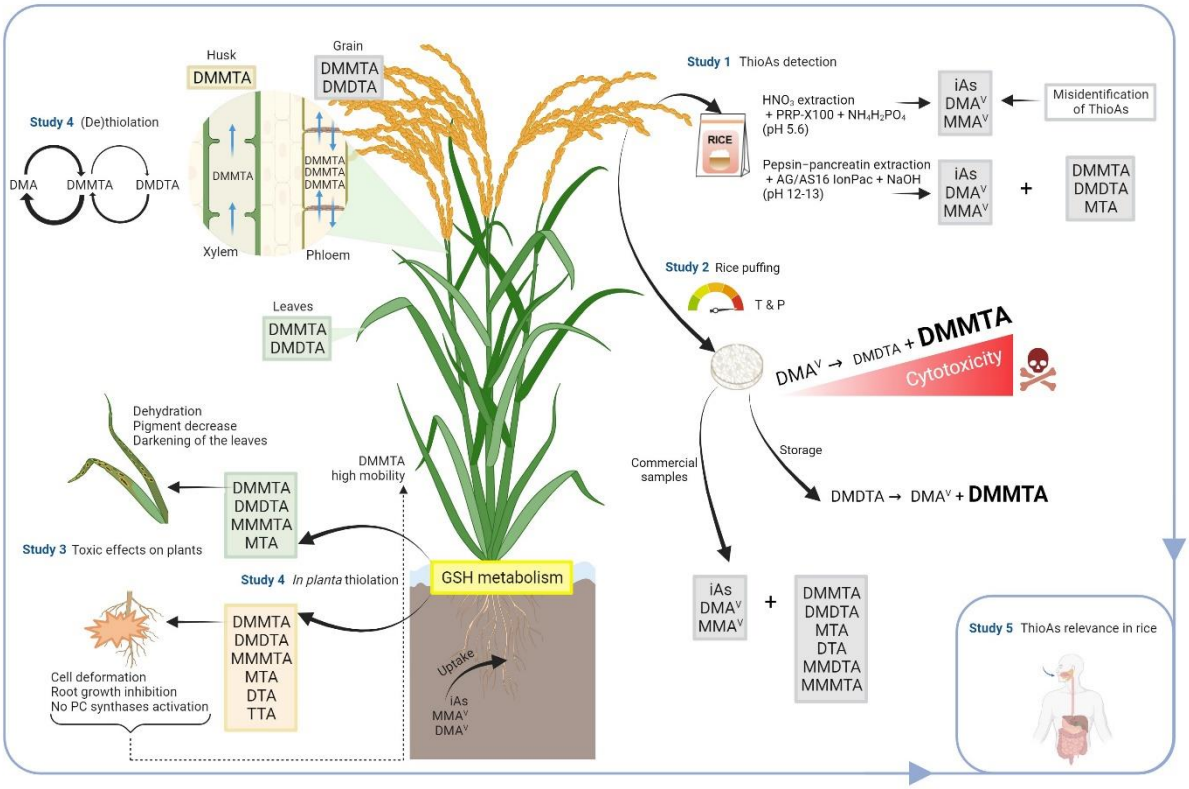

Figure 6. Conceptual model summarizing the main results from studies 1-5. T: temperature, P: pressure, and ThioAs: thioarsenates. Created with BioRender.com

Our research with *A. thaliana* (study 3) demonstrated strong toxic effects of DMMTA in plants. Significant root growth inhibition, deformation of root epidermal cells, and no activation of PC synthases were the primary toxic modes observed in the roots. Similarly, after DMMTA exposure the shoots displayed severe alterations such as dehydration, decrease in photosynthetic pigments, and darkening of the leaves, which ultimately lead to the arrest of shoot growth. Moreover, efficient DMMTA root-to-shoots transfer was observed. Altogether, our results indicate that the presence of DMMTA in soil pore water is a threat for plant health. In the case of rice, our findings raise concerns about DMMTA's potential negative effect on grain yield and mobility to the grains.

For the first time, *in planta* thiolation was observed in arsenite-, MMA^V-, and DMA^V-exposed rice seedlings (study 4). Furthermore, rice plants exposed to DMA^V during the grain filling stage accumulated DMDTA and especially, DMMTA in the leaves and grains. Hence, revealing pore water is not the only source for thioarsenate accumulation in grains. These results strongly suggest that currently unregulated DMA^V should be monitored as it is a precursor of highly toxic DMMTA in rice. Rice plants exposed to DMMTA during the grain filling stage also accumulated DMDTA and DMMTA, however, significant dethiolation to DMA^V occurred. The parameters determining the extent/ratio and kinetics of (de)thiolation in the different rice tissues need to be investigated, however, GSH concentration was shown to play a major role.

Many open questions regarding thioarsenates and rice plants need to be investigated, especially to design mitigation strategies to reduce accumulation in grains:

(1) Mobility inside rice plants: Which transporters are involved in thioarsenates root uptake, xylem and phloem loading, and passage across apoplastic barriers into the grains? Are there detoxification mechanisms for thioarsenates? Efflux, chelation (e.g., DMMTA-GS), storage in roots? Do nodes play an important role in the retention of thioarsenates?

Identification of transporters and detoxification mechanisms would contribute to engineering crop studies. Approaches such as overexpression of proteins mediating sequestration or efflux or the use of loss of function alleles (e.g., genes encoding uptake transporters) would help to overall reduce permeability and mobility of thioarsenates in plants.[23]

(2) Thiolation mechanism: Where is thiolation happening? What influences (de)thiolation ratios in different rice tissues? Do interactions between iAs-S increase thiolation due to generally higher GSH levels, or conversely, decrease thiolation as a result of iAs competing for GSH?

Our kinetic experiment suggested thiolation is not purely abiotic, therefore, investigating the enzyme(s) responsible for catalyzing thiolation is needed (section 3.2; study 4, Table S5). Investigating whether absolute DMA concentrations determine the extent of thiolation could help predict shares of DMMTA in the plants.

(3) Toxic effects in rice: Is DMMTA involved/causing straighthead disease? Do rice varieties have different thiolation capabilities? Is the observed susceptibility or resistance to straighthead disease from certain rice varieties correlated to their thiolation capabilities?

A study addressing DMA phytotoxicity in *A. thaliana* revealed that plants with lower GSH biosynthesis were less sensitive to DMA^V toxicity.[107] Taking into account our *in planta* thiolation results (study 4), the decrease in the toxic effect of DMA^V could be due to lower DMMTA production. Considering these findings and that rice varieties with higher levels of sulfur have

been shown to be more susceptible to straighthead disease,[108] it is worth to assess the potential involvement of DMMTA.

(4) Food safety: Where in the grain is DMMTA distributed (rice bran, OVT, or endosperm?)?

While studying distribution at field relevant concentrations is likely not possible, it might be interesting to assess whether DMMTA permeation in the grain is dose dependent. Arsenate distribution in the grain was shown to be dose dependent with most of the As retained in the OVT when low arsenate concentrations were fed to the plants, while higher As permeation to the grain was observed when using higher arsenate concentrations.[109] However, similar As distribution in the grains have been reported in studies with rice that was dosed with high (133 µmol L^{-1} and 13.3 µmol L^{-1}) and low (8 µmol L^{-1} and 5 µmol L^{-1}) arsenite and DMA^V concentrations, respectively.[20,57]

For the detection of thioarsenates in rice grains and products, we developed an analytical method for As speciation using a pepsin-pancreatin enzymatic extraction followed by chromatographic separation at pH 12-13 (study 1). Analysis of commercial samples showed that thioarsenates, namely DMMTA, DMDTA, and MTA can accumulate in the grains and that puffed rice cakes had a particularly high content of thioarsenates. A further screening of commercial puffed rice cakes confirmed widespread occurrence of thioarsenates, with DMMTA and DMDTA contributing sometimes even more than iAs to total As content, and MTA, DTA, MMMTA, and MMDTA as minor yet widely present species (study 2). The particular accumulation of thioarsenates in puffed rice cakes was shown to be a result of the manufacturing process. Furthermore, species were shown to transform during storage, with most of the DMDTA being dethiolated mainly to DMMTA and some DMA^V. The (post)production conditions responsible for the net thiolation in the rice cakes are not known. Finally, comparison between the As speciation of our method and an acid-based extraction and chromatographic separation revealed the latter is not suitable for the detection of thioarsenates. From a food safety perspective, based on cytotoxicity data, the misidentification of DMMTA as DMA^V could be problematic. Given the ubiquitous detection of thioarsenates in commercial samples, studies are needed to estimate the relevance of their accumulation in rice grains and products (study 5).

Open questions remain regarding thioarsenate formation during food processing and their relevance for food safety:

(1) Rice puffing: What is the exact mechanism underlying the thiolation process during rice puffing?

We confirmed that processing rice at high temperatures enhanced thiolation (study 2). Reduced sulfur is needed to form thioarsenates. After puffing, the rice cakes had a strong sulfuric odor,

originating from the thermal decomposition of S-compounds present in rice (thiamine, methionine, cysteine?), however, the mechanism of thiolation is not yet understood.

(2) Manufacturing: Which food processing parameters determine thiolation of DMA^V during puffing? Do post-processing methods affect thiolation? Can storage conditions be optimized to completely dethiolate?

Several parameters such as raw material (type of rice), rinsing/soaking rice, moisture, temperature, and pressure can be tested to optimize a puffing method with lower thiolation. Furthermore, post-processing methods like drying for preservation, ventilation, storage at warmer/cooler temperatures, and consumer practices (e.g., reheating the rice cakes) can influence dethiolation of DMDTA and DMMTA.

(3) Food safety: Is ingesting thioarsenates-containing rice a risk for human health?

Currently, there are still many open questions regarding occurrence, metabolism, and toxicity of thioarsenates. Fortunately, due to emerging evidence (study 1, study 2, and others[83,102,110]) on the widespread occurrence of DMMTA in food, authorities in Europe have started to address this potential risk. The European Food Safety Authority (EFSA) launched in March 2023 a call for the collection of contaminants occurrence data in food to begin risk assessments.[111] Amongst other substances, the priority list included: "*Organic arsenic [thiolated compounds (DMMTA and MMMTA)] and others (MMA, DMA, arsenolipids, arsenosugars, etc.)*". Furthermore, on the 137th Plenary meeting of the Panel on Contaminants in the Food Chain (CONTAM) held on November 2023 (open for observers), the approach for developing the draft risk assessment on small organoarsenic species in food, DMMTA included, was discussed by the chair of the working group on As in food.[112] During this session, our publication from study 2 (Widespread occurrence of dimethylmonothioarsenate (DMMTA) in rice cakes: effects of puffing and storage) and from study 5 (Dimethylated Thioarsenates: A Potentially Dangerous Blind Spot in Current Worldwide Regulatory Limits for Arsenic in Rice) were mentioned as primary motivators for their recent interest in thioarsenates in food. The 138th (January 2024) and 139th (March 2024) Plenary meetings of the CONTAM will include results on their findings on these small organoarsenic species (occurrence, biomarkers, toxicity, epidemiology, and hazard characterization), which will culminate in a sound risk assessment opinion and subsequent recommendations.

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Contribution to Studies 1-5

STUDY 1: Detection of thioarsenates in rice grains and rice products

Andrea E. Colina Blanco	80%	development of research concept, laboratory work, analyses and data interpretation, preparation of manuscript
Carolin F. Kerl	10%	development of research concept, discussion of results, comments on manuscript
Britta Planer-Friedrich	10%	development of research concept, discussion of results, comments on manuscript

STUDY 2: Widespread occurrence of dimethylmonothioarsenate (DMMTA) in rice cakes: effects of puffing and storage

Andrea E. Colina Blanco	85%	development of research concept, laboratory work, analyses and data interpretation, preparation of manuscript
Alejandra Higa Mori	5%	assistance with laboratory work, comments on manuscript
Britta Planer-Friedrich	10%	development of research concept, discussion of results, comments on manuscript

STUDY 3: Dimethylmonothioarsenate Is Highly Toxic for Plants and Readily Translocated to Shoots

Erik Pischke	35%	development of research concept, laboratory work, analysis and data interpretation, comments on manuscript
Fabrizio Barozzi	35%	development of research concept, laboratory work, analysis and data interpretation, comments on manuscript
Andrea E. Colina Blanco	10%	laboratory work and data analysis, discussion of results, comments on manuscript
Carolin F. Kerl	5%	laboratory work and data analysis, discussion of results, comments on manuscript
Britta Planer-Friedrich	5%	discussion of results, comments on manuscript
Stephan Clemens	10%	development of research concept, discussion of results, writing of manuscript

STUDY 4: *In Planta* Arsenic Thiolation in Rice and *Arabidopsis thaliana*

Andrea E. Colina Blanco	35%	development of research concept, laboratory work, analyses and data interpretation, preparation of manuscript
Erik Pischke	35%	development of research concept, laboratory work, analyses and data interpretation
Alejandra Higa Mori	10%	assistance with laboratory work, comments on manuscript
Carolin F. Kerl	5%	assistance with laboratory work, comments on manuscript
Stephan Clemens	5%	development of research concept, discussion of results, comments on manuscript
Britta Planer-Friedrich	10%	development of research concept, discussion of results, comments on manuscript

STUDY 5: Dimethylated Thioarsenates: A Potentially Dangerous Blind Spot in Current Worldwide Regulatory Limits for Arsenic in Rice

Britta Planer-Friedrich	60%	development of research concept and preparation of manuscript
Carolin F. Kerl	15%	contribution to manuscript writing and comments on manuscript
Andrea E. Colina Blanco	15%	contribution to manuscript writing and comments on manuscript
Stephan Clemens	10%	assistance with development of research concept, comments on manuscript

Appendix: Studies 1-5

STUDY 1

Colina Blanco, A. E.; Kerl, C. F.; Planer-Friedrich, B. Detection of Thioarsenates in Rice Grains and Rice Products. *J. Agric. Food Chem.* **2021**, *69* (7), 2287–2294. https://doi.org/10.1021/acs.jafc.0c06853.

STUDY 2

Colina Blanco, A.E.; Higa Mori, A.; Planer-Friedrich, B. Widespread occurrence of dimethylmonothioarsenate (DMMTA) in rice cakes: effects of puffing and storage. *Food Chem.* **2024**, *436* (2024), 137723. https://doi.org/10.1016/j.foodchem.2023.137723

STUDY 3

Pischke, E.; Barozzi, F.; Colina Blanco, A. E.; Kerl, C. F.; Planer-Friedrich, B.; Clemens, S. Dimethylmonothioarsenate Is Highly Toxic for Plants and Readily Translocated to Shoots. *Environ. Sci. Technol.* **2022**, *56* (14), 10072–10083. https://doi.org/10.1021/acs.est.2c01206.

STUDY 4

Colina Blanco, A.E.; Pischke, E.; Higa Mori, A.; Kerl, C.F.; Clemens, S.; Planer-Friedrich, B. *In Planta* Arsenic Thiolation in Rice and *Arabidopsis thaliana*. *Environ. Sci. Technol.* **2023**, *57* (51), 21846- 21854. https://doi.org/10.1021/acs.est.3c06603.

STUDY 5

Planer-Friedrich, B.; Kerl, C. F.; Colina Blanco, A. E.; Clemens, S. Dimethylated Thioarsenates: A Potentially Dangerous Blind Spot in Current Worldwide Regulatory Limits for Arsenic in Rice. *J. Agric. Food Chem.* **2022**, *70* (31), 9610–9618. https://doi.org/10.1021/acs.jafc.2c02425.

Study 1: Detection of Thioarsenates in Rice Grains and Rice Products

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Detection of Thioarsenates in Rice Grains and Rice Products

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ABSTRACT: Inorganic and methylated thioarsenates have recently been reported to contribute substantially to arsenic (As) speciation in paddy-soil pore waters. Here, we show that thioarsenates can also accumulate in rice grains and rice products. For their detection, a method was developed using a pepsin−pancreatin enzymatic extraction followed by chromatographic separation at pH 13. From 54 analyzed commercial samples, including white, parboiled and husked rice, puffed rice cakes, and rice flakes, 50 contained dimethylmonothioarsenate (DMMTA) (maximum 25.6 $\mu g\ kg^{-1}$), 18 monothioarsenate (MTA) (maximum 5.6 $\mu g\ kg^{-1}$), 14 dimethyldithioarsenate (DMDTA) (maximum 2.8 $\mu g\ kg^{-1}$), and 5 dithioarsenate (DTA) (maximum 2.3 $\mu g\ kg^{-1}$). Additionally, we show that the commonly used nitric acid extraction transforms MTA to arsenite and DMMTA and DMDTA to dimethylarsenate (DMA). Current food guidelines do not require an analysis of thioarsenates in rice and only limit the contents of inorganic oxyarsenic species (including acid-extraction-transformed MTA), but not DMA (including acid-extraction-transformed DMMTA and DMDTA).

KEYWORDS: *rice grains, rice products, arsenic speciation, enzymatic extraction, thioarsenates*

INTRODUCTION

Rice is a dietary staple for half of the world's population. However, it has also been identified as one of the main dietary sources of inorganic arsenic (As), a class 1 carcinogen.[1] Rice is typically grown under flooded conditions which mobilizes naturally occurring As from the soil due to reductive dissolution of Fe(III) (oxyhydr)oxides.[1] Rhizosphere microorganisms can methylate bioavailable inorganic As (iAs: arsenite and arsenate) and form methylated oxyarsenates, such as monomethylarsenate (MMA) and dimethylarsenate (DMA).[2] Methylated oxyarsenates are also cancer promoters but are considered less toxic than iAs.[3−6] Food guidelines in Europe only regulate the content of iAs in rice grains and rice products.[7] The limits are a maximum of 300 $\mu g\ kg^{-1}$ iAs for rice waffles, wafers, crackers, and cakes, 250 $\mu g\ kg^{-1}$ for parboiled and husked rice, 200 $\mu g\ kg^{-1}$ for white rice, and 100 $\mu g\ kg^{-1}$ for rice used in the production of baby foods.[7]

A recent study has detected, that besides the known oxyAs species, also inorganic and methylated thioarsenates are present in paddy soil pore waters.[8] Inorganic thioarsenates (mono(MTA), di(DTA), and trithioarsenate (TTA)) form under reducing conditions, at near-neutral to alkaline pH, from arsenite, zerovalent sulfur, and sulfide.[9] Methylated thioarsenates (monomethylmonothioarsenate (MMMTA), monomethyldithioarsenate (MMDTA), dimethylmonothioarsenate (DMMTA), and dimethyldithioarsenate (DMDTA)) form under acidic pH conditions by the nucleophilic attack of sulfide on MMA or DMA.[10] These species can contribute up to 19% of total As according to rice cultivation mesocosm experiments, and their contribution can increase significantly upon sulfate fertilization.[8]

Uptake of thioarsenates by rice plants has been shown before.[11,12] The presence of MTA in rice roots and shoots has been confirmed by direct analytical evidence,[11] and hydroponic experiments have demonstrated that MTA,[11] MMMTA,[12] and DMMTA[12] can reach the xylem of rice plants. In rice grains, As speciation is dominated by iAs and DMA.[13] Even though the DMA contribution to the total As in pore water typically is low,[13] DMA can contribute up to 90% to total As in grains because it is translocated with greater efficiency in comparison to iAs.[14−16] Hydroponic experiments have revealed that the root to shoot translocation factors of MTA and DMMTA are on the same order of magnitude as those of DMA,[11,12] suggesting that these species can potentially contribute to As accumulation in rice grains.

Detection of thioarsenates in rice grains is only possible when their limited stability during extraction (dependent on, e.g., pH, temperature, and rice matrix) and detection (dependent on, e.g., the chromatographic column and the eluent) is accounted for. For instance, extraction in nitric acid was shown to transform DMMTA to DMA in a rice grain speciation study,[17] and the widely used As speciation on strong anion exchange columns (e.g., Hamilton PRP X-100) with slightly acidic phosphate eluents is not strong enough to elute DMMTA.[18] Thus, routine acid-based grain extractions and chromatographic separations are not suitable for DMMTA detection.

In 2005, DMMTA was identified in two rice samples (accounting for 13 and 20% of total As in the grain) using a two-step enzymatic extraction that mimics cooked rice entering the human digestive tract (stomach and small intestine).[19] The

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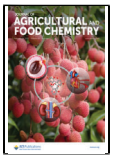

Table 1. Extraction and Chromatographic Conditions Tested for DMMTA and MTA Detection in Rice Grains and Rice Products

	method A	method B	method C
		Extraction Conditions	
extractant	0.28 mol L^{-1} HNO_3	enzymatic extraction using saline solution	enzymatic extraction using PBS
preserved species[a]	DMA	DMA	DMA
	MMA	MMA	MMA
	iAs	iAs	iAs[c]
		DMMTA[b]	DMMTA (11% transformation)
			DMDTA
			MTA (55% transformation)
			DTA[b]
		Chromatographic Conditions	
column	PRP-X100 (Hamilton)	PRP-X100 (Hamilton)	AG/AS16 IonPac (Dionex)
eluent	10−40 mmol L^{-1} $NH_4H_2PO_4$ (gradient), pH 5.6	10−40 mmol L^{-1} $NH_4H_2PO_4$ + NH_4NO_3 (gradient), pH 8.2	2.5−100 mmol L^{-1} NaOH (gradient) + 2.4% methanol, pH 12−13
flow rate	1.0 mL min^{-1}	1.0 mL min^{-1}	1.2 mL min^{-1}
sample volume	50 μL	50 μL	50 μL
eluted species[a]	DMA	DMA	DMA
	MMA	MMA	MMA
	iAs	iAs	iAs
		DMMTA	DMMTA
		MTA	DMDTA
			MTA
			DTA

[a]Only listing species of interest in this study. [b]Only identified (stability not evaluated). [c]Share of arsenate + arsenite.

fact that DMMTA was only detected in two samples at relatively high concentrations (40 and 46 μg kg^{-1}) might be attributable to the use of $(NH_4)_2CO_3$ as the chromatographic eluent, which produced relatively broad and flat DMMTA peaks. DMMTA was also observed in a later rice grain survey using enzymatic extraction.[17] The observed concentrations were lower (5−19 μg kg^{-1}), but since DMMTA recovery was not tested, the extent of transformation to DMA remained unclear (the authors state "at least a partial preservation" for DMMTA). Differentiation of DMMTA from DMA is important because DMMTA has been shown to be about 10 times more cytotoxic than DMA in human HepG2 cells[20] and up to 7 times more cytotoxic than arsenite in human bladder cancer EJ-1 cells.[21] To the best of our knowledge, no study has reported the presence of MTA in rice grains; thus, its stability in grain extracts (acid or enzymatic) has yet not been studied. A toxicity study in human cells (HepG2 and UROtsa cells) showed that MTA is more cytotoxic than arsenate but less cytotoxic than arsenite.[22]

The aim of the present study was to show that thioarsenates, especially DMMTA and MTA, contribute to the total As in rice and demonstrate that they are overlooked by acid-based extractions. For thioarsenate detection, we modified and validated an enzymatic extraction method and the chromatographic conditions for ion chromatography coupled to inductively coupled plasma mass spectrometry (IC-ICP-MS) measurements. The applicability of the modified method was tested using commercial rice grains and rice products. To demonstrate the shortcomings of currently used acid-based extractions and the advantage of our method, a comparison with routine nitric acid extraction was done.

MATERIALS AND METHODS

Chemicals and Standards. Dimethylarsinic acid ($(CH_3)_2AsNaO_2·3H_2O$), pepsin from porcine gastric mucosa, and pancreatin from porcine pancreas were purchased from Sigma-Aldrich Chemical Co. (Steinheim, Germany). Sodium sulfide nonahydrate ($Na_2S·9H_2O$) was supplied by Acros Organics (Geel, Belgium), and arsenic trioxide (As_2O_3) was supplied by Alfa Aesar (Karlsruhe, Germany). Arsenite ($NaAsO_2$) and arsenate ($Na_2HAsO_4·7H_2O$) were purchased from Fluka Analytical, Sigma-Aldrich Chemical Co. (Spain), and MMA ($CH_3AsNa_2O_3·6H_2O$) was obtained from Chem Service (West Chester, PA).

DMMTA ($(CH_3)_2AsS(OH)$) was synthesized according to Lee et al.[23] Briefly, DMA and $Na_2S·9H_2O$ solutions in N_2-purged deionized water (MQ) were mixed under anoxic conditions and then acidified with concentrated sulfuric acid to achieve a final molar ratio of As:S:H_2SO_4 = 1:1.6:1.6. After 1 h, the synthesized DMMTA was separated using a liquid−liquid extraction with diethyl ether. The solvent was evaporated with a constant flow of N_2. The synthesized DMMTA was stored anoxically at 4 °C in the dark and was analyzed by ion chromatography coupled to inductively coupled plasma mass spectrometry (IC-ICP-MS) (purity of 91%; 7% DMDTA and 2% DMA). MTA ($Na_3AsO_3S·2H_2O$) was synthesized according to previous literature methods.[24,25] Briefly, elemental sulfur was mixed with arsenic trioxide and sodium hydroxide to achieve a final molar ratio of As:S:NaOH = 1.1:1:3.3 in MQ. The solution was heated to 65 °C for 2 h and filtered, and the remaining solvent was removed under vacuum (purity of 98.5%; 0.5% arsenite and 1% arsenate). Arsenic species were identified by IC-ICP-MS using the chromatographic conditions of method C (see next section and Table 1).

Development and Test of Conditions for Extraction and Chromatographic Separation. Three different methods were tested to extract and detect DMMTA and MTA (methods A−C). The methods are summarized in Table 1, together with information on which species each extraction method can preserve and which species each chromatographic condition can elute.

Method A was a commonly applied acid-based extraction.[26] Briefly, 1 g of milled rice was mixed with 0.28 mol L^{-1} of HNO_3, digested in a heating block at 95 °C for 90 min, filtered (0.45 μm hydrophilic Teflon filters), and analyzed immediately by IC-ICP-MS. Samples were separated using a PRP-X100 column (Hamilton, using 10−40 mmol L^{-1} of $NH_4H_2PO_4$ eluent, gradient elution, pH 5.6, a flow rate of 1.0 mL min^{-1}, and 50 μL injection volume).[27]

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For method B, an enzymatic extraction that had been described before[19] to partially preserve DMMTA in rice grains was used. Briefly, 1 g of milled rice was mixed with 3.2 mL of deionized water and heated for 1 h at 80 °C. The formed paste was digested in 10 mL of a saline solution (120 mmol L^{-1} of NaCl + 5 mmol L^{-1} of KCl, pH 2) and 0.5 mL of pepsin solution (25 g L^{-1}). The samples were incubated for 1 h at 37 °C. Afterward, 1 mol L^{-1} of $NaHCO_3$ was added to raise the pH to 6. Finally, 2.5 mL of pancreatin solution (1.9 g L^{-1}) was added and the samples were incubated for 2 h at 37 °C. The final mass of the solution was adjusted with saline solution to 15 g, and the samples were filtered. Arsenic species were analyzed immediately by IC-ICP-MS using an ammonium phosphate/nitrate eluent that had been used for DMMTA detection in enzymatic extraction samples before;[17] however, we directly applied a gradient elution to obtain narrower chromatographic peaks (PRP-X100 column, Hamilton, using a 10−40 mmol L^{-1} of $NH_4H_2PO_4$ + NH_4NO_3 eluent, gradient elution, pH 8.2, a flow rate of 1.0 mL min^{-1}, and 50 μL injection volume).

Method C used the same enzymatic extraction as method B with the exception that the saline solution was replaced by a phosphate buffer solution (PBS, 2 mmol L^{-1} of NaH_2PO_4 + 0.2 mmol L^{-1} of Na_2-EDTA, pH 2) to reduce the salt load and because the stability of MTA[11] and DMMTA[12] in PBS had previously been confirmed. For chromatographic separation, a method that had previously been developed to separate inorganic and methylated thioarsenates in aqueous samples was applied,[28] using an AG/AS16 IonPac column (Dionex; 2.5−100 mmol L^{-1} of NaOH + 2.4% methanol eluent, gradient elution, a flow rate of 1.2 mL·min^{-1}, and 50 μL injection volume). To show the effect of changing the extractant (PBS instead of the saline solution) and column (AS16 instead of PRP-X100) on the DMMTA chromatographic peak shape, one rice sample was extracted with both method B and method C for comparison. After extraction, 1 μg L^{-1} of DMMTA was spiked to the samples to evaluate the effect of the new matrix and chromatographic conditions.

To confirm the natural occurrence of DMMTA in the samples and exclude artifact formation, transformation of DMA (the DMMTA precursor) into DMMTA during the extraction had to be ruled out. For this, a rice sample was spiked before the extraction with 10 μg L^{-1} of DMA. Full recovery of the DMA spike and no detection of additional DMMTA were the criteria used to rule out DMMTA artifact formation when method C was used. The extraction efficiency of method C was tested using the rice certified reference material ERM-BC211.

The stabilities of MTA and DMMTA upon extraction were tested for all steps of the HNO_3 extraction (method A) and the enzymatic extraction (method C). For this, 10 μg L^{-1} of MTA and 20 μg L^{-1} of DMMTA were spiked in (1) MQ, (2) HNO_3 0.28 mol L^{-1}, (3) HNO_3 0.28 mol L^{-1}, and heating at 95 °C (90 min), and (4) HNO_3 0.28 mol L^{-1}, heating at 95 °C (90 min), and the rice sample. For analyses of these samples, the chromatographic conditions of method B were used, since MTA and DMMTA would not elute under the chromatographic conditions of method A. Further, 10 μg L^{-1} of MTA and 20 μg L^{-1} of DMMTA were spiked in (1) MQ, (2) PBS, (3) PBS with the enzymes (pepsin and pancreatin), (4) PBS with the enzymes (pepsin and pancreatin) and heating at 80 °C (1 h) and 37 °C (3 h), and (5) PBS with the enzymes (pepsin and pancreatin), heating at 80 °C (1 h) and 37 °C (3 h), and the rice sample and samples were analyzed using the chromatographic conditions of method C.

Analysis of Commercial Rice Grains and Rice Products. To test the applicability of method C, As speciation was determined for 54 commercially available samples purchased in Costa Rica, Germany, and the United States. The sample types included white rice (n = 22), parboiled rice (n = 13), husked rice (n = 8), puffed rice cakes (n = 6), and other (n = 5; rice flakes, wild rice, black rice, a mix of husked, red, and black rice, and a mix of husked rice, black barley, and daikon radish seeds).

To demonstrate that acid-based grain extractions lead to codetermination of DMMTA as DMA, 10 samples with the highest DMMTA concentrations were analyzed to compare the results obtained by method A (routine HNO_3 extraction), method C (modified enzymatic extraction), and a total As determination. To rule out speciation changes due to storage, the samples were freshly milled before extraction and analysis (they will be referred to as replicates A_C). To measure the total As content, 0.2 g of milled rice was digested in 30% H_2O_2 and 65% HNO_3 (ratio 4:3) in a CEM Mars 5 microwave digestion system (CEM Corp., Matthews, NC), and analyzed by ICP-MS.

IC-ICP-MS Conditions. For all methods, As speciation was analyzed by IC (Dionex ICS-3000) coupled to ICP-MS (XSeries2, Thermo-Fisher) using oxygen as the reaction cell gas for As determination (AsO^+, m/z 91). DMMTA and MTA were identified using the synthesized standards. Arsenite, arsenate, DMA, and MMA were identified using commercial standards. DMDTA and DTA were identified by comparison with retention times previously reported in the literature (also using the chromatographic conditions of method C).[28,29] For method C, the recovery of arsenate (106 ± 7%), arsenite (98 ± 10%), DMA (99 ± 3%), and MMA (103 ± 3%) was evaluated by spiking every 15th sample with 5 and 10 μg L^{-1} of each species. Additionally, a 5 μg L^{-1} calibration standard was measured after every 15th sample to account for instrument signal drift. The limit of detection (LOD) was defined as the concentration providing a signal to noise ratio of 3: DMMTA 0.40 μg kg^{-1}, DMDTA 0.41 μg kg^{-1}, MTA 0.37 μg kg^{-1}, and DTA 0.43 μg kg^{-1}.

RESULTS AND DISCUSSION

Comparison of MTA and DMMTA Stability in Acidic vs Enzymatic Extraction Matrices. For a comparison with routine acid-based extractions, a method using HNO_3 was selected because it can preserve arsenate, arsenite, DMA, and MMA[26] and does not contain additional oxidizing agents (i.e., H_2O_2) that would further contribute to thioarsenate transformation.[30] Figure 1 shows that neither MTA nor DMMTA were stable upon extraction in HNO_3.

Monothioarsenate is the most stable species among the inorganic thioarsenates upon acidification,[31] and it was also detected under high-temperature−low-pH conditions in geothermal waters before.[29] However, it has not yet been detected in rice grains using the HNO_3 extraction. The results of our stability tests (Figure 1) show that the MTA-MQ spike originally contained 97% of MTA. In the presence of HNO_3, the MTA percentage decreased to 79% and, once the sample was heated to 95 °C, the percentage decreased to 58%. When MTA was spiked in a rice sample before the HNO_3 extraction, it was almost completely transformed to iAs (94%). The (partial) stability under acidic conditions matches previous reports;[29,31] however, the reason for the almost complete transformation in the presence of the starch matrix in the rice extraction is currently unknown. From a toxicological point of view, the transformation of MTA and misidentification as iAs probably is not critical, since iAs is taken into account by food guidelines and the MTA toxicity is lower than that of arsenite.[22]

For DMMTA, poor stability under acidic conditions has already been briefly mentioned in previous studies.[17,19] The results of our stability tests (Figure 1) show that the DMMTA-MQ spike originally contained 94% of DMMTA. In the presence of HNO_3, the DMMTA percentage decreased to 74% and, once the sample was heated up to 95 °C, the percentage decreased to 7%. When DMMTA was spiked in a rice sample before the HNO_3 extraction, it was transformed to DMA. These results show that DMMTA is codetermined as DMA by acid-based extractions. From a toxicological point of view, the transformation of DMMTA and misidentification as DMA could be critical because DMA is not considered in food

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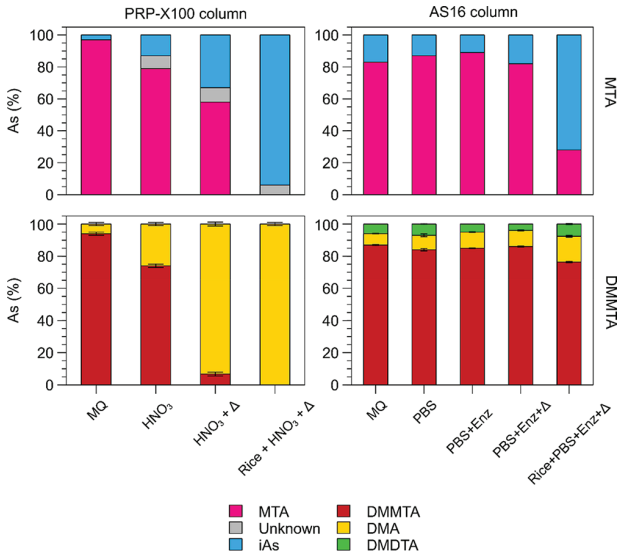

Figure 1. MTA (top row) and DMMTA (bottom row) stability in HNO_3 (method A extraction). Left column: spikes measured with a PRP-X100 column (because chromatographic conditions of method A were not suitable for eluting MTA or DMMTA, method B was used instead). All samples were extracted for 90 min (Δ = 95 °C). Right column: MTA and DMMTA stability in PBS (method C): spikes measured with an AS16 column. All samples were extracted for 4 h (Δ = 80 °C for 1 h and 37 °C for 3 h). Two different MTA standards were used for the PRP-X100 and AS16 measurements, explaining the different initial purities (97% and 83%, respectively). The same DMMTA standard was used in both measurements: 94% purity for the PRP-X100 measurement and 87% (+7% DMDTA) for the AS16 measurement.

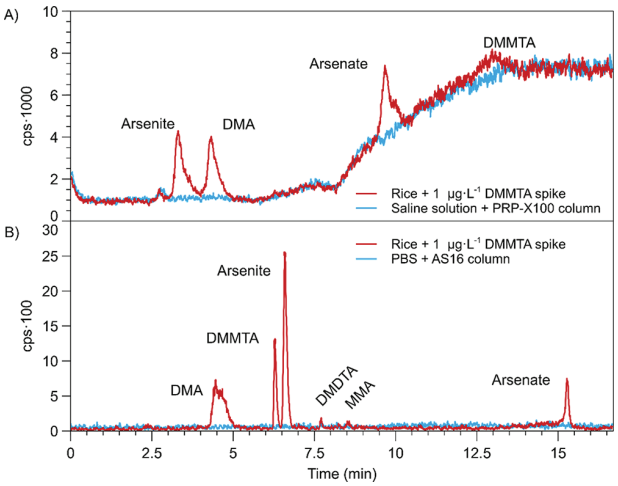

Figure 2. Comparison between method B and method C. IC-ICP-MS chromatogram of rice samples spiked with 1 $\mu g\ L^{-1}$ of DMMTA after enzymatic extractions: (A) method B (saline solution and PRP-X100 column); (B) method C (PBS extractant and AS16 column). The signal intensity is given in counts per second (cps).

guidelines but DMMTA has been found to be more cytotoxic than DMA[20] and arsenite.[21]

In contrast to acidic extraction (method A), enzymatic extraction (method C) preserved MTA and DMMTA. Figure 1 shows that both species were stable in the presence of PBS and enzymes and even upon heating. Once they were spiked in rice samples, the starch matrix again caused a relatively large transformation of MTA (decrease to 28%; 55% transformation from the initial MTA) but relatively little transformation for DMMTA (decrease to 76%; 11% transformation from the initial DMMTA). Again, on the basis of the potential negative effects for human health, the possibility to differentiate DMMTA from DMA with method C is important. Additionally, the detection and quantification of DMDTA were only possible with method C. Figure 1 shows that the percentage of DMDTA present in the synthesized standard (7%) remained between 6 ± 1% in all the measurements, thus suggesting that this species was stable during enzymatic extraction.

Evaluation of Detection Limits, Extraction Efficiency, and Exclusion of DMMTA Artifact Formation with Method C. When we started out with chromatographic separation conditions similar to those used in previous reports[17,19] (method B), no peak was detected when 0.25, 0.5, and 0.75 $\mu g\ L^{-1}$ of DMMTA were spiked in a rice sample after extraction. When 1 $\mu g\ L^{-1}$ of DMMTA was spiked (this concentration in solution would translate to 16.6 $\mu g\ kg^{-1}$ of DMMTA in the rice grain), a broad peak could be detected at the end of the chromatogram (Figure 2A). The gradient elution did not provide a narrow DMMTA peak as expected but caused an increase of the baseline, and the LOD obtained with this method (16.6 $\mu g\ kg^{-1}$ DMMTA) was not sufficient. Since the outcome of the chromatographic conditions of method B (saline solution + PRP X-100) was not satisfactory, we tested the same enzymatic extraction of method B (with the saline solution) using the AS16 column; however, the peak separation was poor (Figure S1). Therefore, we replaced the saline solution with PBS when the AS16 column was used (method C). Figure 2B shows that, when 1 $\mu g\ L^{-1}$ of DMMTA was spiked in the same rice sample after extraction with method C, a narrower DMMTA peak was detected. With method C, the detection limits were substantially improved (LOD of 0.40 $\mu g\ kg^{-1}$).

To evaluate the extraction efficiency of method C, a rice certified reference material (ERM-BC211)[32] was used to compare the concentration of DMA, iAs, and the sum of As species. Additionally, we also show values obtained for the same reference material using method A. The speciation results are summarized in Table 2.

The concentration of DMA, iAs, and sum of As species determined by method C were 115 ± 1, 121 ± 1, and 260 ± 2 $\mu g\ kg^{-1}$, respectively. Within the range of stated uncertainties, these results are all in agreement with the certified values. The sum of As species recovery of method C was 100 ± 1%. These results show that with method C we were able to extract the As species present in the reference material while the speciation of iAs was maintained (regulated species). The results obtained with method A are also in agreement with the certified values. Table 2 shows that the concentrations of MMA obtained with both speciation methods are also in agreement; however, a comparison with the reference material was not possible, since it does not provide a certified value for this species. Thioarsenates cannot be detected using method A. The characterization of DMA and iAs of ERM-BC211 was done mainly using extraction procedures with acid solvents such as HNO_3, HNO_3/H_2O_2, trifluoroacetic acid, HNO_3, HCl, and HCl/H_2O_2;[32] thus, no thioarsenate could be detected. With method C, 11 ± 1 $\mu g\ kg^{-1}$ of DMMTA was detected in this reference material; however, no reference material has reported a certified value for DMMTA to compare the recovery against.

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Table 2. Concentration of As Species (μg kg^{-1}) and Extraction Recoveries (%) of the Rice Certified Reference Material (ERM-BC211)

method	DMA	DMMTA	iAs[b]	MMA	∑As	recovery
ERM-BC211 (certified values)	119 ± 13		124 ± 11		260 ± 13	
method A[a] (measured values)	114 ± 5		124 ± 10	12 ± 2	252 ± 15	97 ± 6
method C[a] (measured values)	115 ± 1	11 ± 1	121 ± 1	13.5 ± 0.1	260 ± 2	100 ± 1

[a] n = 3. [b] Arsenate and arsenite.

To prove the natural occurrence of DMMTA, a rice sample was spiked before the extraction according to method C with 10 μg L^{-1} of DMA (the DMMTA precursor) and was compared against the nonspiked sample (to show that the DMA spike does not transform into DMMTA during extraction). Figure 3A shows that the DMA-spiked sample

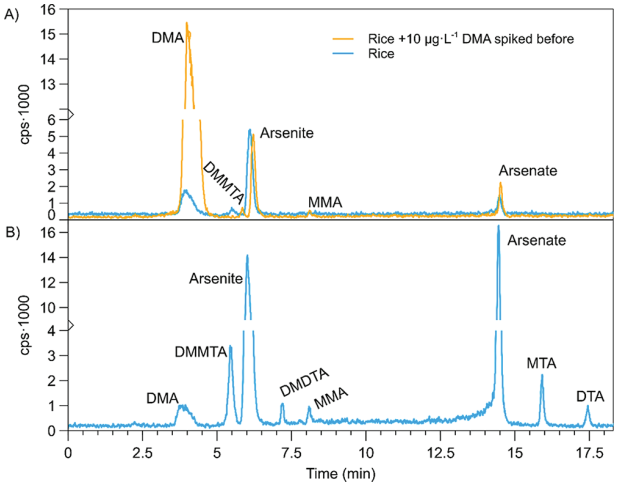

Figure 3. (A) IC-ICP-MS chromatogram of a rice sample spiked before enzymatic extraction according to method C (PBS extractant and AS16 column) with 10 μg L^{-1} of DMA spike. (B) IC-ICP-MS chromatogram of a puffed rice cake sample containing all 4 thioarsenates detected in the present study using method C. The signal intensity is given in counts per second (cps).

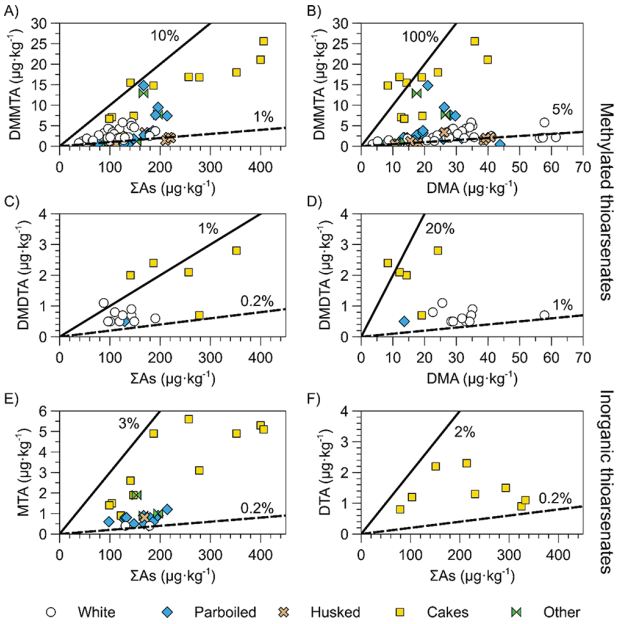

Figure 4. Concentration of methylated thioarsenates (DMMTA and DMDTA) in relation to ∑As (A, C) and in relation to DMA concentration (B, D). Concentration of inorganic thioarsenates (MTA and DTA) in relation to ∑As (E, F). The solid and dotted lines represent percentage lines. The values displayed are above the LOD and include all the samples from Table S1. Low DMMTA concentration points from (A) and (B) are provided with a larger scale in Figure S3.

(yellow line) does not have additional DMMTA ($DMMTA_{spiked\_rice}$(yellow line) = 0.12 ± 0.1 μg L^{-1}, n = 3; $DMMTA_{rice}$(blue line) = 0.11 μg L^{-1}). From the 10.4 μg L^{-1} of DMA that was spiked, 10.7 ± 0.9 μg L^{-1} of DMA (n = 3) was recovered, thus ruling out any DMMTA artifact formation.

Analysis of As Speciation in Commercial Rice Grains and Rice Products Using Method C. The applicability of method C was tested in 54 commercial samples. The sum of As species in the samples ranged from 21 to 406 μg kg^{-1} (Table S1). Inorganic As was the predominant component, and for most samples, DMA was the second most dominant component (Figure S2). DMMTA was found in 50 samples (maximum 25.6 μg kg^{-1}), MTA in 18 samples (maximum 5.6 μg kg^{-1}), and DMDTA in 14 samples (maximum 2.8 μg kg^{-1}) (Figure 4). During this analysis, we also detected DTA in 5 samples (maximum 2.3 μg kg^{-1}) (identified by a retention time match of DTA previously reported in the literature using the same chromatographic conditions as in the present method C).[29] Since the stability of DTA in the method C extract was not tested, we only know that it can be partially preserved throughout this procedure because it was detected after extraction (Figure 3B).

Altogether, thioarsenates contributed up to 15% of total As. DMMTA contributed between 0.3 and 11% to ∑As (Figure 4A), and in relation to DMA (Figure 4B), the share was between 1 and 176%. Using an acid-based extraction, DMMTA would be transformed and determined as DMA and we would not be able to see that, for certain samples (3 puffed rice cakes above the 100% line in Figure 4B), the DMMTA share can actually even be higher than that of DMA. In the case of DMDTA, this species contributed between 0.2% and 1.4% to ∑As (Figure 4C) and, in relation to DMA (Figure 4D), the share was between 1 and 28% (share that would also be determined as DMA in acid-based extractions, according to the results of Figure 1). With regard to the inorganic thioarsenates, it can be seen that MTA contributed between 0.2 and 2.6% to ∑As (Figure 4E) and DTA between 0.2 and 1.2% (Figure 4F). These results are the first evidence that MTA can reach the rice grains and that DTA can be found in rice products. Interestingly, puffed rice cakes (yellow squares) had a particularly high content of thioarsenates in comparison to the rest of the samples. However, since the samples were not selected systematically, we do not yet know how representative the results are.

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Comparison of Results for Commercial Samples between Method A and Method C. To show that acid-based grain extractions codetermine DMMTA as DMA, 10 samples with the highest DMMTA concentrations were analyzed to compare the results between method A and method C (Table S2). The extraction efficiency of both methods was determined relative to the As totals. The extraction efficiency for method A was 105 ± 6% and for method C was 107 ± 13%. Similar extraction efficiency ranges have been reported for the 0.28 mol L^{-1} HNO_3 extraction of As species in rice grains (method A), where approximately 95% of the samples had recoveries of between 81% and 120% (n = 121).[33] With regard to the As speciation, both methods yielded the same percentages of iAs for all of the samples (differences between 0 and 5.0%, $\bar{x}$ = 1.9%) (Table S3). Method C partially oxidized arsenite to arsenate. Healthwise, this is not critical because food guidelines consider the sum of both iAs species. When the DMA percentages of the methods are compared (Figure 5 and Table S4), it can be seen that all of the DMA

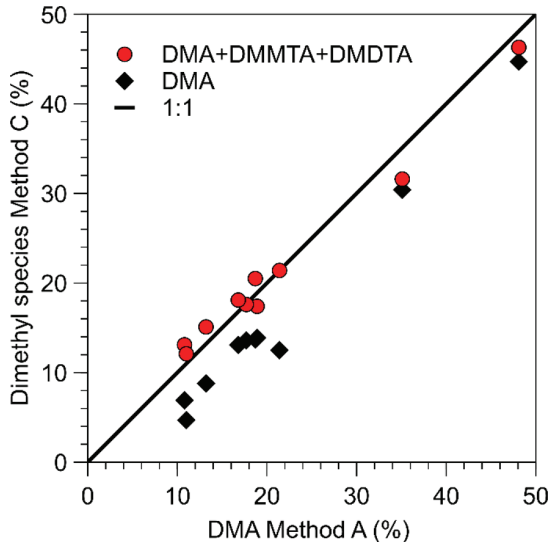

Figure 5. Comparison between the DMA percentage from method A and the dimethyl species percentage from method C. The solid line represents the 1:1 line.

values obtained with method C are below the 1:1 line (black diamonds). This means that, in all of the samples, the DMA percentage of method C was lower in comparison to that obtained with method A (differences between 2.1% and 8.8%, $\bar{x}$ = 4.7%). Once all of the dimethyl species (DMA + DMMTA + DMDTA) from method C were added (red points), the values come closer to the 1:1 line (differences between 0 and 3.5%, $\bar{x}$ = 1.4%). This result shows that the advantage of method C over acid-based extractions (such as method A) is that it does not summarize all the dimethylated species as DMA but can distinguish the concentration of DMMTA. Discovering "hidden" DMMTA will be especially relevant in rice samples that have previously been reported to contain high DMA concentrations.

In the present study, we introduce a novel analytical method for the speciation of As in rice grains and rice products using enzymatic extraction followed by chromatographic separation with 2.5−100 mmol L^{-1} of NaOH as eluent. In addition to the commonly investigated species iAs, MMA, and DMA, this method enables detection of MTA, DTA, DMMTA, and DMDTA. DMMTA was the most frequently encountered thioarsenate species, detected in 50 out of 54 commercial samples. When routine acid-based extractions are applied, DMMTA is "hiding" as currently nonregulated DMA. In particular, the high DMMTA concentrations observed in puffed rice cakes, accounting for a fourth of the maximum amount of toxic As allowed in baby foods (100 $\mu g\ kg^{-1}$), might be of concern. Additionally, our results are the first evidence that MTA, DTA, and DMDTA can be present in rice products.

Further investigations are needed to understand, on the one hand, how widespread thioarsenate occurrence in rice grains and rice products is and how agricultural managing practices or food processing treatments influence thioarsenate accumulation in rice. On the other hand, the fate of inorganic and methylated thioarsenates upon ingestion of rice or rice products in the human digestive tract needs to be investigated to allow a risk assessment for their potential consideration in food guidelines. To date there have been, to the best of our knowledge, no studies following thioarsenate species transformation upon ingestion of thioarsenate-containing rice. However, the enzymatic extraction applied in the present study mimics cooked rice (heating for 1 h at 80 °C) entering the human digestive tract and passing the stomach (incubation for 1 h at 37 °C in a pepsin solution at pH 2) and small intestine (incubation for 2 h at 37 °C in a pancreatin solution at pH 6). We therefore hypothesize that, upon human ingestion of thioarsenate-containing rice, these species will, similarly, be (at least partially) preserved during digestion. Monothioarsenate and DMMTA have been shown before to be able to pass through the gastrointestinal barrier,[34] and their cytotoxicity would therefore have to be considered in risk assessments. Especially DMMTA, even if it remains only partially intact, will have to be addressed because of its higher cytotoxicity in comparison to DMA[20] and arsenite.[21]

Alternatively, on consideration that species of higher toxicity might be overlooked by inappropriate extraction or analytical methods (DMMTA misidentified as DMA (results from the present study)) or species of lower toxicity might transform in the human digestive tract into species of higher toxicity (DMA being methylthiolated to DMMTA[35]), food guidelines might just be changed to limit total As contents in rice, instead of individual species. To err on the side of caution, i.e. overestimation of an As-exposure risk, e.g. by misidentifying less toxic MTA as more toxic arsenite (results from the present study) or overestimating DMMTA stability in the human digestive tract while it is in fact partially transformed to less toxic DMA (to be investigated), will be the safer solution, since in the end all As species are cancer promoters.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c06853.

Peak resolution with saline solution vs PBS on an AS16 column, iAs and DMA speciation of rice products, low DMMTA concentration points, raw data of As speciation of rice products, total As and As speciation from replicates A_C, raw data of iAs speciation of samples from replicates A_C, and raw data of dimethyl species from replicates A_C (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Detection of thioarsenates in rice grains and rice products

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(5 pages, 3 figures, and 4 tables)

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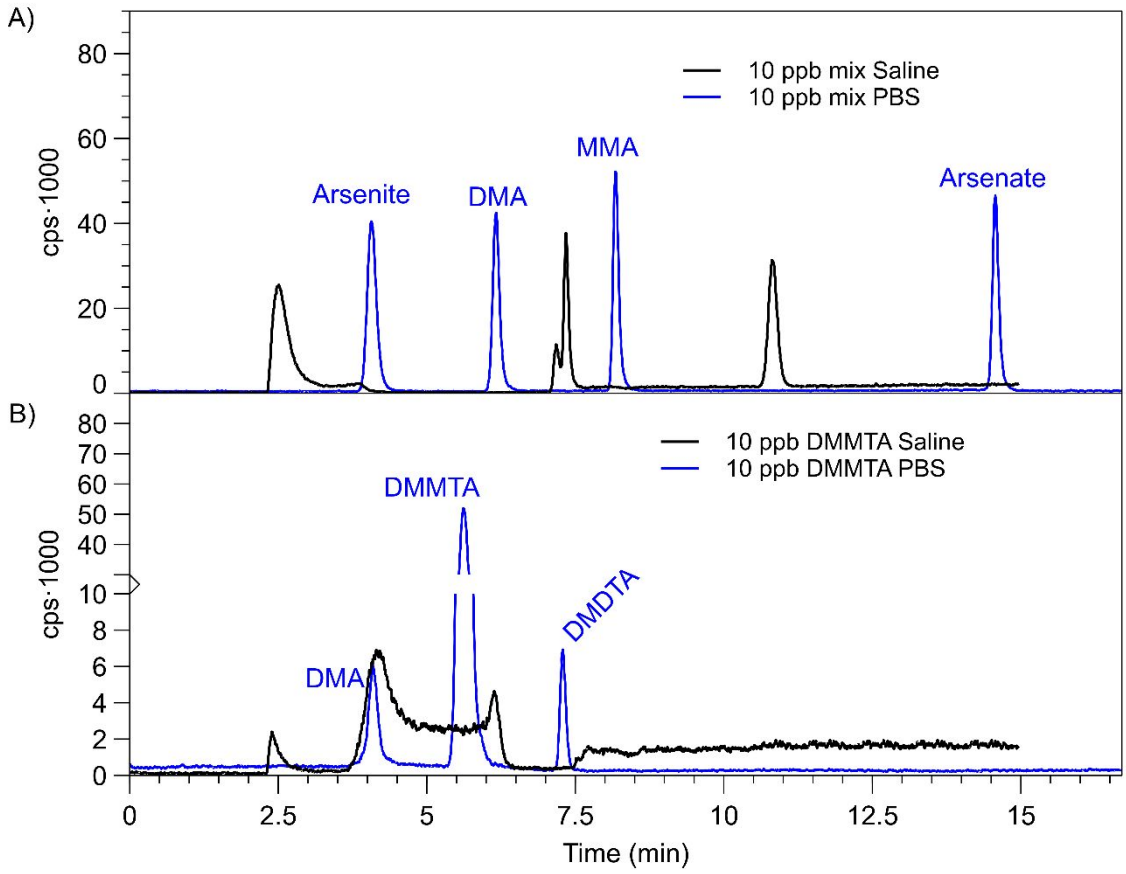

Figure S1. Peak resolution with the saline solution vs phosphate buffer solution (PBS) on an AG/AS16 IonPac column. (A) Mixed standard with 10 ppb arsenite, DMA, MMA, and arsenate and (B) Standard with 10 ppb DMMTA.

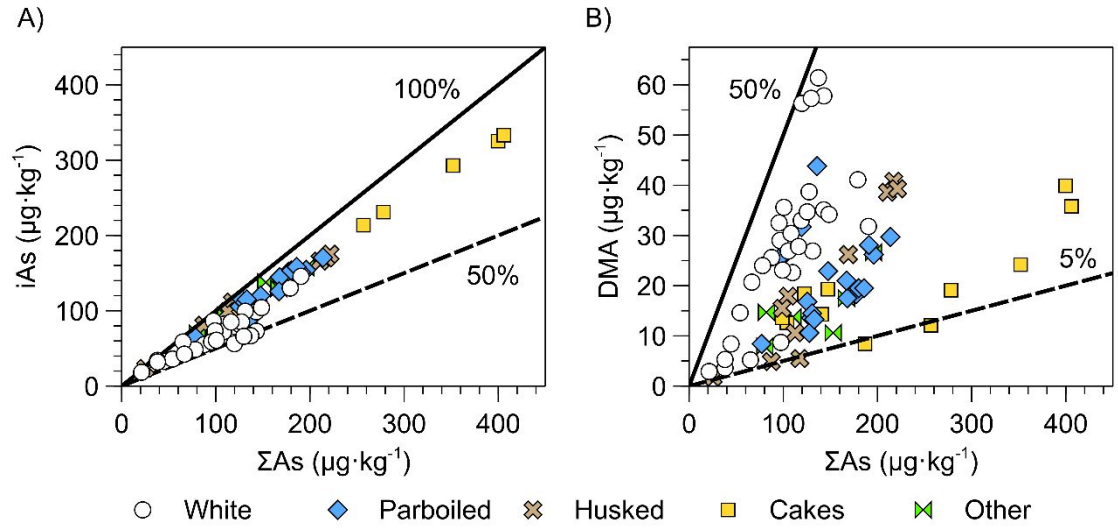

Figure S2. Concentration of (A) iAs and (B) DMA in relation to ΣAs concentration. The solid and dotted lines represent percentage lines.

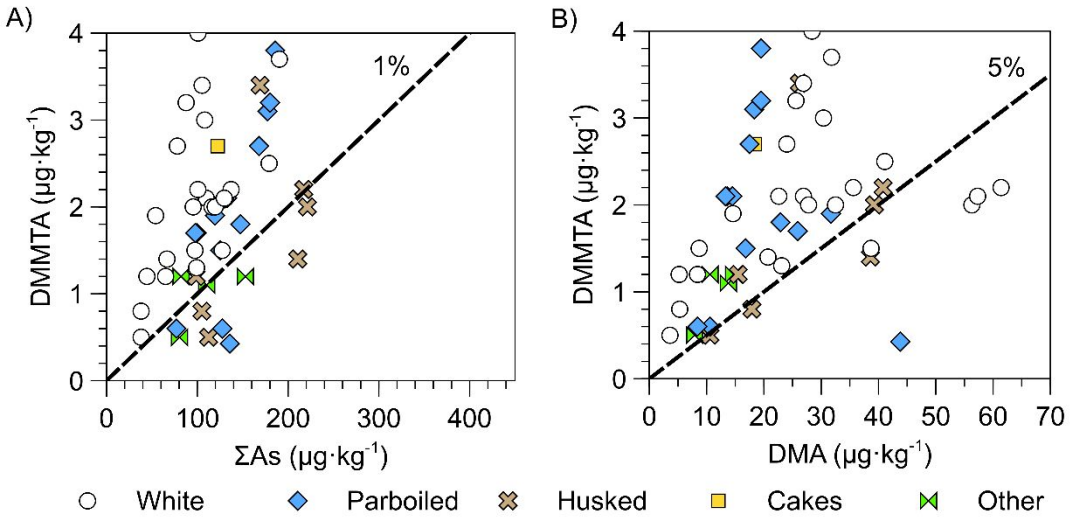

Figure S3. Low DMMTA concentration points from Figure 3A and 3B in a larger scale. (A) In relation to ΣAs and (B) in relation to DMA. The dotted lines represent percentage lines.

Table S1. Summary of As speciation obtained with method C (PBS extractant and AS16 column). All values are given in μg·kg^{-1}.

Type	#	Sample name	Country of origin	Unknown	DMA	DMMTA	Arsenite	DMDTA	MMA	Arsenate	MTA	DTA	ΣAs
White	1	Sample_1	Costa Rica	0.55	8	1.18	26	n.d	1.6	6	n.d	n.d	45
	2	Sample_2_I	Costa Rica	n.d	23	2.15	73	0.77	1.8	10	n.d	n.d	110
	3	Sample_3	Costa Rica	0.73	58	5.76	65	0.69	5.1	8	n.d	n.d	143
	4	Sample_4	Costa Rica	n.d	3	n.d	17	n.d	n.d	2	n.d	n.d	21
	5	Sample_5	Costa Rica	0.67	26	3.22	50	1.08	2.1	5	n.d	n.d	88
	6	Sample_6	Costa Rica	n.d	4	0.49	31	n.d	n.d	3	n.d	n.d	38
	7	Sample_7	Costa Rica	0.79	35	5.04	85	0.93	2.3	13	n.d	n.d	143
	8	Sample_8	Costa Rica	n.d	15	1.87	33	n.d	1.7	3	n.d	n.d	54
	9	Sample_9	Costa Rica	0.63	33	4.33	70	0.46	1.3	10	n.d	n.d	119
	10	Sample_10	Costa Rica	n.d	24	2.74	39	n.d	2.7	9	n.d	n.d	78
	11	Sample_11	Costa Rica	n.d	9	1.45	71	n.d	1.0	15	n.d	n.d	97
	12	Sample_12	Costa Rica	n.d	5	0.79	26	n.d	n.d	6	n.d	n.d	38
	13	Sample_13	Costa Rica	n.d	35	5.81	67	0.74	2.5	14	n.d	n.d	125
	14	Sample_14	Costa Rica	n.d	28	3.95	50	0.47	2.3	16	n.d	n.d	101
	15	Sample_15	Costa Rica	n.d	5	1.24	48	n.d	n.d	11	n.d	n.d	65
	16	Sample_16	USA	0.71	34	4.59	85	0.53	4.3	20	n.d	n.d	148
	17	Sample_17_I	USA	n.d	29	4.26	48	0.49	1.8	13	n.d	n.d	96
	18	Sample_18	USA	0.50	27	2.13	74	n.d	1.9	26	0.39	n.d	131
	19	Sample_19	USA	0.87	32	3.72	115	0.63	7.8	30	n.d	n.d	190
	20	Sample_20	Italy	0.63	41	2.54	102	n.d	4.4	28	0.37	n.d	179
	21	Sample_21	Cambodia	0.80	27	3.38	58	n.d	2.2	14	n.d	n.d	105
	22	Sample_22	USA	0.79	30	2.97	57	n.d	1.8	15	n.d	n.d	108
Parboiled	23	Sample_23	Costa Rica	0.49	26	1.73	53	n.d	2.9	16	n.d	n.d	100
	24	Sample_24	Costa Rica	0.74	26	1.65	53	n.d	3.0	13	0.55	n.d	98
	25	Sample_25_I	[c]	n.d	18	3.08	117	n.d	3.6	35	0.44	n.d	177
	26	Sample_26	[c]	0.51	17	2.68	107	n.d	2.3	37	0.47	n.d	168
	27	Sample_27	[c]	1.59	26	9.52	106	n.d	3.5	49	0.92	n.d	196
	28	Sample_28_I	[d]	n.d	14	2.14	67	0.51	2.0	43	0.66	n.d	129
	29	Sample_29	USA	0.51	44	0.43	49	n.d	2.7	39	n.d	n.d	136
	30	Sample_30	USA	n.d	17	1.46	81	n.d	1.3	25	n.d	n.d	125
	31	Sample_31	[d]	n.d	11	0.60	81	n.d	1.3	34	n.d	n.d	128
	32	Sample_32	India	0.43	8	0.60	42	n.d	0.8	24	n.d	n.d	77
	33	Sample_33	[c]	0.69	23	1.83	76	n.d	1.7	44	0.47	n.d	148
	34	Sample_34	[c]	0.68	32	1.86	65	n.d	2.0	18	n.d	n.d	120
	35	Sample_35	[c]	0.67	28	7.64	97	n.d	2.6	53	0.76	n.d	191
Husked	36	Sample_36	Costa Rica	n.d	2	n.d	19	n.d	n.d	5	n.d	n.d	25
	37	Sample_37_I	Costa Rica	1.15	41	2.15	128	n.d	3.0	42	n.d	n.d	217
	38	Sample_38	Costa Rica	1.02	18	0.80	63	n.d	3.2	20	n.d	n.d	105
	39	Sample_39	Costa Rica	n.d	5	n.d	89	n.d	0.5	23	n.d	n.d	118
	40	Sample_40	Italy	0.74	26	3.42	70	n.d	2.9	65	0.76	n.d	169
	41	Sample_41	USA	0.93	11	0.52	70	n.d	1.1	29	n.d	n.d	112
	42	Sample_42	USA	0.77	5	n.d	60	n.d	0.8	21	n.d	n.d	87
	43	Sample_43	USA	0.71	16	1.24	59	n.d	0.9	21	n.d	n.d	99
Puffed rice cakes[a]	44	Sample_44	[d]	0.97	18	2.67	23	n.d	1.6	75	0.89	n.d	122
	45	Sample_45	[d]	0.98	13	7.07	39	n.d	1.8	40	1.53	0.85	104
	46	Sample_46	Belgium	1.93	40	21.13	88	n.d	5.4	238	5.27	0.88	400
	47	Sample_47	[d]	4.06	24	17.95	96	2.84	3.8	197	4.89	1.53	352
	48	Sample_48	[d]	0.97	14	15.52	44	2.02	1.7	58	2.64	1.21	141
	49	Sample_49	[c]	0.61	8	14.81	81	2.39	2.1	70	4.90	2.18	187
Other	50	Sample_50	Germany	0.45	11	1.24	51	n.d	0.9	86	1.91	n.d	153
	51	Sample_51	USA	2.82	18	12.91	82	n.d	3.3	47	0.93	n.d	167
	52	Sample_52	USA	0.62	15	1.20	27	n.d	n.d	39	n.d	n.d	82
	53	Sample_53	USA	0.65	8	0.46	49	n.d	1.0	21	n.d	n.d	80
	54	Sample_54	USA	0.62	14	1.12	37	n.d	1.0	57	n.d	n.d	110
Replicates A_C	55	Sample_3_A_C		2.22	61	2.15	54	n.d	4.5	12	n.d	n.d	137
	56	Sample_8_ A_C		1.09	21	1.39	36	n.d	1.0	7	n.d	n.d	67
	57	Sample_13_ A_C		0.63	39	1.49	73	n.d	1.5	12	n.d	n.d	127
	58	Sample_27_ A_C		1.28	30	7.41	101	n.d	3.4	70	1.25	n.d	214
	59	Sample_35_ A_C		0.96	27	7.73	101	n.d	2.2	56	1.01	n.d	196
	60	Sample_45_ A_C		1.38	14	6.68	36	n.d	1.4	38	1.42	n.d	99
	61	Sample_46_ A_C		0.94	36	25.58	103	n.d	4.2	230	5.10	1.14	406
	62	Sample_47_ A_C		2.81	19	16.76	92	0.67	3.3	139	3.09	1.28	278
	63	Sample_48_ A_C		1.78	19	7.44	40	n.d	2.3	75	1.87	n.d	147
	64	Sample_49_ A_C		1.51	12	16.85	97	2.09	2.6	116	5.55	2.34	257
	65	Sample_51_ A_C		0.94	21	14.76	87	n.d	3.8	39	0.91	n.d	167
Replicates[b]	66	Sample_2_II		n.d	23	1.25	57	n.d	1.5	16	n.d	n.d	99
	67	Sample_2_III		n.d	28	2.04	67	n.d	1.3	19	n.d	n.d	116
	68	Sample_17_II		n.d	33	1.99	48	n.d	2.2	11	n.d	n.d	95
	69	Sample_17_III		n.d	36	2.19	48	n.d	1.8	13	n.d	n.d	101
	70	Sample_25_II		0.35	19	3.19	92	n.d	2.6	61	0.84	n.d	180
	71	Sample_25_III		0.63	19	3.75	97	n.d	3.1	62	0.56	n.d	186
	72	Sample_28_II		n.d	14	2.09	66	n.d	1.6	45	0.80	n.d	130
	73	Sample_28_III		n.d	13	2.06	69	n.d	1.4	46	0.78	n.d	133
	74	Sample_37_II		1.16	39	1.42	118	n.d	3.7	48	n.d	n.d	211
	75	Sample_37_III		1.11	39	1.98	126	n.d	3.6	49	n.d	n.d	221
	76	Sample_3_A_C		0.84	56	2.02	44	n.d	4.2	12	n.d	n.d	120
	77	Sample_3_A_C		0.96	57	2.10	50	n.d	3.4	16	n.d	n.d	130

n.d indicates not detected; LOD: unknown 0.35 μg·kg^{-1}, DMMTA 0.40 μg·kg^{-1}, DMDTA 0.41 μg·kg^{-1}, MMA 0.49 μg·kg^{-1}, MTA 0.37 μg·kg^{-1}, and DTA 0.43 μg·kg^{-1}. Column recovery was >98% in all samples. [a] no additives reported; [b] done for quality control; [c] not indicated; [d] label only reports "not from the EU".

Table S2. Summary of As total concentration ($91AsO^+$, $\mu g \cdot kg^{-1}$), ΣAs obtained with method A ($\mu g \cdot kg^{-1}$), ΣAs obtained with the method C ($\mu g \cdot kg^{-1}$), and extraction recoveries (%) for the replicates A_C.

Sample	$91AsO^+$	ΣAs_Method A	ΣAs_Method C	Recovery_Method A	Recovery_Method C
Sample_3_A_C	148	139	137	94	93
Sample_8_ A_C	69	73	67	106	97
Sample_13_ A_C	150	154	127	103	85
Sample_27_ A_C	188	197	214	104	114
Sample_35_ A_C	185	208	196	112	106
Sample_45_ A_C	101	102	99	101	98
Sample_46_ A_C	349	381	406	109	116
Sample_47_ A_C	256	289	278	113	108
Sample_48_ A_C	119	117	147	99	124
Sample_49_ A_C	206	226	257	110	125
Sample_51_ A_C	159	156	167	98	105
Mean	**-**	**-**	**-**	**105**	**107**
std	**-**	**-**	**-**	**6**	**13**

Table S3. Summary of the iAs, arsenite, and arsenate percentage (%) obtained with method A and method C for the replicates A_C.

Sample	iAs_Method A	iAs_ Method C	Arsenite_ Method A	Arsenate_ Method A	Arsenite_Method C	Arsenate_Method C
Sample_3_A_C	50	49	47	3	40	9
Sample_8_ A_C	64	67	59	5	57	9
Sample_13_ A_C	64	64	63	1	54	10
Sample_27_ A_C	80	80	60	20	47	33
Sample_35_ A_C	81	80	67	14	51	29
Sample_45_ A_C	78	75	56	22	37	39
Sample_46_ A_C	86	82	66	20	25	57
Sample_47_ A_C	88	83	73	15	33	50
Sample_48_ A_C	80	78	49	31	27	51
Sample_49_ A_C	87	83	73	14	38	45
Sample_51_ A_C	75	75	64	12	52	23

Table S4. Summary of the DMA and dimethyl species percentage (%) obtained with method A and method C for the replicates A_C.

Sample	DMA_Method A	DMA_Method C	DMMTA+DMDTA_ Method C	DMA+DMMTA+DMDTA_Method C
Sample_3_A_C	48.1	44.7	1.6	46.3
Sample_8_ A_C	35.1	30.4	1.2	31.6
Sample_13_ A_C	33.2	31.1	2.1	33.2
Sample_27_ A_C	18.9	13.9	3.5	17.4
Sample_35_ A_C	17.7	13.6	4.0	17.6
Sample_45_ A_C	18.7	13.7	6.8	20.5
Sample_46_ A_C	13.2	8.8	6.3	15.1
Sample_47_ A_C	10.8	6.9	6.3	13.1
Sample_48_ A_C	16.8	13.1	5.0	18.1
Sample_49_ A_C	11.0	4.7	7.4	12.1
Sample_51_ A_C	21.4	12.5	8.8	21.4

Study 2: Widespread occurrence of dimethylmonothioarsenate (DMMTA) in rice cakes: effects of puffing and storage

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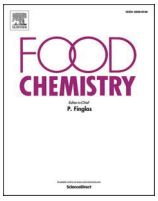

Short communication

Widespread occurrence of dimethylmonothioarsenate (DMMTA) in rice cakes: Effects of puffing and storage

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ABSTRACT

Thioarsenates have recently been detected in rice and rice-based products, with particularly high contents in puffed rice cakes. Here, we show that puffing rice can cause almost complete transformation of dimethylarsenate (DMA) to dimethyldithioarsenate (DMDTA) and dimethylmonothioarsenate (DMMTA). Analysis of puffed rice cakes after 3 months of non-sealed storage at room temperature showed transformation of DMDTA mainly into DMMTA. From a food safety perspective, this likely represents an increased risk because DMMTA is highly cytotoxic and misidentified as non-regulated DMA by routine acid extractions. Analysis of 80 commercial puffed rice cakes confirmed widespread occurrence of thioarsenates. The sum of non-regulated, but potentially toxic DMMTA and DMDTA reached values up to 537 $\mu g \cdot kg^{-1}$ and 241 $\mu g \cdot kg^{-1}$ for generic and infant-labeled rice cakes, respectively. Our results highlight the importance of better understanding (de)thiolation processes along the rice cake-production chain and potentially revising current thresholds set for iAs to include DMMTA and DMDTA.

1. Introduction

Puffed rice cakes are snacks prepared using rapid heating methods. High temperatures are applied to pregelatinized rice to release the moisture inside the grains, causing the kernels to expand (Joshi & Mohapatra, 2014). They have gained popularity in the food industry because of their low serving weight, low-calorie content (Hsieh et al., 1989), and compatibility with celiac diets (Munera-Picazo et al., 2014). Rice-based products are commonly fed to young children due to their mild flavor, nutritional properties, and low allergen potential (Signes-Pastor et al., 2016).

However, rice and rice-based products have also been identified as one of the main dietary sources of arsenic (As) (Munera-Picazo et al., 2014; Signes-Pastor et al., 2016). Arsenic speciation is typically reported to be dominated by arsenite and arsenate (summarized as inorganic arsenic (iAs)) and dimethylarsenate (DMA) (Zhao et al., 2013). Food guidelines in Europe limit carcinogenic iAs in polished rice with a threshold of 150 $\mu g \cdot kg^{-1}$ and in rice-based products with a threshold of 300 $\mu g \cdot kg^{-1}$ for rice waffles, wafers, crackers, and cakes, and 100 $\mu g \cdot kg^{-1}$ iAs for rice used in the production of food intended for infants and young children (European Commission, 2023). Dimethylarsenate, classified as possibly cancerogenic, is not regulated. Analytically, typically total As and iAs are determined and DMA is assumed to be the calculated difference between both.

Recent studies have, however, also shown the widespread occurrence of inorganic and methylated thioarsenates, especially dimethylmonothioarsenate (DMMTA), in rice grains (Colina Blanco et al., 2021; Dai et al., 2022) and, in even higher contents, in a small number of tested commercial puffed rice cakes (n = 6) where thioarsenate contents (DMMTA, dimethyldithioarsenate (DMDTA), monothioarsenate (MTA), and dithioarsenate (DTA)) up to 26 $\mu g \cdot kg^{-1}$ were detected (Colina Blanco et al., 2021). The reason for their previous non-detection is that routinely applied acid-based extraction and detection methods transform inorganic thioarsenates to arsenite and methylated thioarsenates to the respective methylated oxyarsenates (Colina Blanco et al., 2021).

Even though a risk assessment of ingesting thioarsenate-containing rice or rice products including absorption, metabolism, and human biomonitoring studies is lacking, yet (Planer-Friedrich et al., 2022), different pieces of evidence point out that from a food safety perspective DMMTA is the most important species to be addressed. It is stable under conditions that mimic the human digestive tract (Ackerman et al., 2005; Colina Blanco et al., 2021; Glahn et al., 2002), it has been shown to pass the gastrointestinal barrier (Hinrichsen et al., 2015), and it is highly cytotoxic (e.g. for human lung and bladder cells, cytotoxicity decreased

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in the order DMMTA ≥ arsenite ≫ DMDTA > DMA (Moe et al., 2016)). For inorganic thioarsenates, cytotoxicity in human liver and bladder cells decreased in the order arsenite > trithioarsenate > DTA > MTA > arsenate (Hinrichsen et al., 2014). Inorganic thioarsenates therefore likely pose no additional health risk, if they are wrongly determined as arsenite since iAs is regulated (Planer-Friedrich et al., 2022). Methylated thioarsenates, especially DMMTA and likely also DMDTA, in contrast could be a potentially dangerous blind spot if hidden as DMA (Planer-Friedrich et al., 2022).

Whether puffed rice cakes really are a more significant source of DMMTA and DMDTA than polished rice, could not be clearly deduced from our previous study (Colina Blanco et al., 2021) because of the small number of samples and a lack of comparison between puffed rice cake and the original rice used to produce them. Therefore, the purpose of the present study was to show the effect of puffing on rice As speciation, focusing on transformations between DMA, DMDTA, and DMMTA, to analyze their stability upon storage, and to assess the occurrence of thioarsenates in a larger number of commercial cakes, separating between those intended for generic and infant consumption due to different vulnerability (and implemented iAs thresholds) of the target groups.

2. Materials and methods

2.1. Acquisition of rice samples and their respective puffed rice cake

To evaluate the effect of puffing rice on the As speciation, we analyzed rice samples before and after puffing. To do so, a private company (from now on referred to as "the company" for confidentiality reasons) provided us with 10 rice samples (1.5 – 2 kg per sample) and their respective puffed rice cake (1 – 1.5 kg per sample). The puffed rice cakes were prepared with the company's standard method applied to produce commercial rice cakes. The production details are confidential, however, the method followed commonly reported procedures for puffing rice. First, the rice was soaked until ideal moisture (13 – 15%) and then a high-temperature treatment was applied (200 – 300 °C) (Hoke et al., 2005).

2.2. Collection of commercial samples

To assess the occurrence of thioarsenates in commercial puffed rice cakes, a total of 80 samples were purchased in retail and online German stores in 2021 and 2023. All information available on the packages regarding raw ingredient origin, type of rice, type of agriculture, production date (and a calculated storage time before analysis in our laboratories), and the best-before date is given in Table S4. Of the 80 samples, 52 were generic and 28 labeled with an age indication for infants from 7 − 8 months of age. Some cakes contained flavored coatings (e.g. chocolate or yogurt), which were manually removed with a sharp knife to obtain the plain puffed rice.

2.3. Sample preparation

All the samples were first mixed, and a representative subsample was collected for homogenization. Rice received from the company was homogenized by milling using an ultra-centrifugal mill (Retsch MM 2000) at 30 Hz for 2 min, pausing every 30 s, to prevent them from heating. The puffed rice cakes (the company's and the commercial samples) were homogenized by blending using a Ninja 2-in-1 Mixer (stainless steel blades and 1200-watt motor) for 1 min, also pausing every 30 s. To exclude speciation changes due to storage, the samples were freshly blended before extraction.

The As speciation of the company's rice and puffed rice cake samples was measured immediately upon arrival from the company. After 3 months of storage at room temperature (22 – 25 °C), another subsample of puffed rice cakes was collected and homogenized (Figure S1) to evaluate the stability of the As species over time. The samples were stored in closed bags; however, these were not sealed airtight bags.

2.4. Sample extractions, digestion, and analyses

To detect the presence of thioarsenates, the rice grains and puffed rice cakes were extracted following our previously published method (Colina Blanco et al., 2021) that mimics food entering the digestive tract. Briefly, 0.2 g of powdered sample was soaked in 3.2 mL of deionized water and heated for 1 h at 80 °C (this cooking step was omitted for the puffed rice cakes as they are already cooked). Then, the samples were incubated at 37 °C for 1 h in 10 mL of a phosphate buffer solution (2 $mmol{\cdot}L^{-1}$ of NaH_2PO_4 + 0.2 $mmol{\cdot}L^{-1}$ of Na_2-EDTA, adjusted to pH 2 with 5 $mol{\cdot}L^{-1}$ HCl) and 0.5 mL of pepsin solution (125 g per L of 0.1 $mol{\cdot}L^{-1}$ HCl; ≥ 250 $units{\cdot}mg\ solid^{-1}$). After that, 1 $mol{\cdot}L^{-1}$ of $NaHCO_3$ was added to raise the pH to 6, and 2.5 mL pancreatin solution (1.9 g per L of 0.1 $mol{\cdot}L^{-1}$ $NaHCO_3$; 4 × USP specifications) was added. The samples were incubated at 37 °C for 2 h. The final mass of the solution was adjusted to 15 g with phosphate buffer solution (pH 6), and the samples were filtered (0.45 μm hydrophilic Teflon filters). The rice flour-certified reference material ERM-BC211 was used to evaluate the extraction efficiency (extraction recoveries for DMA, iAs, and total As were 102 ± 4%, 91 ± 6%, and 98 ± 5%, respectively (n = 6)). Arsenic speciation was analyzed immediately by ion chromatography coupled to inductively coupled plasma-mass spectrometry (IC-ICP-MS) using a Metrohm 940 Professional IC Vario for the company samples and Dionex ICS-3000 for the commercial samples with an AG/AS16 IonPac column (Dionex; 2.5 – 100 $mmol{\cdot}L^{-1}$ of NaOH with gradient elution, a flow rate of 1.2 $mL{\cdot}min^{-1}$, and 50 μL injection volume) and a 8900 ICP-MS Triple Quad (Agilent) for the company samples and XSeries2 (Thermo-Fisher) for the commercial samples, using oxygen as the reaction cell gas (AsO^+, *m/z* 91).

To demonstrate the limitation of acid extractions, the commercial puffed rice cake samples were additionally extracted using a routine nitric acid extraction (Huang et al., 2010). For this, 0.5 g of sample were incubated for 90 min at 95 °C with 10 mL of 0.28 $mol{\cdot}L^{-1}$ HNO_3. The rice flour-certified reference material ERM-BC211 was used to evaluate the extraction efficiency (extraction recoveries for DMA, iAs, and total As were 102 – 110%, 110%, and 105 – 108%, respectively (n = 2)). The samples were filtered (0.45 μm hydrophilic Teflon filters) and immediately analyzed by IC-ICP-MS using a PRP-X100 column (Hamilton, 10 – 40 $mmol{\cdot}L^{-1}$ of $NH_4H_2PO_4$ with gradient elution, pH 5.6, a flow rate of 1.0 $mL{\cdot}min^{-1}$, and 50 μL injection volume) (Muehe et al., 2019).

To determine total As (all samples) and sulfur (company's rice and puffed rice cakes) contents, 0.2 g of sample were digested in 4 mL of 30% H_2O_2 and 3 mL 65% HNO_3 in a CEM Mars5 microwave digestion system (CEM Corp., Matthews, NC). The rice flour-certified reference material IRMM- 804 was used to assess the digestion performance (extraction recovery for total As was 98 ± 5%, n = 3). The samples were filtered using a 0.2 μm cellulose-acetate filter and analyzed by ICP-MS as AsO^+ (*m/z* 91) and SO^+ (*m/z* 48) using rhodium (Rh^+, *m/z* 103) as internal standard.

2.5. Statistical analysis

A two-sample *t*-test was used to evaluate differences between the means of samples. Differences were considered significant when $p < 0.001$.

3. Results and discussion

3.1. Effect of puffing on rice As speciation

The As speciation of 10 rice samples before and after puffing was studied to assess speciation changes induced by the high-temperature treatment. Table S1 shows the summary of the As speciation and total

As contents in the samples. The recovery of sum of As species from the enzymatic extraction versus total digested As was 95 ± 7%. Additionally, Figure S2 shows that the As content measured in the rice samples was in line with the content measured in their respective puffed rice cake samples.

In the original rice samples, speciation was dominated by iAs (ranging between 84 and 264 $\mu g \cdot kg^{-1}$), followed by DMA (ranging between 6 – 46 $\mu g \cdot kg^{-1}$). Regarding thioarsenates, only very low contents of DMMTA and DMDTA (1 – 3 $\mu g \cdot kg^{-1}$) were detected in only 5 out of 10 rice samples (Table S1). Fig. 1A shows that the puffing treatment significantly changed the As speciation. Most of the DMA present in the rice samples was transformed to DMMTA (4 – 24 $\mu g \cdot kg^{-1}$) and especially DMDTA (11 – 56 $\mu g \cdot kg^{-1}$). Furthermore, all the puffed rice samples additionally contained small amounts of MTA and DTA (2 – 5 $\mu g \cdot kg^{-1}$), products of iAs thiolation. Figure S3 shows a chromatogram of a rice sample before and after the puffing treatment.

Our results clearly show that As thiolation is promoted by processing rice at high temperatures. The exact mechanism is unknown, yet. But it is known that reduced sulfur is needed to react with arsenite or methylated oxyarsenates to form thioarsenates. The company's rice samples had total sulfur contents of 1.0 ± 0.1 $g \cdot kg^{-1}$, in line with previously reported values (Hagan et al., 2003). A large share of these sulfur-containing compounds in rice grains are compounds such as cysteine, methionine, and thiamine (Müller-Fischer, 2012), which upon thermal degradation, produce hydrogen sulfide (Dwivedi & Arnold, 1973; Güntert et al., 1994; Weiss et al., 2018). In fact, when opening the bags containing the freshly produced puffed rice cakes in our laboratories, we perceived an intense sulfuric odor typically associated with volatile compounds such as hydrogen sulfide or dimethyl sulfide found in the aroma of cooked rice (Hu et al., 2020; Verma & Srivastav, 2020). We therefore postulate that reduced sulfur causes the observed thiolation of DMA to DMMTA, which can further be thiolated to DMDTA, during rice cake production. Fig. 1B shows that, as expected, there is a high positive correlation between the DMA content in rice and the DMMTA and DMDTA content in the puffed rice cakes. These trends highlight the potential risk of using rice grains with high DMA contents, deemed safe by food guidelines, to produce puffed rice. The extent of thiolation likely depends on the type of original rice, the exact processing conditions, and potentially also on post-processing methods such as drying for preservation, which might also sorb hydrogen sulfide (Watanabe, 2012), and conditions for storage.

3.2. Effect of storage in non-sealed bags on the stability of DMMTA and DMDTA

Three months after first opening the rice cakes bags, then storing them just in tied bags, as consumers would in their households, the sulfuric smell initially perceived had disappeared. We therefore re-analyzed As speciation. Fig. 2 shows that after this time of non-sealed storage, most of the DMDTA was dethiolated (the range decreased from 11 to 56 $\mu g \cdot kg^{-1}$ to 0 – 8 $\mu g \cdot kg^{-1}$). It had transformed mainly to DMMTA (the range increased from 4 to 24 $\mu g \cdot kg^{-1}$ to 10 – 57 $\mu g \cdot kg^{-1}$) and some DMA (the range increased from 0 to 7 $\mu g \cdot kg^{-1}$ to 4 – 28 $\mu g \cdot kg^{-1}$). We therefore hypothesize that DMDTA dethiolation is driven by the loss of excess hydrogen sulfide during this type of storage. These results suggest that despite the lower cytotoxicity of DMDTA (IC_{50}: 230 $\mu mol \cdot L^{-1}$ for human lung cells at 48 h) compared to DMMTA (IC_{50}: 21 $\mu mol \cdot L^{-1}$ for human lung cells at 48 h) (Moe et al., 2016), monitoring

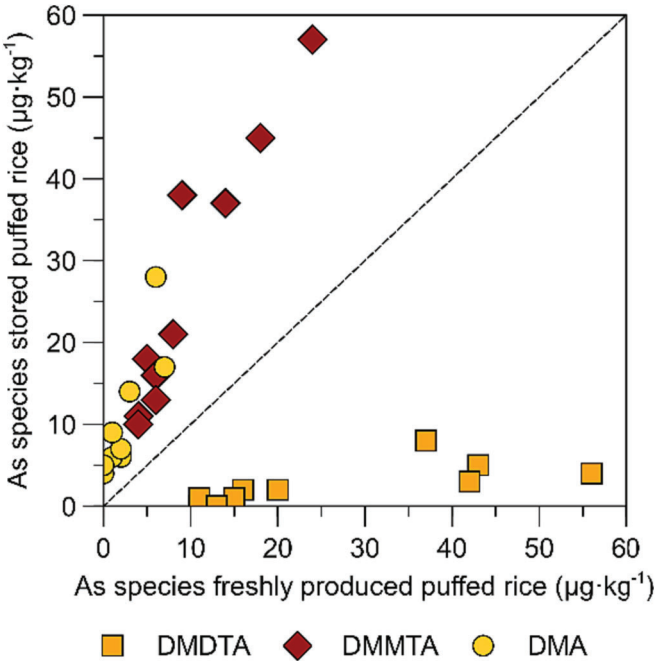

Fig. 2. Comparison between As species in rice cakes, analyzed directly upon arrival from the company and after 3 months of storage at room temperature (the dotted line represents a 1:1 line).

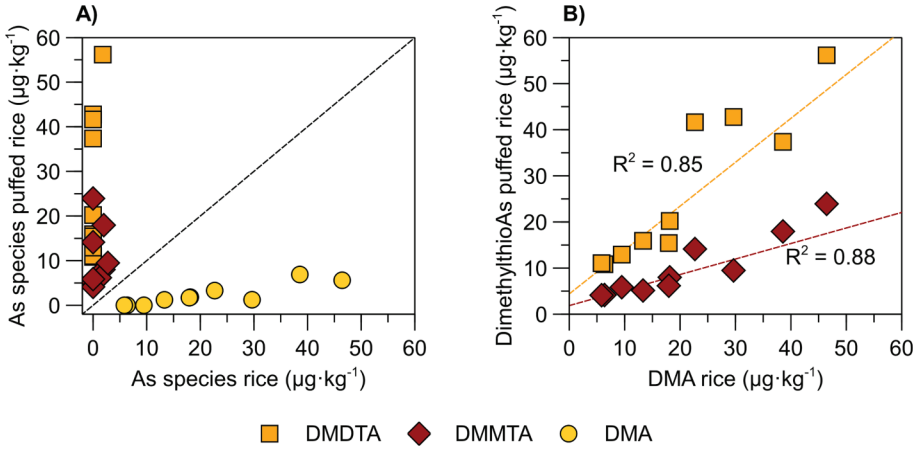

Fig. 1. A) Comparison between dimethylated As species in rice samples and their respective puffed rice cake sample (the black dotted line represents a 1:1 line) and B) Correlation between the DMA content in the rice samples and the content of dimethylthioarsenates in the corresponding puffed rice samples (the orange and red dotted lines represent regression lines).

DMDTA in freshly produced puffed rice cakes is also relevant as it can be a precursor for DMMTA due to dethiolation during storage.

Further dethiolation of DMMTA to DMA might occur upon longer storage, which from a health perspective would be beneficial. However, rice cake-producing companies cannot make safe predictions on the extent and duration for complete dethiolation without knowing original reduced sulfur contents, extent of thiolation upon production, sulfur oxidation depending on duration between production and packaging, type of packaging, and storage duration and conditions (e.g. temperature). Storage temperature has previously been shown to impact the stability of volatile components (Tsugita et al., 1983) and sulfur-containing compounds such as thiamine (Goulette et al., 2020) in rice. Nevertheless, storage conditions do not only depend on producers and retail but largely on individual consumer behavior (following best-before-date, re-use of bags already opened, heating of cakes to increase crispiness, etc.). So, whether storage leads to an increase of the food safety threat by DMDTA transformation to DMMTA or a decrease by further dethiolation of DMMTA to DMA must be established.

3.3. Thioarsenates occurrence in commercial puffed rice cakes

To assess occurrence and relevance of thioarsenates, specifically DMMTA and DMDTA, in a larger dataset of samples, a total of 80 commercial puffed rice cakes was analyzed. Of the 80 samples, 28 were specifically labelled as suitable for infants from 7 to 8 months of age. The sum of As species in the commercial puffed rice cakes ranged between 24 and 747 $\mu g \cdot kg^{-1}$ (Table S2 and Figure S4). This range is comparable to previously reported values in rice cakes (32 – 620 $\mu g \cdot kg^{-1}$, n = 59) (U.S. Food and Drug Administration, 2016). Similarly, the observed ranges of iAs (14 – 314 $\mu g \cdot kg^{-1}$) and ∑dimethylAs species (DMA + DMMTA + DMDTA) (5 – 576 $\mu g \cdot kg^{-1}$) are in line with previously reported values, iAs: 23 – 425 $\mu g \cdot kg^{-1}$ with n = 121 (German Federal Institute for Risk Assessment, 2015; Kollander & Sundström, 2015; U.S. Food and Drug Administration, 2016) and DMA: 15 – 477 $\mu g \cdot kg^{-1}$ with n = 59, determined as ∑dimethylAs species using acid extraction (U.S. Food and Drug Administration, 2016).

Regarding the thioarsenate content, Fig. 3 shows that DMMTA was detected in 79 samples, ranging between 2 – 256 $\mu g \cdot kg^{-1}$ (accounting for up to 38% of total As, Table S3). DMDTA was detected in 77 samples, ranging between 1 – 343 $\mu g \cdot kg^{-1}$ (accounting for up to 46% of total As). MTA (67 samples, up to 8 $\mu g \cdot kg^{-1}$) and DTA (64 samples, up to 7 $\mu g \cdot kg^{-1}$) were widely detected as minor species. For the first time, monomethylmonothioarsenate (MMMTA, 10 samples, up to 3 $\mu g \cdot kg^{-1}$) and monomethyldithioarsenate (MMDTA, 16 samples, up to 7 $\mu g \cdot kg^{-1}$) were detected in rice products (identification was done by comparison with reported retention times using the same chromatographic conditions) (Planer-Friedrich et al., 2007; Wallschläger & London, 2008). Furthermore, Fig. 3C shows that maximum contents observed in our study for DMMTA + DMDTA (up to 576 $\mu g \cdot kg^{-1}$) even exceeded those for iAs (up to 314 $\mu g \cdot kg^{-1}$) and that many samples with low iAs content had high contents of DMMTA + DMDTA, usually hidden as non-regulated DMA.

3.4. Evaluation of a potential food safety threat of thioarsenates occurrence in commercial rice cakes

Lacking a regulation for DMMTA + DMDTA, we discuss here a potential food safety threat based on the thresholds for iAs, which can of course only be a first proxy, until further risk assessment. This proxy might even be too lax, considering that cytotoxicity of DMMTA was reported to be 3.2 times higher than that of arsenite (Moe et al., 2016). Cytotoxicity of DMDTA was reported to be 3.4 times lower than that of arsenite (Moe et al., 2016), but with the uncertainties of transformation during storage, a considerate estimate is to assume the higher risk of DMMTA. Fig. 4 shows box plots of the 80 rice cakes, divided in generic and infant-labeled cakes with their respective thresholds at 300 and 100 $\mu g \cdot kg^{-1}$ iAs, considering only iAs, only DMMTA + DMDTA, or iAs + DMMTA + DMDTA as a risk for human consumption. While for iAs only 1 generic sample and none of the infant-labeled samples exceeded the respective thresholds for adults and infants, for DMMTA + DMDTA, it would have been 6 and 3 samples, respectively, and considering iAs + DMMTA + DMDTA it would have been 10 samples on each category.

The risk for infants increases when they are fed generic puffed rice cakes, frequently marketed to them using packaging with cartoons or appealing flavored coatings. Considering only iAs, more than half of the generic samples are not adequate for their consumption (35 of the 52 samples, median at 124 $\mu g \cdot kg^{-1}$; Fig. 4A "generic" exceeding 100 $\mu g \cdot kg^{-1}$). If hidden DMMTA + DMDTA are added to iAs, 41 out of 52 samples (median at 188 $\mu g \cdot kg^{-1}$) would not be adequate for their consumption (Fig. 4C "generic" exceeding 100 $\mu g \cdot kg^{-1}$). One uniform, low threshold for all types of rice cakes could decrease this threat. However, it was also pointed out before that even the current threshold is potentially problematic (Carey et al., 2018). When a 1 year-old child (9.25 kg body weight) consumes 2 to 3 units of rice cakes (20 g) containing 100 $\mu g \cdot kg^{-1}$, the exposure is 0.22 $\mu g \cdot kg$ body $weight^{-1}$, which is 1.5 fold more than a 70 kg adult would receive by drinking 1 L of water at the EU drinking water limit of 10 $\mu g \cdot L^{-1}$ (0.142 $\mu g \cdot kg$ body $weight^{-1}$ per day).

No further estimate to predict net thiolation or type of species formed (DMMTA vs. DMDTA) could be derived from information provided on the packages (Table S4). Much of the information was very unspecific, e.g. the raw material origin ("EU/non-EU agriculture"), or lacking completely, e.g. details on whether whole grain or white rice was used or whether products came from organic agriculture. Likely, multiple processes govern thiolation and dethiolation in rice cakes, making it challenging for scientists and producers, let alone for consumers, to estimate a potential food safety threat based on currently available data.

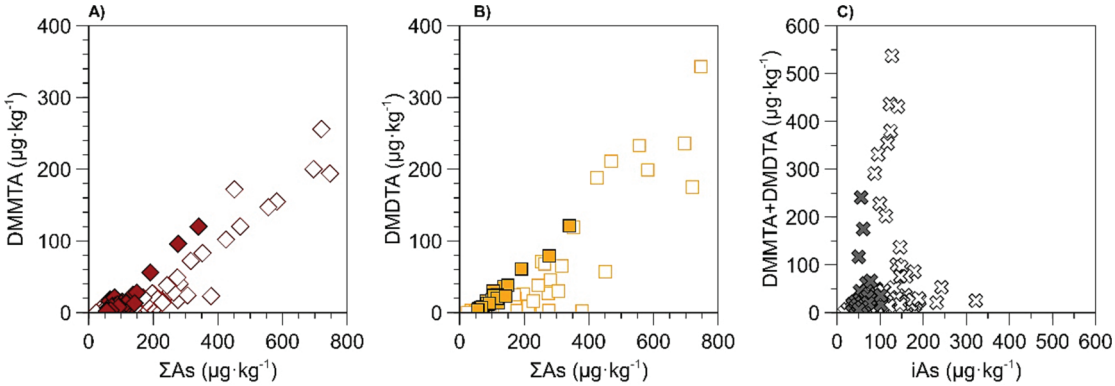

Fig. 3. A) Content of DMMTA in relation to ΣAs, B) Content of DMDTA in relation to ΣAs, and C) Content of DMMTA + DMDTA in relation to iAs of the commercial puffed rice cake samples. Filled symbols correspond to the infant-labeled samples (n = 28) and open symbols to the generic samples (n = 52).

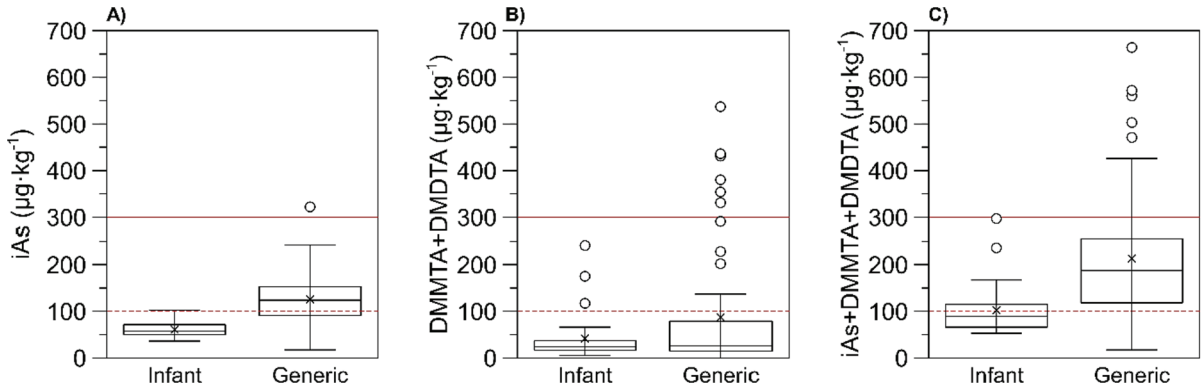

Fig. 4. Box plots of As content in commercial samples of infant (n = 28) and generic (n = 52) puffed rice cakes: A) Evaluation of the iAs content (toxic and officially regulated), B) Evaluation of the DMMTA + DMDTA content (potentially toxic, currently non-regulated As species), and C) Evaluation of the iAs + DMMTA + DMMTA content (toxic and potentially toxic). The red solid and dotted lines represent the EU limit for iAs in generic puffed rice cakes and rice used in the production of food intended for infants and young children, respectively. The whisker position represents 1.5 x interquartile range (IQR) from the edge of the box (Tukey style).

4. Conclusions

Our results show that previously observed high contents of thioarsenates, particularly of DMMTA and DMDTA, in puffed rice cakes are no coincidence, but a result of thiolation during the cake production process. Storage of non-sealed rice cakes at room temperature can lead to dethiolation of DMDTA to DMMTA, likely increasing a potential health risk based on the specie's cytotoxicities. Analysis of commercial puffed rice cake samples confirmed the widespread presence of DMMTA, DMDTA, MTA, and DTA with DMMTA and DMDTA contributing significantly, and sometimes even more than iAs, to total As contents in rice cakes.

Considering the limited currently available data, it is not feasible to provide an estimate for net thiolation in rice cakes. A first step should be to understand the influence of different producing conditions to ideally avoid thiolation at all or, if thiolation during production is unavoidable, to optimize post-processing conditions for complete dethiolation before release for sale. Urgent further tasks are adaptation of approved routine analytical methods to ensure DMMTA and DMDTA do not remain unrecognized anymore, adaptation of thresholds based on a full risk assessment of DMMTA and DMDTA, or, as a conservative option, limit total As; at which contents needs to be discussed. The value of rice cakes specifically as "healthy" food snack might need re-consideration, specifically for infants. New formulations by mixing grains (Carey et al., 2018) such as maize, quinoa, oats, or millet could help to reduce the overall dietary As exposure (iAs and thioarsenates) from rice-based foods.

CRediT authorship contribution statement

Andrea E. Colina Blanco: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft. **Alejandra Higa Mori:** Investigation. **Britta Planer-Friedrich:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137723.

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Supplementary material

Widespread occurrence of dimethylmonothioarsenate (DMMTA) in rice cakes: effects of puffing and storage

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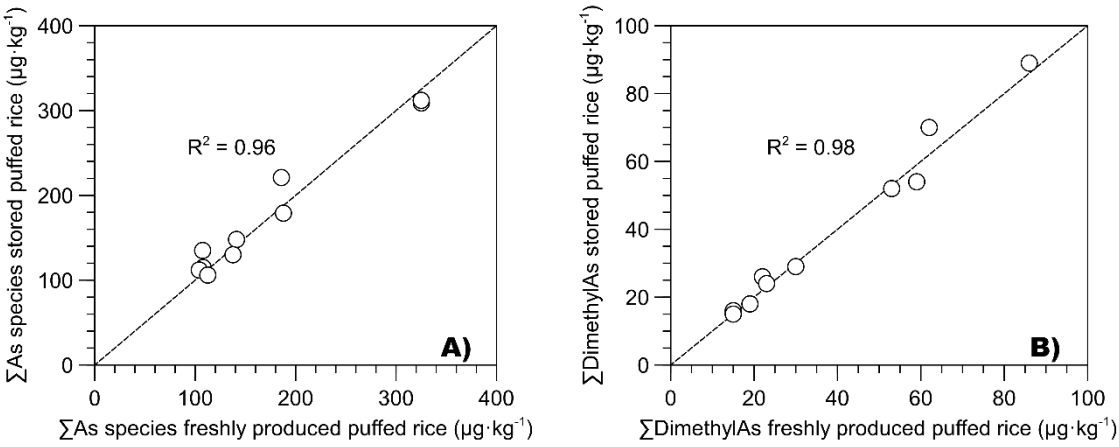

Figure S1. A) Correlation between sum of As species (*t*-test, p=0.93) and B) sum of dimethylAs species (DMA+DMMTA+DMDTA) (*t*-test, p= 0.96) in the freshly produced puffed rice cakes and after 3 months of storage at room temperature (these puffed rice cakes were sampled and homogenized in different batches) (dotted line represents a 1:1 line).

Table S1. Summary of the As speciation from the enzymatic extraction and total As content of the company's rice samples and their respective puffed rice cake. All values are given in µg As·kg dry* sample^{-1}.

Sample Name	TMAO	DMA	DMMTA	DMDTA	MMA	iAs**	MTA	DTA	ΣAs	As total	Recovery (%)
Rice_1	0	18	2	0	0	90	0	0	110	105	105
Rice_2	0	13	0	0	0	106	0	0	119	111	107
Rice_3	2	39	2	0	0	121	0	0	163	173	94
Rice_4	0	18	1	0	0	84	0	0	103	95	109
Rice_5	0	30	3	0	0	114	0	0	146	149	98
Rice_6	0	6	0	0	0	153	0	0	159	169	94
Rice_7	0	23	0	0	2	264	0	0	288	302	96
Rice_8	0	6	0	0	0	90	0	0	96	103	93
Rice_9	0	46	0	2	1	208	0	0	257	316	81
Rice_10	0	9	0	0	1	111	0	0	122	122	100
Puffed rice cake_1	0	2	8	20	0	73	2	3	108	113	95
Puffed rice cake_2	0	1	5	16	0	80	2	3	107	114	94
Puffed rice cake_3	0	7	18	37	0	118	2	3	186	195	95
Puffed rice cake_4	0	2	6	15	0	76	2	3	104	107	98
Puffed rice cake_5	0	1	9	43	0	82	2	3	141	170	83
Puffed rice cake_6	0	0	4	11	0	166	3	4	188	211	89
Puffed rice cake_7	0	3	14	42	0	257	4	5	325	349	93
Puffed rice cake_8	0	0	4	11	0	93	2	2	113	132	85
Puffed rice cake_9	0	6	24	56	0	231	4	4	325	367	89
Puffed rice cake_10	0	0	6	13	0	115	2	3	138	146	94

Drying factor: for 1 kg of puffed rice cakes, 1.026 kg of rice is needed
** iAs= arsenite + arsenate

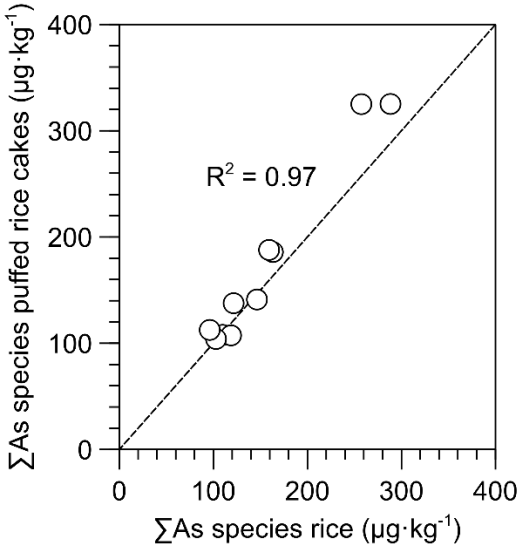

Figure S2. Correlation between As content in the rice and their respective puffed rice cake samples (*t*-test, p= 0.62) (dotted line represents a 1:1 line).

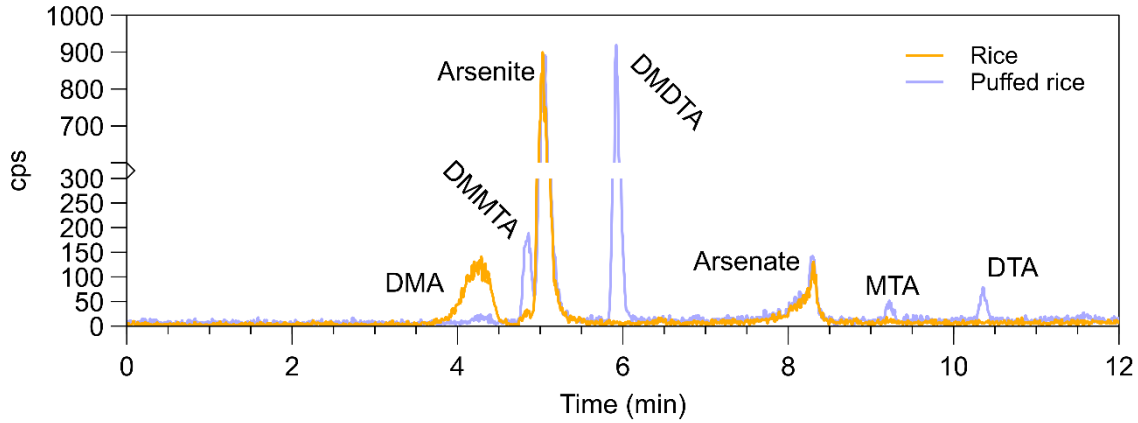

Figure S3. Chromatogram of Rice_5 (146 µg·kg^{-1}) and Puffed rice cake_5 (141 µg·kg^{-1}). The signal intensity is given in counts per second (cps).

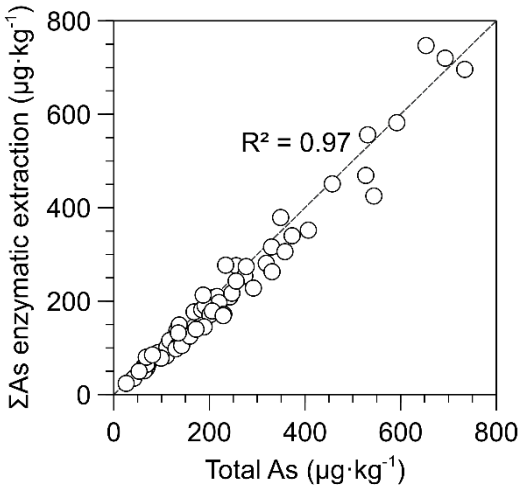

Figure S4. Correlation between total As content and sum of As species from the enzymatic extraction (*t*-test, p=0.48) (dotted line represents a 1:1 line). The enzymatic extraction recovery was 92 ± 12%.

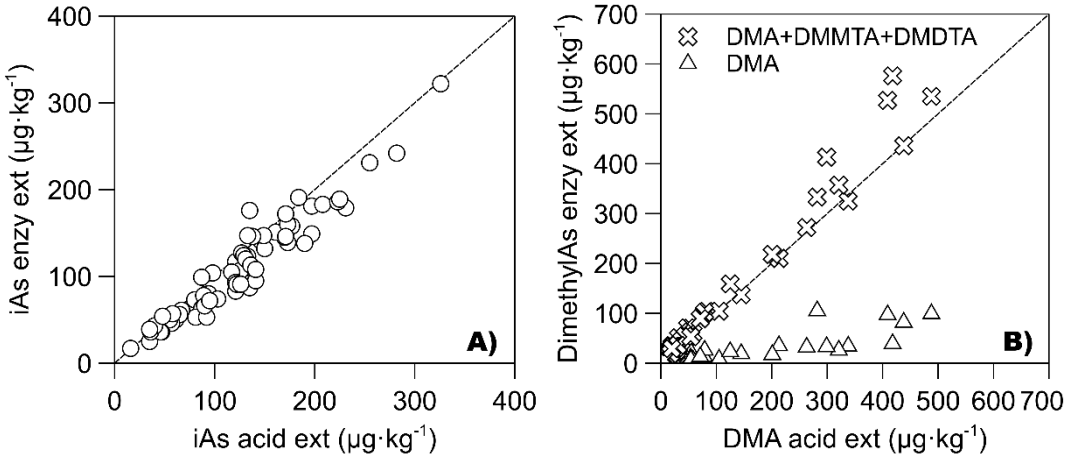

Figure S5. A) Correlation between iAs species (*t*-test, p= 0.14) and B) DMA and dimethylAs species (DMA+DMMTA+DMDTA) (*t*-test DMA, p<0.001 and *t*-test dimethyl species, p= 0.56) in the commercial puffed rice cakes determined by acid and enzymatic extraction (dotted line represents a 1:1 line).

Table S2. Summary of As speciation from enzymatic extraction and total As content of commercial puffed rice cakes. All values are given in µg As·kg sample^{-1}.

Sample #	TMAO	DMA	DMMTA	Arsenite	DMDTA	MMA	MMMTA	MMDTA	Arsenate	MTA	DTA	Sum	Totals
1	0	12	7	42	0	0	0	0	31	0	0	92	101
2	3	17	18	115	3	3	0	0	109	5	2	276	256
3	2	4	29	96	71	5	0	5	35	3	6	254	274
4	1	15	6	36	0	1	0	0	42	2	0	104	119
5	0	11	12	106	5	0	0	0	66	5	4	207	206
6	0	30	23	117	2	3	0	0	197	5	3	379	349
7	1	13	40	102	46	1	0	0	69	4	4	281	319
8	2	4	9	109	11	0	0	0	64	4	5	209	243
9 [i]	0	4	9	50	15	0	0	0	9	2	2	90	93
10 [i]	0	9	25	46	36	0	0	0	16	2	3	137	132
11 [i]	0	6	15	39	30	0	0	0	12	0	3	104	132
12 [i]	0	6	28	51	38	0	0	0	21	2	3	149	137
13 [i]	0	4	10	40	16	0	0	0	10	1	2	83	110
14 [i]	0	3	13	42	18	0	0	0	20	2	3	101	111
15 [i]	0	4	14	53	25	0	0	0	16	2	3	116	117
16	0	9	25	93	11	0	0	0	61	2	2	203	209
17	3	82	155	100	199	24	0	3	13	1	2	582	592
18	4	99	200	97	236	30	0	3	22	2	3	696	734
19	0	3	11	85	17	0	0	0	51	3	6	177	168
20	0	0	9	105	14	0	0	0	74	2	5	210	216
21 [i]	0	35	96	53	79	6	0	0	8	0	0	277	234
22 [i]	0	19	56	46	61	5	0	0	1	2	1	191	220
23	0	7	13	97	9	1	0	0	48	3	3	182	184
24	2	8	28	104	68	5	0	2	38	3	5	263	331
25	10	97	256	108	175	36	2	4	29	2	1	720	693
26 [i]	2	32	120	41	121	7	0	3	11	2	2	340	373
27 [i]	2	4	12	62	14	0	0	0	7	1	2	104	134
28	0	3	4	19	3	0	0	0	5	0	0	35	42
29	4	34	102	70	188	7	0	2	11	3	3	425	544
30	0	0	8	80	10	0	0	0	23	3	3	126	154
31	0	0	8	84	8	0	0	0	18	3	3	123	151
32	2	10	27	82	26	0	0	0	45	3	3	197	220
33	3	26	49	102	27	6	0	3	49	4	5	274	277
34	3	23	72	97	65	6	0	2	38	5	6	316	330
35	5	39	194	93	343	30	3	7	27	3	4	747	653
36	0	0	11	113	7	0	0	0	47	6	6	190	191
37	2	6	24	155	30	2	0	0	74	6	7	306	358
38	2	3	6	29	4	0	0	0	17	0	0	60	63
39	5	26	120	73	211	9	0	4	17	2	3	469	527
40	4	33	147	89	233	11	1	4	29	2	4	556	531
41	2	2	4	62	6	0	0	0	17	1	2	98	130
42	2	4	6	78	5	0	0	0	23	2	2	121	141
43	0	0	16.9	100.1	6.2	0	0	0	38.9	5	3.7	171	201
44	1	2	10	95	20	2	0	0	36	3	5	172	230
45	0	0	6	36	2	0	0	0	13	1	0	57	69
46	2	4	6	29	3	0	0	0	5	1	1	51	64
47	0	0	5	67	7	0	0	0	20	2	3	105	142
48	0	4	15	61	13	2	0	0	25	2	3	125	159
49	0	0	18.8	127.9	6.9	0	0	0	47.9	8	7	217	247
50 [i]	4	8	18	22	1	0	0	0	14	0	0	66	72
51 [i]	2	10	16	20	1	0	0	0	16	0	0	65	69
52 [i]	3	11	21	21	2	0	0	0	22	0	0	80	68
53	1	6	8	34	4	2	0	0	21	1	1	78	99
54	1	2	5	14	2	0	0	0	25	0	0	50	53
55	0	7	14	63	7	0	0	0	38	2	1	132	135
56	2	105	172	60	57	12	3	2	35	3	1	451	457
57	0	13.1	14.5	92.7	2.9	1.8	0	0	46.5	5	2.3	179	206
58	0	3	24	61	26	0	0	0	23	3	4	145	189
59	1	7	19	119	8	3	0	0	54	3	1	213	187
60	1	4	7	78	7	0	0	0	38	1	1	140	172
61	0	6	14	108	16	4	0	0	72	4	5	228	292
62	0	7	0	8	0	0	0	0	7	3	0	24	26
63	3	17	83	62	119	15	0	3	46	2	3	352	407
64	2	7	21	57	25	5	0	2	45	3	3	169	229
65	2	13	38	71	38	3	1	1	70	3	2	243	256
66	0	10	14	32	4	1	0	0	22	1	0	85	81
67 [i]	0	0	3	74	14	0	0	0	22	2	4	120	106
68 [i]	0	0	4	55	24	0	3	0	15	2	4	106	112
69 [i]	0	7	9	35	7	0	0	0	15	0	0	73	63
70 [i]	0	8	13	50	20	0	4	0	17	2	2	116	103
71 [i]	0	7	11	42	11	0	2	0	14	2	0	89	73
72 [i]	0	5	13	73	23	0	0	0	22	2	3	142	139
73 [i]	0	0	4	48	13	0	0	0	15	2	2	84	96
74 [i]	0	3	6	30	6	0	2	0	14	0	0	60	59
75 [i]	0	5	8	35	8	0	2	0	13	0	0	71	68
76 [i]	0	0	8	52	13	0	0	0	16	2	2	92	100
77 [i]	0	0	1	33	4	0	0	0	13	0	4	56	65
78 [i]	0	0	3	37	7	0	0	0	15	0	4	65	82
79 [i]	0	0	3	39	7	0	0	0	14	2	2	68	89
80 [i]	0	0	2	32	4	0	0	0	13	2	3	56	61

[i]: samples labeled with an age indication for infants from 7 - 8 months of age

Table S3. Summary of As species percentage (%) obtained from the enzymatic extraction of commercial puffed rice cakes (calculated as percent from the sum of As species).

Sample #	TMAO	DMA	DMMTA	Arsenite	DMDTA	MMA	MMMTA	MMDTA	Arsenate	MTA	DTA
1	0	13	8	46	0	0	0	0	34	0	0
2	1.1	6	7	42	1	1.1	0	0	39	1.8	0.7
3	0.8	2	11	38	28	2.0	0	2.0	14	1.2	2.4
4	1.0	14	6	35	0	1.0	0	0	40	1.9	0
5	0	5	6	51	2	0	0	0	32	2.4	1.9
6	0	8	6	31	1	0.8	0	0	52	1.3	0.8
7	0.4	5	14	36	16	0.4	0	0	25	1.4	1.4
8	1.0	2	4	52	5	0	0	0	31	1.9	2.4
9 [i]	0	4	10	56	17	0	0	0	10	2.2	2.2
10 [i]	0	7	18	34	26	0	0	0	12	1.5	2.2
11 [i]	0	6	14	38	29	0	0	0	12	0	2.9
12 [i]	0	4	19	34	26	0	0	0	14	1.3	2.0
13 [i]	0	5	12	48	19	0	0	0	12	1.2	2.4
14 [i]	0	3	13	42	18	0	0	0	20	2.0	3.0
15 [i]	0	3	12	46	22	0	0	0	14	1.7	2.6
16	0	4	12	46	5	0	0	0	30	1.0	1.0
17	0.5	14	27	17	34	4.1	0	0.5	2	0.2	0.3
18	0.6	14	29	14	34	4.3	0	0.4	3	0.3	0.4
19	0	2	6	48	10	0	0	0	29	1.7	3.4
20	0	0	4	50	7	0	0	0	35	1.0	2.4
21 [i]	0	13	35	19	29	2.2	0	0	3	0	0
22 [i]	0	10	29	24	32	2.6	0	0	1	1.0	0.5
23	0	4	7	53	5	0.5	0	0	26	1.6	1.6
24	0.8	3	11	40	26	1.9	0	0.8	14	1.1	1.9
25	1.4	13	36	15	24	5.0	0.3	0.6	4	0.3	0.1
26 [i]	0.6	9	35	12	36	2.1	0	0.9	3	0.6	0.6
27 [i]	1.9	4	12	60	13	0	0	0	7	1.0	1.9
28	0	9	11	54	9	0	0	0	14	0	0
29	0.9	8	24	16	44	1.6	0	0.5	3	0.7	0.7
30	0	0	6	63	8	0	0	0	18	2.4	2.4
31	0	0	7	68	7	0	0	0	15	2.4	2.4
32	1.0	5	14	42	13	0	0	0	23	1.5	1.5
33	1.1	9	18	37	10	2.2	0	1.1	18	1.5	1.8
34	0.9	7	23	31	21	1.9	0	0.6	12	1.6	1.9
35	0.7	5	26	12	46	4.0	0.4	0.9	4	0.4	0.5
36	0	0	6	59	4	0.0	0	0.0	25	3.2	3.2
37	0.7	2	8	51	10	0.7	0	0	24	2.0	2.3
38	3.3	5	10	48	7	0.0	0	0	28	0	0
39	1.1	6	26	16	45	1.9	0	0.9	4	0.4	0.6
40	0.7	6	26	16	42	2.0	0.2	0.7	5	0.4	0.7
41	2.0	2	4	63	6	0	0	0	17	1.0	2.0
42	1.7	3	5	64	4	0	0	0	19	1.7	1.7
43	0	0	10	59	4	0	0	0	23	2.9	2.2
44	0.6	1	6	55	12	1.2	0	0	21	1.7	2.9
45	0	0	11	63	4	0	0	0	23	1.8	0
46	3.9	8	12	57	6	0	0	0	10	2.0	2.0
47	0	0	5	64	7	0	0	0	19	1.9	2.9
48	0	3	12	49	10	1.6	0	0	20	1.6	2.4
49	0	0	9	59	3	0	0	0	22	3.7	3.2
50 [i]	6.1	12	27	33	2	0	0	0	21	0	0
51 [i]	3.1	15	25	31	2	0	0	0	25	0	0
52 [i]	3.8	14	26	26	3	0	0	0	28	0	0
53	1.3	8	10	44	5	2.6	0	0	27	1.3	1.3
54	2.0	4	10	28	4	0	0	0	50	0	0
55	0	5	11	48	5	0	0	0	29	1.5	0.8
56	0.4	23	38	13	13	2.7	0.7	0.4	8	0.7	0.2
57	0	7	8	52	2	1.0	0	0	26	2.8	1.3
58	0	2	17	42	18	0	0	0	16	2.1	2.8
59	0.5	3	9	56	4	1.4	0	0	25	1.4	0.5
60	0.7	3	5	56	5	0	0	0	27	0.7	0.7
61	0	3	6	47	7	1.8	0	0	32	1.8	2.2
62	0	29	0	33	0	0	0	0	29	12.5	0
63	0.9	5	24	18	34	4.3	0	0.9	13	0.6	0.9
64	1.2	4	12	34	15	3.0	0	1.2	27	1.8	1.8
65	0.8	5	16	29	16	1.2	0.4	0.4	29	1.2	0.8
66	0	12	16	38	5	1.2	0	0	26	1.2	0
67 [i]	0	0	3	62	12	0	0	0	18	1.7	3.3
68 [i]	0	0	4	52	23	0	2.8	0	14	1.9	3.8
69 [i]	0	10	12	48	10	0	0	0	21	0	0
70 [i]	0	7	11	43	17	0	3.4	0	15	1.7	1.7
71 [i]	0	8	12	47	12	0	2.2	0	16	2.2	0.0
72 [i]	0	4	9	51	16	0	0	0	15	1.4	2.1
73 [i]	0	0	5	57	15	0	0	0	18	2.4	2.4
74 [i]	0	5	10	50	10	0	3.3	0	23	0	0
75 [i]	0	7	11	49	11	0	2.8	0	18	0	0
76 [i]	0	0	9	57	14	0	0	0	17	2.2	2.2
77 [i]	0	0	2	59	7	0	0	0	23	0	7.1
78 [i]	0	0	5	57	11	0	0	0	23	0	6.2
79 [i]	0	0	4	57	10	0	0	0	21	2.9	2.9
80 [i]	0	0	4	57	7	0	0	0	23	3.6	5.4

[i]: samples labeled with an age indication for infants from 7 - 8 months of age

Table S4. Summary of information provided in the commercial puffed rice cakes labels (as stated in the packaging).

Sample #	Origin of raw material	Type of rice	Type of agriculture	Production day	Analysis day	Calculated storage time (days)	Best-before-date
1	EU/non EU agriculture	Brown	Organic	11.02.19	12.02.21	732	11.02.20
2	EU/non EU agriculture	-	Organic	10.01.19	12.02.21	764	11.01.20
3	EU/non EU agriculture	-	Organic	04.12.20	22.02.21	80	05.12.21
4	EU/non EU agriculture	Brown	Organic	17.07.19	12.02.21	576	16.07.20
5	-	-	-	10.04.19	12.02.21	674	10.04.20
6	Italy	Brown	Organic	04.12.18	12.02.21	801	05.12.19
7	Italy	Brown	Organic	24.11.20	12.02.21	80	25.11.21
8	Italy	Brown	Organic	21.12.20	22.02.21	63	22.12.21
9 [i]	EU/non EU agriculture	-	Organic	30.09.20	12.02.21	135	31.08.21
10 [i]	EU/non EU agriculture	-	Organic	29.10.20	12.02.21	106	30.09.21
11 [i]	EU/non EU agriculture	-	Organic	06.11.20	12.02.21	98	08.10.21
12 [i]	EU/non EU agriculture	-	Organic	06.11.20	12.02.21	98	08.10.21
13 [i]	EU/non EU agriculture	-	Organic	25.11.20	12.02.21	79	27.10.21
14 [i]	EU/non EU agriculture	-	Organic	25.11.20	12.02.21	79	27.10.21
15 [i]	EU/non EU agriculture	-	Organic	22.10.20	12.02.21	113	22.09.21
16	EU/non EU agriculture	Brown	Organic	10.11.20	12.02.21	94	11.08.21
17 *	EU/non EU agriculture	Brown	Organic	25.11.20	11.03.21	106	24.11.21
18 *	EU/non EU agriculture	Brown	Organic	24.11.20	22.02.21	90	24.11.21
19 *	EU/non EU agriculture	Brown	Organic	-	12.02.21	-	01.12.21
20	Italy	Brown	Organic	14.12.20	12.02.21	60	15.12.21
21 [i]	Non EU agriculture	-	Organic	11.10.20	11.03.21	151	12.10.21
22 [i]	EU/non EU agriculture	-	Organic	04.11.20	11.03.21	127	06.08.21
23	EU/non EU agriculture	-	-	24.08.20	12.02.21	172	25.08.21
24	EU/non EU agriculture	-	Organic	08.12.20	11.03.21	93	09.12.21
25	EU/non EU agriculture	Brown	Organic	03.11.20	22.02.21	111	05.09.21
26 [i]	EU/non EU agriculture	White	Organic	08.12.20	22.02.21	76	09.12.21
27 [i]	EU/non EU agriculture	White	Organic	04.10.20	22.02.21	141	05.10.21
28	EU/non EU agriculture	Brown	Organic	30.11.20	11.03.21	101	01.09.21
29	EU/non EU agriculture	Brown	Organic	17.12.20	22.02.21	67	18.12.21
30	EU/non EU agriculture	Brown	Organic	12.12.20	11.03.21	89	13.12.21
31	EU/non EU agriculture	-	Organic	17.12.20	11.03.21	84	17.12.21
32	USA	Brown	Organic	-	22.02.21	-	08.06.21
33	-	Brown	-	-	22.02.21	-	07.02.21
34	-	Brown	-	-	22.02.21	-	26.03.21
35 *	EU/non EU agriculture	Brown	Organic	20.12.20	22.02.21	64	21.12.21
36 *	EU/non EU agriculture	Brown	Organic	-	22.02.21	-	08.12.21
37 *	EU/non EU agriculture	Brown	Organic	19.11.20	22.02.21	95	19.11.21
38 *	EU/non EU agriculture	Brown	Organic	17.11.20	22.02.21	97	17.11.21
39 *	EU/non EU agriculture	-	Organic	19.12.20	22.02.21	65	19.12.21
40 *	EU/non EU agriculture	Brown	Organic	23.12.20	22.02.21	61	23.12.21
41 *	EU/non EU agriculture	Brown	Organic	16.12.20	22.02.21	68	12.12.21
42 *	EU/non EU agriculture	Brown	Organic	16.12.20	22.02.21	68	12.12.21
43 *	EU/non EU agriculture	-	Organic	16.11.20	11.03.21	115	17.11.21
44 *	EU/non EU agriculture	-	Organic	-	01.03.21	-	12.11.21
45 *	EU/non EU agriculture	-	Organic	13.11.20	11.03.21	118	13.11.21
46 *	EU/non EU agriculture	-	Organic	14.11.20	01.03.21	107	13.11.21
47 *	EU/non EU agriculture	-	Organic	17.12.20	11.03.21	84	17.12.21
48 *	EU/non EU agriculture	-	-	17.11.20	01.03.21	104	18.11.21
49 *	EU/non EU agriculture	-	-	03.12.20	11.03.21	98	04.12.21
50 [i]	-	-	-	-	01.03.21	-	21.08.21
51 [i]	-	-	-	-	01.03.21	-	21.08.21
52 [i]	-	-	-	-	01.03.21	-	21.08.21
53	EU/non EU agriculture	Brown	Organic	14.04.20	01.03.21	321	15.04.21
54	-	-	Organic	19.02.20	01.03.21	376	19.02.21
55	EU/non EU agriculture	-	Organic	18.05.20	01.03.21	287	19.05.21
56	EU/non EU agriculture	Brown	Organic	22.03.20	01.03.21	344	23.03.21
57	EU/non EU agriculture	Brown	Organic	-	01.03.21	-	08.02.21
58	Italy	-	Organic	-	11.03.21	-	01.02.22
59	Thailand	-	Organic	-	01.03.21	-	04.11.21
60	EU agriculture	Brown	Organic	30.12.20	01.03.21	61	30.06.22
61	EU agriculture	Brown	Organic	-	01.03.21	-	15.12.21
62	EU/non EU agriculture	-	Organic	-	01.03.21	-	05.06.21
63	EU/non EU agriculture	Brown	Organic	25.08.20	01.03.21	188	10.10.21
64	EU/non EU agriculture	Brown	Organic	24.08.20	01.03.21	189	25.08.21
65	Italy	-	Organic	31.10.20	01.03.21	121	30.09.21
66	EU agriculture	-	Organic	13.11.20	01.03.21	108	13.12.21
67 [i]	EU/non EU agriculture	-	Organic	-	06.07.23	-	06.08.23
68 [i]	EU/non EU agriculture	-	Organic	-	06.07.23	-	03.10.23
69 [i]	EU/non EU agriculture	White	Organic	31.01.23	06.07.23	156	30.01.24
70 [i]	EU/non EU agriculture	White	Organic	23.03.23	06.07.23	105	21.05.24
71 [i]	EU/non EU agriculture	-	Organic	24.03.23	06.07.23	104	23.03.24
72 [i]	-	-	-	30.03.23	06.07.23	98	28.01.24
73 [i]	EU/non EU agriculture	White	Organic	16.02.23	06.07.23	140	18.11.23
74 [i]	EU/non EU agriculture	White	Organic	18.05.23	06.07.23	49	16.05.24
75 [i]	EU/non EU agriculture	White	Organic	28.03.23	06.07.23	100	27.03.24
76 [i]	EU/non EU agriculture	-	Organic	20.02.23	06.07.23	136	21.02.24
77 [i]	EU/non EU agriculture	-	Organic	24.05.23	06.07.23	43	24.05.24
78 [i]	EU/non EU agriculture	-	Organic	11.05.23	06.07.23	56	11.05.24
79 [i]	EU/non EU agriculture	-	Organic	06.04.23	06.07.23	91	06.04.24
80 [i]	EU/non EU agriculture	-	Organic	20.04.23	06.07.23	77	20.04.24

[i]: samples labeled with an age indication for infants from 7 - 8 months of age

* samples containing flavored coating

Study 3: Dimethylmonothioarsenate Is Highly Toxic for Plants and Readily Translocated to Shoots

Erik Pischke, Fabrizio Barozzi, Andrea E. Colina Blanco, Carolin F. Kerl, Britta Planer-Friedrich, and Stephan Clemens

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Article

Dimethylmonothioarsenate Is Highly Toxic for Plants and Readily Translocated to Shoots

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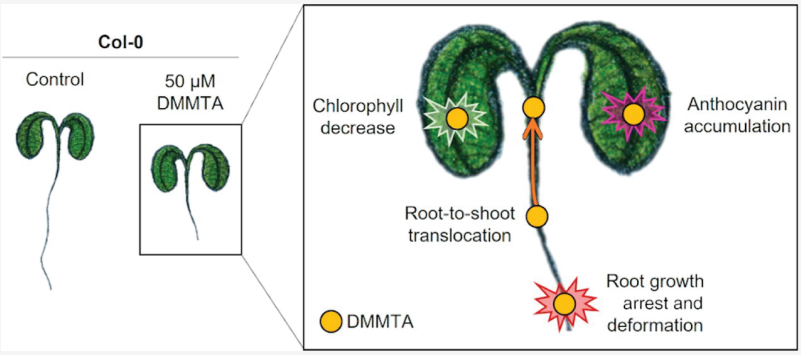

ABSTRACT: Arsenic is one of the most relevant environmental pollutants and human health threats. Several arsenic species occur in soil pore waters. Recently, it was discovered that these include inorganic and organic thioarsenates. Among the latter, dimethylmonothioarsenate (DMMTA) is of particular concern because in mammalian cells, its toxicity was found to exceed even that of arsenite. We investigated DMMTA toxicity for plants in experiments with *Arabidopsis thaliana* and indeed observed stronger growth inhibition than with arsenite. DMMTA caused a specific, localized deformation of root epidermal cells. Toxicity mechanisms apparently differ from those of arsenite since no accumulation of reactive oxygen species was observed in DMMTA-exposed root tips. Also, there was no contribution of the phytochelatin pathway to the DMMTA detoxification as indicated by exposure experiments with respective mutants and thiol profiling. RNA-seq analysis found strong transcriptome changes dominated by stress-responsive genes. DMMTA was taken up more efficiently than the methylated oxyarsenate dimethylarsenate and highly mobile within plants as revealed by speciation analysis. Shoots showed clear indications of DMMTA toxicity such as anthocyanin accumulation and a decrease in chlorophyll and carotenoid levels. The toxicity and efficient translocation of DMMTA within plants raise important food safety issues.

KEYWORDS: *arsenic speciation, arsenic toxicity, food safety, phytochelatins, thioarsenates*

INTRODUCTION

Ubiquitously present environmental pollutants such as arsenic (As) negatively impact the health of plants, animals, and humans.[1,2] Organisms can be exposed to As via different routes as various chemical forms of As occur in both groundwater and soils due to natural processes or anthropogenic activities.[3] Besides drinking water, human dietary uptake is mainly attributable to the consumption of rice and can thus cause chronic poisoning in regions with rice-based diets.[4] A major reason is the cultivation of rice in anaerobic paddy field conditions which strongly promote the bioavailability of As.[5,6] The inorganic forms arsenate and arsenite are present in most soils, with the latter being favored in the reducing environment of flooded soils and associated with greater health risks.[7,8] In addition to the dominant inorganic species, methylation processes result in the formation of organic As species in soil pore waters.[9,10] Methylation of arsenite, mediated by sulfate-reducing bacteria and methanogens,[11,12] yields monomethylarsenate (MMA) and dimethylarsenate (DMA), which are considered the most abundant methylated organic As species in paddy soil pore waters. Recently, additional As species resulting from thiolation processes were discovered in paddy soils, namely, pentavalent inorganic monothioarsenate (MTA) and methylated thioarsenates such as monomethylmonothioarsenate (MMMTA), dimethylmonothioarsenate

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(DMMTA), monomethyldithioarsenate (MMDTA), and dimethyldithioarsenate (DMDTA).[13,14] Mesocosm experiments detected inorganic and organic thioarsenates at all stages of rice cultivation. Incubation experiments with a range of European and Chinese soils found the ubiquitous presence of thioarsenates and concentrations that were on average higher than those of the well-studied methylated oxyarsenates.[13] In a survey of As-contaminated soils and paddy fields, DMMTA was the dominant methylated thioarsenate in pore waters.[14] Until then, thioarsenates had escaped detection in paddy soils, one reason being the commonly used acidic conditions of sample stabilization and chromatography, which do not preserve these species.[13,15] Their apparent widespread occurrence raises the question as to how they interact with plants. Uptake, metabolism, and accumulation of thioarsenates need to be investigated in order to better understand possible negative impacts on plant growth as well as food safety threats associated with the exposure to thioarsenates. First studies demonstrated uptake and metabolic conversion of inorganic MTA in *Arabidopsis thaliana*[16] and *Oryza sativa* (rice).[17] Pilot experiments with rice seedlings found uptake of methylated thioarsenates too.[18]

Among the methylated thioarsenates, DMMTA appears to be of particular relevance for two reasons, namely, accumulation in plants and known toxicity to human and other mammalian cells. First, DMMTA is the one thiolated As species that was previously reported to occur in rice grains. An analysis of commercial rice samples found DMMTA following an enzymatic extraction to mimic digestion of cooked rice.[19] Furthermore, recent analyses of commercial rice samples and rice products found widespread occurrence of DMMTA.[20,21] This indicated uptake, translocation, and grain loading of organic thioarsenates. The potential accumulation of DMMTA in plants was recently confirmed after exposure of rice seedlings to MMMTA and DMMTA[18] and in grains harvested from rice plants cultivated in paddy fields.[14] Thus, DMMTA impact on plants appears to be relevant regarding risks of human exposure.

Evidence derived from studies with human and animal cells indicates that DMMTA is among the most toxic As species even though it belongs to the pentavalent forms which are generally regarded less toxic.[22] Detection of DMMTA in human urine samples prompted investigations of thioarsenate metabolism and toxicity.[23,24] Experiments with rats and hamsters revealed that the methylated thioarsenates MMMTA and DMMTA, and in hamsters also DMDTA, can be found after arsenite treatments.[25] Experiments with human bladder cancer EJ-1 cells determined the highest toxicity for trivalent DMA^{III} and DMMTA with IC_{50} values about 2 orders of magnitude lower than for arsenate and DMA.[26] Similarly, reports on epidermoid carcinoma A431 cells or hepatocarcinoma-derived HepG2 cells showed comparable toxicity of DMMTA and the trivalent As species arsenite and DMA^{III}.[24,27] A comparison of 14 different As species tested for toxicity on two human cell lines determined IC_{50} values for DMMTA that were up to 3-fold lower than for arsenite and 300-fold lower than for DMA.[28] In contrast, IC_{50} values were 30-fold higher for MMMTA and >200-fold higher for DMDTA. More efficient uptake and different modes of action of DMMTA were observed in comparison to trivalent As species. For example, formation of reactive oxygen species was highest after DMMTA treatments, and the cell cycle was affected.[27] These observations raise the question as to the toxicity of DMMTA for plants and further highlight the importance of studies on the interaction of DMMTA with plants with respect to food safety.

We therefore initiated a comparative study to determine the toxicity of DMMTA for plants relative to that of arsenite, a highly toxic As species, and to that of DMA as a pentavalent organic As species known to be toxic to plants as well.[29] Like several previous studies,[30] we used *A. thaliana* as a model system. Assays are well established, a wide range of genotypes are readily available, and current knowledge on the transport, metabolism, and detoxification of As species shows that the major mechanisms are conserved between dicots and monocots. For example, arsenite uptake is mediated by transporters of the nodulin 26-like family (NIP, e.g., AtNIP5;1[31] for *A. thaliana* and OsNIP2;1, OsLSI1 for *O. sativa*[32]). Glutathione (GSH) and especially phytochelatins (PCs), synthesized by phytochelatin synthases (PCSs), play a crucial role in the complexation and thus detoxification of arsenite (AtPCS1,[33] OsPCS1 and OsPCS2[34,35]). Transporters of the ABCC family mediate the sequestration of As-PC complexes in vacuoles (AtABCC1, AtABCC2,[36] and OsABCC1[37]). Uptake and translocation of arsenate occur through phosphate transporters (AtPHT1[38] or OsPHT1[39]), and reduction of arsenate to arsenite is catalyzed by arsenate reductases in both *A. thaliana* and *O. sativa* (AtHAC1[40,41] and OsHAC4[42]). Knowledge on the metabolic fate of methylated species such as MMA and DMA is more limited to date.[43] Uptake was shown in rice to occur through OsNIP2;1 (OsLsi1) like for arsenite.[44] While reduction of MMA^{V} to MMA^{III} and further complexation by PCs was observed, this could not be confirmed for DMA^{V}.[45]

We report here on experiments comparing the toxicity of DMMTA to that of arsenite and DMA. To test the potential function of the PC pathway and the arsenate reductase HAC1 for DMMTA tolerance, experiments with the loss of function mutants *cad1-3* (defective in AtPCS1),[46] *abcc1/2*,[36] and *hac1*[41] were performed. RNA-seq analyses revealed genome-wide responses to DMMTA. Imaging of reactive oxygen species (ROS) formation and propidium iodide staining to assess cell integrity were performed to shed light on modes of toxicity of DMMTA in comparison to the other tested As species arsenite and DMA. Arsenic speciation and thiol profiling assessed the *in planta* mobility of DMMTA. In leaves, pigment concentrations and stress marker gene expression were compared as toxicity indicators.

MATERIAL AND METHODS

Arsenic Species. Commercially available arsenite (Riedel-de Haën) and pentavalent DMA (Sigma-Aldrich) were used. DMMTA was synthesized according to a protocol by Lee et al.[47] IC-ICP-MS analysis detected 2% DMA and 8% DMDTA (±1%) as impurities (Table S1).

Plant Growth Assays. For root growth analyses, plants were grown in a liquid seedling system with a modified 1/10 Hoagland medium[48] over a growth period of 9 days[49] with the following adjustments. Seedlings were grown in 12-well plates and exposed to different As species on day 6 for 24 h. Treatment was terminated by washing the wells with 1.5 mL of 1/10 Hoagland medium and the addition of 3.5 mL of fresh medium. On day 8, the medium was again exchanged, and on day 9, root lengths were measured. For ROS and propidium iodide staining, plants were grown as described for root growth assays. For qRT-PCR and RNA-seq analyses, plants were

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grown in Petri dishes containing 20 mL of medium with shaking at 75 rpm and the medium was changed on day 5 prior to addition of As on day 6. Seedlings were harvested after different times of exposure (2−24 h).

For the analysis of As accumulation and the determination of As effects on leaves, plants were grown in hydroponic culture[48] at 22 °C and short-day conditions (8 h day/16 h night). After 3−4 weeks, plants were transferred to 50 mL Falcon tubes, cultivated for another 10 days, and then treated by adding different As species as indicated either for 5 days (medium exchange after 72 h) or for 24 h, followed by 4 days of recovery.

As Measurements. The speciation of As in medium and plant samples was analyzed using an ion chromatograph (Dionex ICS-3000) coupled to an inductively coupled plasma mass spectrometer (XSeries 2, Thermo Scientific) using an AS16 column (Dionex AG/AS16 IonPac column; 2.5−100 mM NaOH; flow rate, 1.2 mL/min)[50] and oxygen as the reaction cell gas (AsO^+, m/z 91). For speciation analysis of tissues, about 0.01−0.08 g of frozen plant material was ground and transferred into 2 mL tubes containing 0.4 g of glass beads. For extraction of arsenic species, 1.5 mL of PBS buffer (2 mmol L^{-1} of NaH_2PO_4 + 0.2 mmol L^{-1} of Na_2-EDTA, pH 6) was added. Samples were boiled for 5 min, cooled on ice for 2 min, and then vortexed for 53 min under anaerobic conditions. The extract was filtered using 0.2 μm cellulose-acetate filters (Macherey-Nagel), immediately followed by As speciation via IC-ICP-MS analysis as described above.[18,20]

The analysis of As total plant content was performed using an inductively coupled plasma mass spectrometer (AsO^+, m/z 91) using rhodium (Rh+, m/z 103) as an internal standard. Root material was washed first at 4 °C for 10 min each with Milli-Q water, two times with 20 mM $CaCl_2$, 10 mM EDTA (pH 5.7), and again Milli-Q water. Samples were then dried at 60 °C for at least 3 days, weighed, and microwave-digested (START 1500, MLS GmbH) using 65% HNO_3 and 30% H_2O_2 solutions.

Thiol Analysis. Thiols were extracted from the material stored at −80 °C using 3 volumes of extraction buffer [trifluoroacetic acid (0.1%, v/v) + 6.3 mM diethylene triamine pentaacetic acid (DTPA) + 40 μM of the internal standard *N*-acetylcysteine] per milligram of fresh weight. Extracts were labeled using 50 mM monobromobimane in a master mix containing 200 mM EPPS + 6.3 mM DTPA (pH 8.2) and 20 μM tris-(2-carboxyethyl)-phosphine dissolved in 200 mM EPPS (pH 8.2) at 45 °C for 30 min. The reaction was stopped by the addition of 1 M methanesulfonic acid. Analysis of GSH, PC2, and PC3 was performed by HPLC as described elsewhere.[49]

ROS Staining of *A. thaliana* Roots. Formation of ROS was analyzed by labeling with 25 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) in 1× PBS buffer (pH 7.4) for 15 min. For cell wall integrity and cell death assessment, propidium iodide staining solution (10 mg/L) was used, and seedlings were incubated for 2 min. For analysis of fluorescence signals, H_2DCFDA and propidium iodide were visualized with a confocal laser-scanning microscope (Leica TCS SP5 Microsystems). H_2DCFDA emission was detected in the range of 522−543 nm and propidium iodide in the range of 590−670 nm. The excitation wavelength for H_2DCFDA was 488 nm and for propidium iodide 514 nm. Confocal images were analyzed using the function Plot Profile in ImageJ.[51] Each single profile was processed with the OriginPro 2017 software (OriginPro). The resulting profiles contain the absolute fluorescence intensity detectable from the root tip up to 260 μm from the root tip with a resolution of ca. 2 μm. Red fluorescent protein (RFP) emission was detected at 575−637 nm with an excitation wavelength of 561 nm. Data were graphically rendered using R software (R Core Team, 2021) and Adobe Photoshop CS6 Version 13.0 × 64 (Adobe Photoshop CS).

Extraction and Quantification of Leaf Pigments. Leaf samples were harvested after hydroponic culture with short-term exposure for 24 h and 4 days of recovery. Chlorophyll and carotenoids were extracted with 80% acetone overnight at 4 °C and constant shaking. Quantification was performed using a spectrophotometer (SPECORD 200 PLUS, Analytik Jena AG) and respective equations.[52] Anthocyanins were extracted using an adaptation of a protocol[53] with acidified methanol. Extraction was performed by the addition of methanol solution to ground samples, followed by inverting and centrifuging at 4 °C and 18,000*g* for 5 min. Quantification was done by photometric analysis of extracts at A_{530} and A_{657} and normalization to fresh weight.

RNA Extraction and qRT-PCR Analysis. RNA was extracted using TriSure (Bioline). The RNA concentration was determined with a nanophotometer. For qRT-PCR analysis, an equal amount of RNA for each sample was treated with DNase I (Thermo Scientific) and reverse-transcribed using PrimeScript RT Master Mix (Takara). Primers were designed with the Primer3 web tool[54] and validated using Beacon Designer Free Edition tool (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1). A list of the primers used is provided in Table S2. qRT-PCR analysis was performed in 96-well plates using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and CFX Connect Real-Time system equipment (Bio-Rad). Bio-Rad CFX Maestro software was used to retrieve and analyze the data. Transcript level was calculated as the "relative expression" ($\Delta\Delta C_t$ method).

RNA-Seq Analysis. Prior to RNA-seq analysis, RNA was cleaned using an RNeasy Mini Kit (QIAGEN). The RNA quantity was measured using a Qubit-Photometer (Invitrogen), and the quality was checked using a Fragment Analyzer (Agilent 2100 Bioanalyzer). Strand-specific libraries were prepared from total RNA (NEBNext Ultra II Directional library preparation kit) after PolyA-selection (NEBNext Poly(A) mRNA Magnetic Isolation Module). The average insert size was 350 bp. Libraries were sequenced on the Illumina NovaSeq platform using paired-end mode (2 × 150 bp). Library preparation, sequencing, adaptor trimming, and filtering of low-quality reads were performed by GENEWIZ. For further analyses, Geneious-Prime software (version 2021.2.2) was used. Raw sequence reads were trimmed and mapped onto the *A. thaliana* genome (TAIR 11). The same software was used to identify differentially expressed genes (DEGs), applying a $\log_2$ fold change equal to or lower than −1 or equal to or higher than 1 and an adjusted *p*-value cut-off of 0.05. Subsequent analyses were performed with R (R Core Team, https://www.r-project.org/). RNA-seq data were deposited in the genomics data repository GEO (https://www.ncbi.nlm.nih.gov/geo/) (acc. no. GSE201786).

GO term enrichment analyses were performed using Panther.[55] Only terms with a false discovery rate (FDR) < 10^{-5}, annotated gene number ≥ 40, and an enrichment value >

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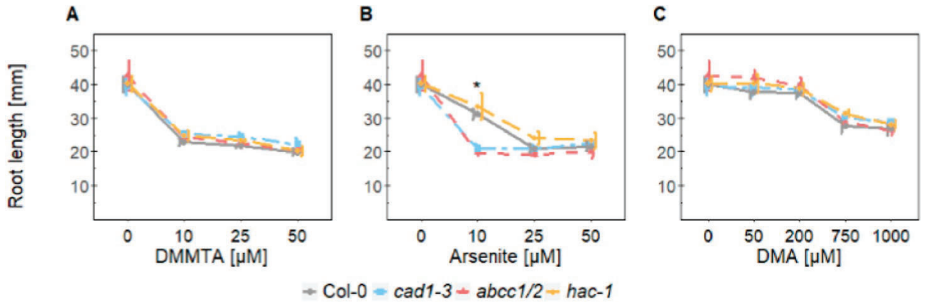

Figure 1. Growth-inhibitory effects of DMMTA on *A. thaliana* seedlings and the role of known As tolerance mechanisms. Plants were cultivated for 9 days in the liquid seedling system. Different concentrations of DMMTA (A), arsenite (B), and DMA (C) were added on day 6 for 24 h, followed by a recovery phase. The wild type Col-0 (grey symbols) was compared to the PCS mutant *cad1-3* (blue symbols), the *abcc1/2* mutant lacking the two most important As-PC transporters (red symbols), and the arsenate reductase-deficient *hac1* mutant (yellow symbols). Please note that the root length at the onset of exposure was about 20 mm (see Figure S2). Data of three replicates with a total of 34−40 plants. Statistical analysis was performed by a Kruskal Wallis test with a post hoc test ($p < 0.05$); * highlights statistically significant differences between the PC pathway mutants and the two other genotypes.

1.5 were considered. For each term hierarchically organized in a category, only the most specific one was taken.

Raw data of available experiments[56−58] submitted to GEO were downloaded and analyzed. For analysis of cellular localization, DEGs were sorted into clusters according to previously published data.[59,60] Longitudinal and radial clusters of genes were defined based on a spatiotemporal map of the *A. thaliana* root[59] and a correlation heatmap was created. In addition, DEGs were sorted into clusters based on a single-cell *A. thaliana* root atlas.[60] Cluster definition, data analysis, and graphical representation were performed using R software (R Core Team, 2021, https://www.r-project.org/).

Statistical Analysis. Statistical analysis was performed with R studio. General characteristics of boxplots are as follows: the box of each boxplot shows the interquartile range (from the 25th percentile to the 75th percentile) with the horizontal black line indicating the 50th percentile (Median). Black vertical lines at ends of boxes indicate the largest or lowest values within the 1.5 time interquartile above the 75th percentile or below the 25th percentile. Black dots represent outliers (>1.5 times and <3 times the interquartile range beyond either end of the box). Statistical analysis was performed by a Kruskal Wallis test with a post hoc test using Fisher's least significant difference and a p value of 0.05. "Bonferroni" was used for adjusting the p values.

RESULTS AND DISCUSSION

DMMTA Stability in Growth Medium. Stability of the synthesized DMMTA was analyzed first in 1/10 Hoagland medium. Transformation was checked in both assay systems at two time points (24 and 72 h) and three different concentrations. Like in a previous study employing different conditions,[18] DMMTA was found to be stable in medium alone (Figure S1). Even in the presence of plants, only a minor conversion of DMMTA to DMA was detected in either assay system. Conversion of DMMTA was highest in samples from liquid seedling assays but never exceeded 6%.

Repression of *A. thaliana* Root Growth by DMMTA. Root growth assays are an efficient tool to assess the toxicity of a compound and to identify mechanisms and pathways involved in coping with stress factors. We tested the toxicity of DMMTA first in a liquid seedling assay. Seedlings were treated on day 6 for 24 h and allowed to recover until day 9. A comparison of root lengths showed a concentration-dependent inhibition of growth by DMMTA at concentrations from 10 to 25 μM and nearly complete growth arrest upon exposure to 50 μM DMMTA (Figure 1A) (please note that root lengths at the onset of exposure were about 20 mm; see Figure S2). Equivalent arsenite concentrations were less inhibitory (Figure 1B). The difference was most pronounced at 10 μM with <50% inhibition by arsenite and about 80% inhibition by DMMTA. In contrast, DMA was much less growth-inhibitory. Even in the presence of 1000 μM DMA, roots were able to grow (Figure 1C). These observations demonstrate that, like for human cells and animals, also for plants, DMMTA is far more toxic than other pentavalent As species and even slightly more toxic than arsenite,[26,27,61] which, to date, has been regarded the most problematic As species plants are exposed to. Accordingly, germination assays suggested higher toxicity of DMMTA relative to that of arsenite as well. The emergence of cotyledons was completely inhibited in the presence of 25 μM DMMTA, while some seedling growth was still detectable in the presence of an equal concentration of arsenite (Figure S3A).

Highest DMMTA concentrations in soil pore water reported to date are around 0.5 μM,[14] that is only about 1 order of magnitude lower than the concentrations chosen here to enable the detection of rapidly developing toxicity symptoms. This suggests that acute DMMTA toxicity may sporadically occur.

ROS Accumulation and Morphological Changes in Roots after As Exposure. Specific primary targets of As toxicity are not known in any eukaryotic organism. One major mechanism hypothesized to be involved in As toxicity is the formation of ROS.[2,62] We therefore assayed ROS formation in roots exposed to DMMTA, arsenite, or DMA. Strong ROS accumulation was apparent in cortex cells of roots treated for 4 h with 25 μM arsenite (Figure 2). A similar picture was observed after 24 h (Figure S4). Pattern and intensity of ROS staining were clearly different for roots exposed to DMMTA. No ROS accumulation was detected in cortex cells. The only quantitative difference to untreated roots was a slight fluorescence increase in the top 100 μm in roots treated with 10 μM DMMTA. DMA had little effect on roots even at much higher concentrations. The pattern of staining was indistinguishable from that of untreated roots. Propidium iodide staining did not reveal any signs of cell death for any of the treatments (Figure S5).

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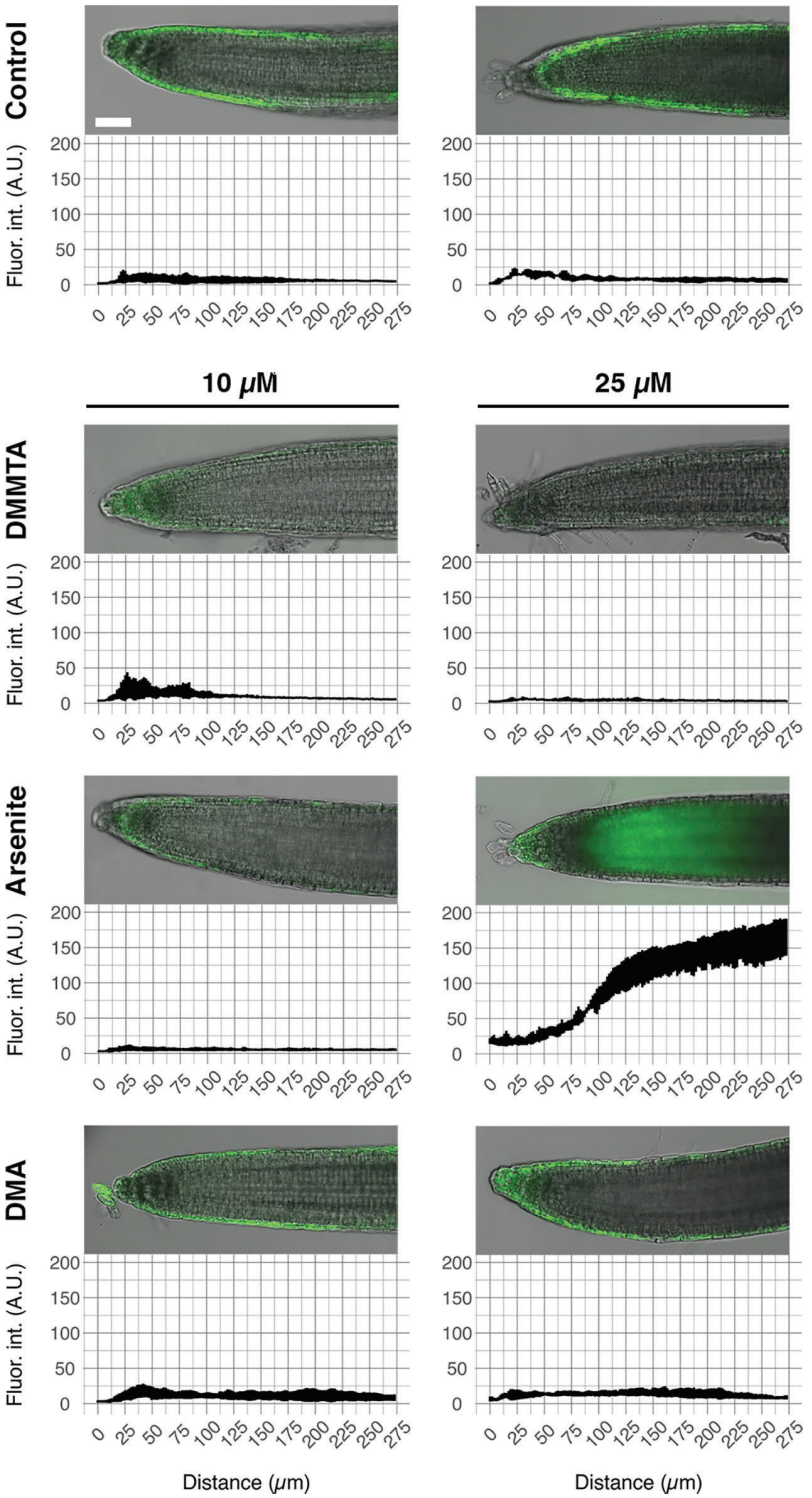

Figure 2. Quantification of ROS accumulation in Col-0 root tips after 4 h of exposure to 10 and 25 μM DMMTA, arsenite, or DMA. Roots were stained with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). The signal strength in the confocal microscopy images (9−12 per condition) was quantified along 275 μm of the root tip. Representative images are shown.

An effect specifically observed in DMMTA-treated roots was a deformation of root epidermal cells, which strongly increased in size (Figure 3A). This happened predominantly in the elongation zone and was never seen under any other condition. In dose−response and time-course experiments, the effect was clearly discernible in the presence of 2 μM DMMTA and as early as 6 h when treated with 5 μM DMMTA (Figure 3B,C). We made use of the reporter line RFP:AtSYP51[63] to visualize the tonoplast of root cells. Imaging showed a massive expansion of the vacuoles in the deformed cells (Figure S6).

Transcriptome Changes in Response to DMMTA. Several studies identified As-responsive genes and gene

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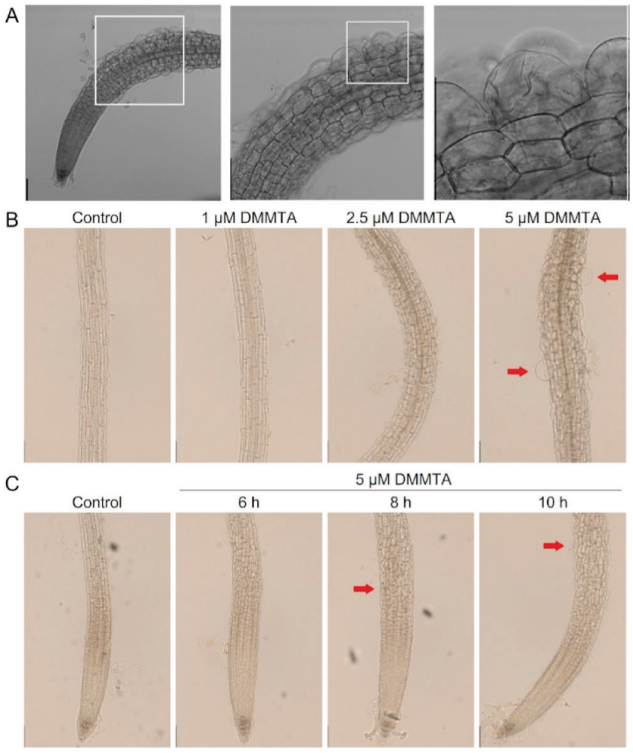

Figure 3. DMMTA exposure causes a deformation of root epidermal cells. (A) Confocal images of a root tip treated with 10 μM DMMTA for 24 h. Highlighted are abnormally shaped epidermal cells. (B) Dose−response analysis documented with light microscopic images. In (C), a corresponding time-course analysis is shown. Red arrows indicate thickening of roots (C) and abnormally shaped single cells (B,C). Scale bars: 100 μm.

networks via expression and transcriptome analyses in the model organisms *A. thaliana*[57,64] and rice.[65] In order to gain insights into genome-wide responses to DMMTA, we performed RNA-seq analyses on seedlings in a liquid culture, treated for 6 h with 15 μM DMMTA. Responsiveness under these conditions had been tested in qRT-PCR pilot experiments targeting two strongly As-activated genes, namely, *DRG1-3* (AT1G72660) and *RBL14* (AT3G17611).[57] Equal concentrations of arsenite and DMMTA both elicited a massive up-regulation of the marker genes (Figure S7). RNA-seq data analysis revealed 2508 DEGs in DMMTA-treated seedlings. 1367 DEGs were up-regulated, and 1141 DEGs were down-regulated (Figure 4A). Strongest responses were detected for several small heat shock protein genes and a few genes known to be upregulated under biotic stress (e.g., *PIP3*). For five genes, the RNA-seq results were confirmed by qRT-PCR (Figure S8). GO terms enriched among the DEGs were mostly related to stress responses (Figure 4B). For example, among the biological process categories enriched in up-regulated DEGs were toxin metabolism, phenylpropanoid biosynthesis, GSH metabolism, and heat stress response (Figure S9). Cellular component categories enriched in down-regulated DEGs indicated changes in the extracellular space.

In order to compare the observed DMMTA responses with known arsenic effects in *A. thaliana*, we analyzed the published data sets.[56−58] In these experiments, different concentrations of arsenate were used (30, 100, 200, and 250 μM). First, an arsenic core response comprising 287 genes was defined as the overlap between the DEGs reported in all three studies. Fifty-five % of them were responsive to DMMTA as well (Figure S10). GO terms enriched among the DEGs that are part of the As core response and responsive to DMMTA as well are mostly categories related to protein folding, general stress responses, and sulfur metabolism (Figure S10). Next, we searched for DEGs apparently responsive only to DMMTA. A total of 1114 DEGs were not reported as arsenic-responsive in *A. thaliana* before (Figure S10). Two of the four GO terms enriched in this group are related to biotic stress responses.

Furthermore, making use of recently published transcriptome data with cellular resolution for *A. thaliana* roots, we analyzed the localization of DMMTA responses. Both approaches converged on detection of stronger responses in epidermal cells (Figure S11), which corresponds with the localized deformation of cells observed in DMMTA-treated roots (Figure 3).

Uptake, Translocation, and Shoot Toxicity of DMMTA. The observed toxicity of DMMTA and a pronounced transcriptional response had indicated uptake of DMMTA by *A. thaliana*. For arsenite and DMA, it is known that aquaglyceroporins mediate passage across the plasma membrane. In *A. thaliana*, NIP1;1 was shown to account for a substantial fraction of arsenite uptake.[66] We therefore tested a *nip1* mutant and a line overexpressing an RFP:NIP1;1 fusion protein[63] for arsenite and DMMTA tolerance. The expected effects could be observed for arsenite-treated seedlings, namely, better growth of the *nip1;1* loss-of-function mutant relative to that of Col-0 and higher sensitivity of an NIP1;1 overexpressor (Figure S12). In contrast, no significant differences between the genotypes were found after DMMTA treatment. Thus, NIP1 and possibly other aquaglyceroporins are apparently not involved in DMMTA uptake.

In order to study the root-to-shoot translocation of As upon DMMTA exposure, plants were grown in hydroponic culture. Again, DMMTA, arsenite, and DMA treatments were compared at two different concentrations. After 5 days of exposure to 10 μM, total As concentrations in roots were much higher in DMMTA-treated plants than in DMA-treated plants (about 10-fold) but significantly lower than after arsenite treatment (44-fold) (Figure 5A, Table S3). Upon addition of 50 μM to the medium, the relative differences were similarly high (Figure S13, Table S3), showing that, as for organic arsenicals in general, DMMTA uptake is lower compared to that of inorganic As.[44,67] It is, however, noteworthy that DMMTA was taken up more efficiently than DMA, which could in part explain the much lower DMA toxicity we observed. Corresponding differences in uptake rates for DMA and DMMTA were previously reported for rice plants,[18] suggesting similar membrane permeabilities for the two methylated As species in monocots and dicots.

In contrast to the findings for roots, shoot As accumulation was highest for DMMTA-treated plants at both tested concentrations (Figure 5A, Table S3). The translocation factors for the three tested As species were around 0.4 for DMMTA and DMA but below 0.002 for arsenite (Table S3), indicating efficient root-to-shoot transfer for both methylated forms. Experiments with rice plants had suggested a higher mobility of DMMTA than that of DMA, too, albeit indirectly. Both total As and xylem sap As were much higher in DMMTA-treated plants.[18]

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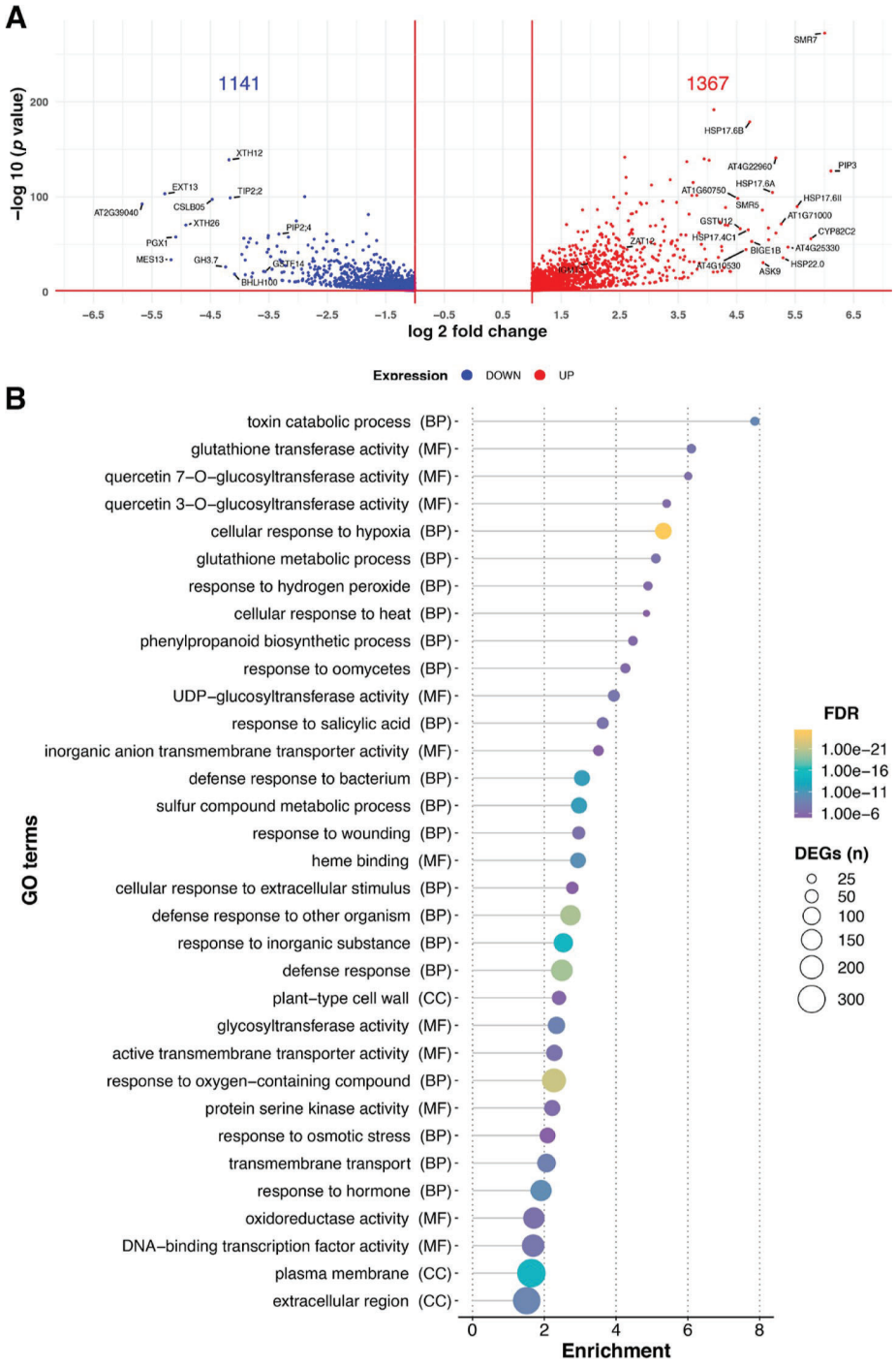

Figure 4. Genome-wide transcriptome changes in response to DMMTA exposure. Seedlings treated with 15 μM DMMTA for 6 h were analyzed by RNA-seq. (A) Volcano plot displaying the DEGs. (B) GO term enrichment analysis indicating fold enrichment and FDR. BP: biological process, CC: cellular component, MF: molecular function.

Next, we performed As speciation analysis to determine how much of the externally applied DMMTA remains intact inside plant organs. Root-to-shoot ratio of total As was again around 3. In roots, about one-third of the detected As was DMMTA. The rest was converted to either DMA or DMDTA (Figure 5B). Importantly, no arsenite was detected. The pattern in leaves was similar, with DMA being the dominant As species. About 25% of the total As was present as DMMTA.

Taken together, the data suggest DMMTA sequestration in roots at even lower rates than for DMA and comparatively high rates of transport across plant membranes. Transporters involved in the xylem loading of dimethylated As species (DMMTA and DMA) represent an open question awaiting further studies.[20,65,66]

The well-documented higher mobility of DMA relative to that of arsenite can be largely explained by the efficient trapping of arsenite in root cells through the PC pathway,[65] that is, PC synthesis, complexation, and transport of As-PC complexes into the vacuole. We therefore compared the thiol profiles of plants exposed to DMMTA, arsenite, and DMA.

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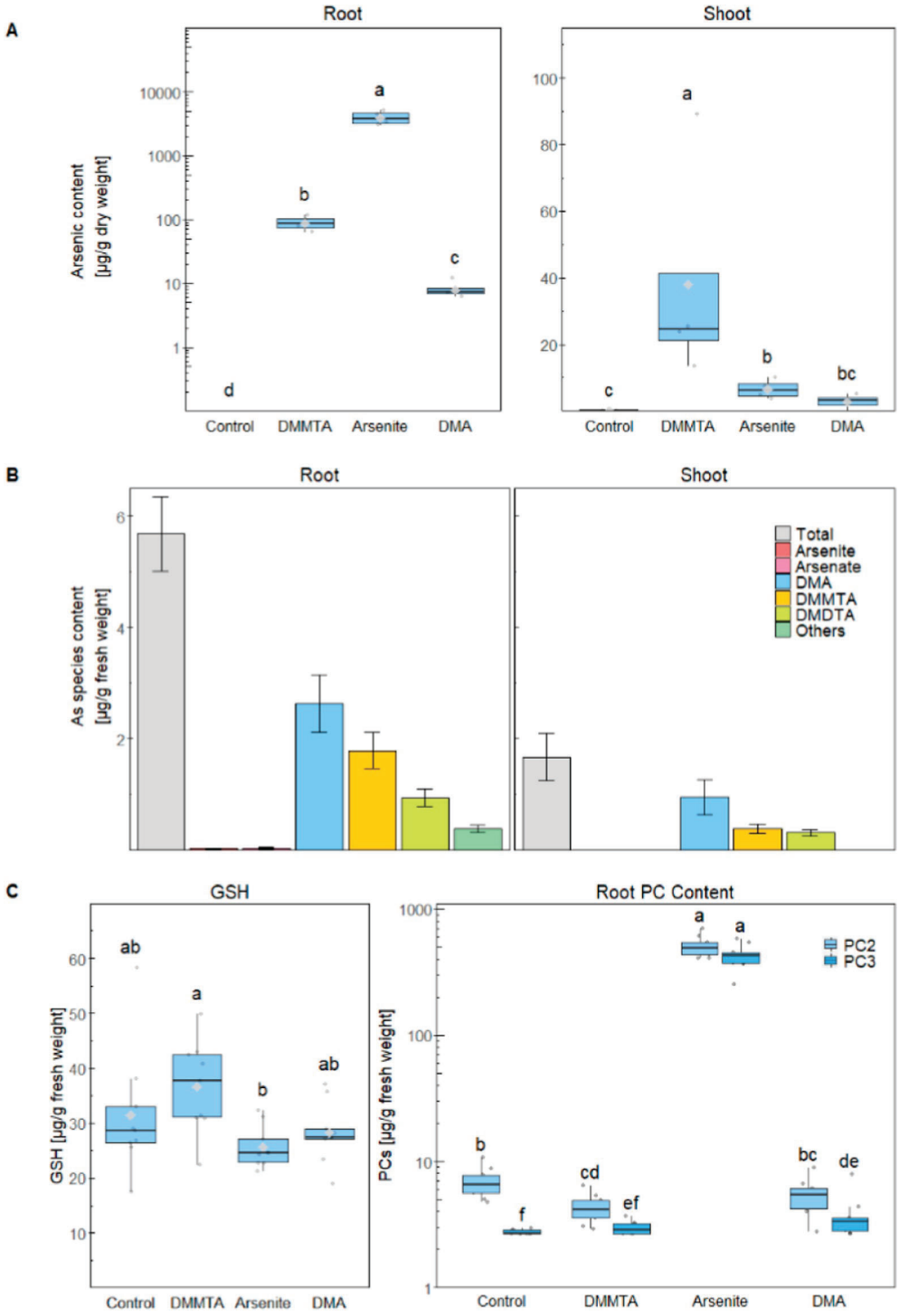

Figure 5. Arsenic accumulation, arsenic speciation, and thiol profiles in *A. thaliana* Col-0 exposed to different As species. Five- to six-week-old hydroponically grown plants were exposed to 10 μM for 5 days. Total As was determined separately in roots and shoots by ICP−MS analysis (A). Data represent three independent replicates with pools of 10 plants each. (B) For DMMTA-treated plants, As speciation in roots and shoots was analyzed by IC-ICP-MS (n = 5). Traces of trimethylarsine oxide, MMA, MMMTA, MMDTA, and three unknown species were detected (in roots) and are summarized as "others". (C) Concentrations of GSH and PCs (PC2 and PC3) were determined via HPLC. Data represent four independent replicates with three plants each. Mean values for each condition are indicated by a gray rhombus; statistical analysis by a Kruskal Wallis test with a post hoc test with $p < 0.05$; letters indicate statistically significant differences.

GSH concentrations (Figure 5C) were slightly elevated in DMMTA-exposed roots. This observation corresponded with the up-regulation of GSH metabolism genes and may be interpreted as indirect evidence for the complexation of DMMTA by GSH as reported once before in *Brassica oleracea*.[67] Complexes of DMMTA and GSH were also identified in human 8226/S multiple myeloma cell lines exposed to darinaparsin (dimethylarsino-GSH, DMA^{III}-GS)[68]

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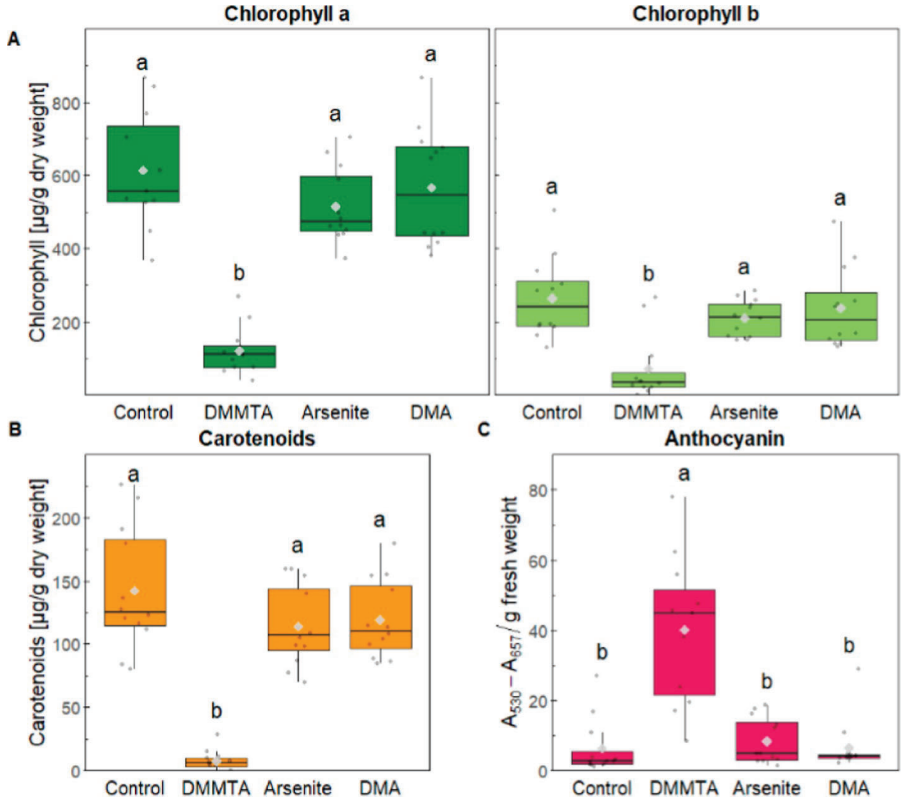

Figure 6. Pigment concentrations in *A. thaliana* Col-0 exposed to different As species. Five- to six-week-old hydroponically grown plants were exposed to DMMTA, arsenite, or DMA at a concentration of 50 μM for 24 h, followed by a recovery period of 4 d. Chlorophyll *a* and *b* (A), carotenoid (including xanthophyll) (B), and anthocyanin (C) content was determined by spectrophotometric analysis. Data represent four independent replicates (n = 11 or 12 plants total). Mean values for each condition are indicated by a gray rhombus; statistical analysis by a Kruskal Wallis test with $p < 0.05$; letters show statistically significant differences.

and in vitro.[69] Arsenite-treated roots expectedly[35,67] accumulated PC2 and PC3 (Figure 5C), while no PC accumulation above the background level was found in DMA- or DMMTA-treated roots. Thus, DMMTA, like DMA,[30] apparently does not activate PC synthases (Figure 5C). This is in line with the absence of any difference in DMMTA sensitivity between wild-type seedlings and PC pathway mutants (Figure 1A). Furthermore, the lack of PC accumulation in DMMTA-treated plants confirmed the As speciation data since any substantial conversion of DMMTA to arsenite would elicit PC synthesis.

Impact of DMMTA on Shoot Development. Shoot growth and development of hydroponically grown plants displayed severe alterations, indicating strong toxic effects of the translocated DMMTA. Exposure to each arsenic species for 24 h resulted in a curling of leaves specifically in DMMTA-treated plants, which was almost completely reversed after a 24 h recovery period in the control medium. Similar curling and rolling phenotypes of leaves represent a previously reported symptom of arsenic toxicity and are probably due to dehydration.[68] After 4 days of recovery, leaf pigment concentrations were analyzed for all treatments (Figure 6). Chlorophyll *a* and *b* content of arsenite- and DMA-exposed plants did not show differences from control conditions (Figure 6A). In contrast, DMMTA exposure led to a significant decrease in chlorophyll *a* and *b* content. The same was observed for carotenoids (Figure 6B), which were again not affected in arsenite- or DMA-treated plants. The comparatively minor impact of arsenite on photosynthetic pigments was reported before for rice.[69]

No recovery of growth was observed for DMMTA-exposed plants after transfer to the control medium. The arrest of shoot growth was accompanied by a darkening of leaves (Figure S14), suggesting the accumulation of anthocyanins, a well-known indicator of abiotic stress.[70] To quantify the effects, anthocyanins were extracted and analyzed. Concentrations were indeed found to be massively elevated in DMMTA-treated shoots of Col-0 (Figure 6C), while no effects of DMA were observed. Arsenite exposure resulted in a slight elevation of anthocyanin levels. When transcript abundance of the two stress marker genes *ZAT12* and *HSP17.4* was assayed, a strong up-regulation in the leaves of DMMTA-treated plants was apparent (Figure S15).

Long-term effects on plant fitness were investigated by growing plants in the presence of 1−3 μM DMMTA until seed set. Seed yield was not significantly affected relative to that of untreated controls (Figure S16). Germination rates of seeds harvested from DMMTA-exposed plants were not affected either (Figure S3B).

Significance of DMMTA Toxicity and Mobility. While direct comparisons of shoot sensitivity are not possible for the tested arsenic species because of different kinetics and rates of translocation, the observations made on the leaves of DMMTA-exposed plants all indicate severe stress caused by the presence of DMMTA. No arsenite was detected in the speciation analysis. Also, the typical activation of PC synthesis by arsenite was absent from the leaves of DMMTA-treated plants. At the same time, DMA, the species that some of the DMMTA is converted to, did not elicit toxicity symptoms and

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only a weak induction of stress marker genes. Taken together with the data showing root growth inhibition (Figure 1), DMMTA present in soils may cause toxic effects even under field conditions. Sensitivity is likely to be dependent on plant age as exposure to 3 μM DMMTA during the flowering stage did not cause a reduction in seed yield (Figure S16).

As emphasized earlier, methylated thioarsenates should be considered and studied as a major arsenic species.[71] The mechanisms underlying DMMTA toxicity have to date only been addressed in mammalian cells. A propensity to elicit strong ROS formation is one possible explanation.[58,71] Our data, however, suggest a minor role of ROS formation in DMMTA toxicity when comparing it to arsenite. Another lead is provided by the evidence for—previously unsuspected—binding of the pentavalent methylated thioarsenates to thiols in proteins and peptides.[22] Genome-wide transcriptome data presented here suggest strong effects of DMMTA on protein folding as indicated, for example, by the up-regulation of small heat shock protein genes, thus supporting the evidence derived from studies on mammalian cells.

The persistence and mobility of DMMTA inside plant tissues that we report here for *A. thaliana* are supported by recent data confirming the observation that DMMTA can be detected in rice grains.[14,20,21] The presence of DMMTA in edible plant tissues and organs raises serious food safety and regulatory issues, which arguably are more urgent than the toxicity of DMMTA for plants. Currently, limits exist only for inorganic As because arsenite is much more toxic than the other major species found in grains, DMA.[72] However, during routine analysis, DMMTA is converted to DMA and, thus, not detected even though it may be present in significant quantities.[20] This calls for adjustments of analytical guidelines as well as maximum allowed concentrations of the most toxic As species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c01206.

DMMTA stability in medium samples; inhibition of root growth by different arsenic species for *A. thaliana* wild-type and mutant seedlings; effects of arsenite, DMMTA, and DMA on seed germination; quantification of ROS accumulation in Col-0 root tips after 24 h of exposure to 10 and 25 μM DMMTA, arsenite, or DMA; propidium iodide staining of roots exposed to 10 and 25 μM DMMTA, arsenite, or DMA; visualization of the tonoplast in root cells exposed to DMMTA; relative transcript levels of the As-responsive genes *DRG1-3* and *RBL14* in *A. thaliana* Col-0 after exposure to different As species; confirmation of RNA-seq results by qRT-PCR for selected genes; GO term enrichment among DEGs in DMMTA-exposed *A. thaliana* seedlings; the arsenic core response and genes specifically induced by DMMTA; cellular localization of DMMTA responses in *A. thaliana* roots; the aquaglyceroporin NIP1;1 not contributing to DMMTA uptake in *A. thaliana* seedlings; arsenic accumulation in *A. thaliana* Col-0 exposed to different As species; DMMTA exposure inhibiting leaf growth; DMMTA exposure activating stress marker gene expression in leaves; effect of DMMTA exposure during flowering; quality of DMMTA synthesis; primers used for qRT-PCR analysis; and total arsenic contents and translocation factors (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Supporting Information

Environmental Science & Technology

Dimethylmonothioarsenate (DMMTA) is highly toxic for plants and readily translocated to shoots

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(19 pages, 16 figures, 3 tables)

Content.

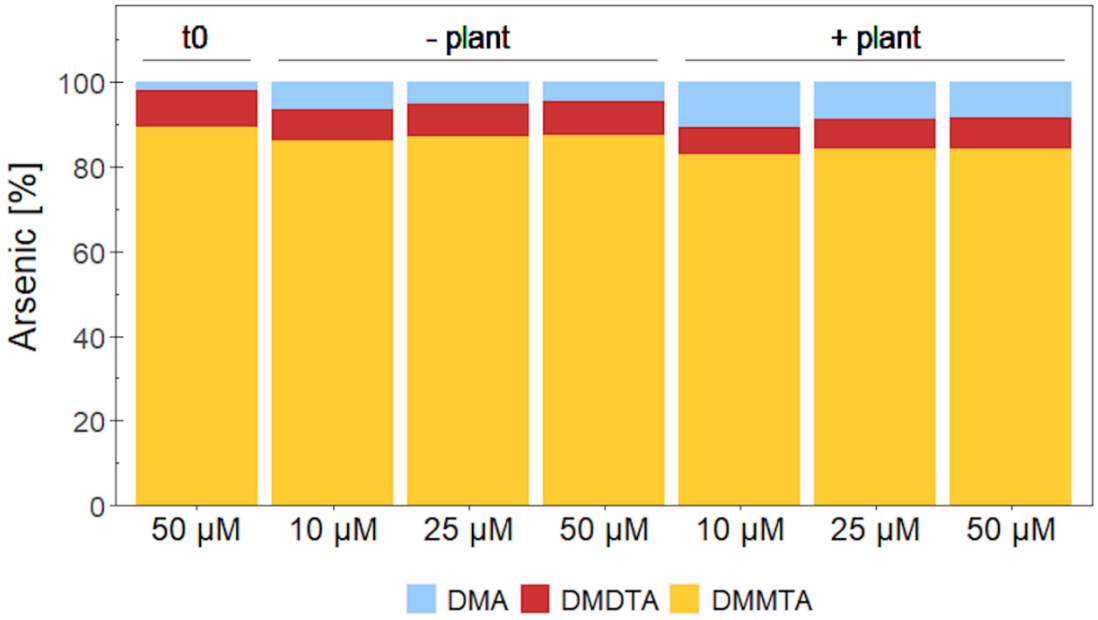


Figure S1. DMMTA stability in medium samples. Speciation analysis detected DMMTA, DMA and DMDTA in medium samples from liquid seedling assays. Time point t0 shows speciation at the beginning of the experiments. Transformation over time without plants in the medium (- plant) and with (+ plant) is shown. Shown are mean values from three replicates.

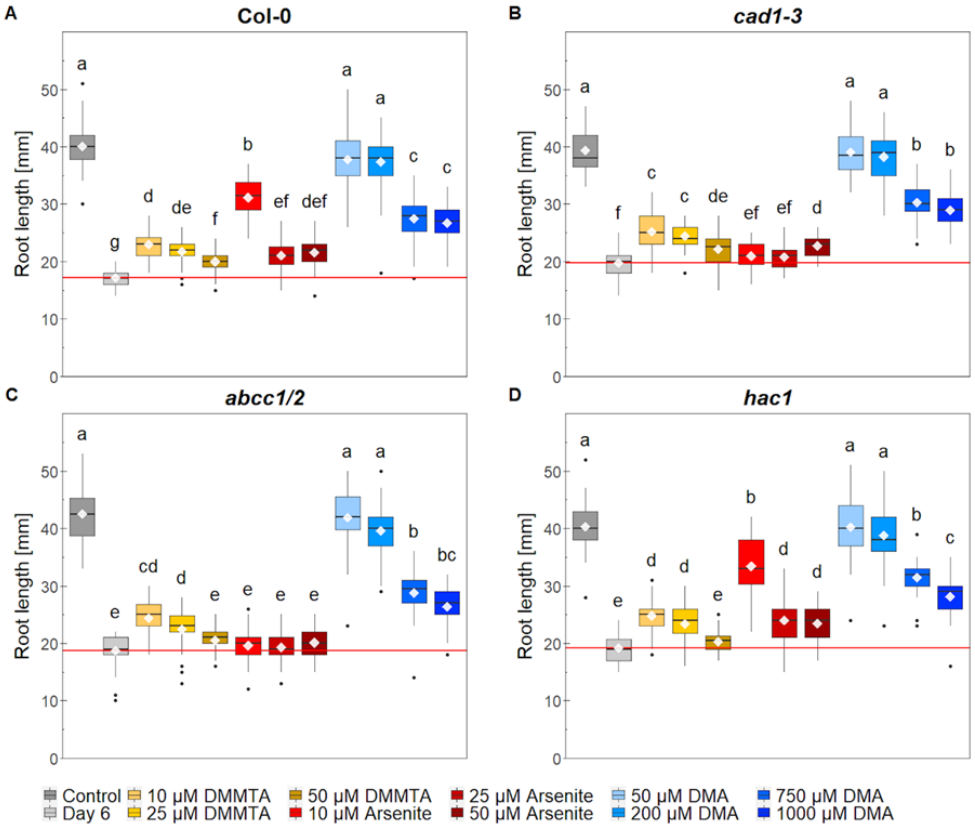


Figure S2. Inhibition of root growth by different arsenic species for *A. thaliana* wild-type and mutant seedlings. Plants were cultivated for 9 days in the liquid seedling system. Different concentrations of DMMTA (yellow bars), arsenite (red bars), and DMA (blue bars) were added on day 6 for 24 h, followed by a recovery phase. The light grey bar shows root length at the start of the exposure, the dark grey bar at the end of the recovery phase. The wild type Col-0 (A) was compared to the phytochelatin synthase mutant *cad1-3* (B), the *abcc1/2* mutant lacking the two most important As-PC transporters (C), and the arsenate reductase-deficient *hac1* mutant (D). The red horizontal line indicates the mean value of root length at day 6 (start of As exposure). A white rhombus indicates mean values per condition. Data of three replicates with in total 34-40 plants. Statistical analysis by Kruskal Wallis test with Post hoc test with $p<0.05$; letters show statistically significant differences. Please note that data are the same as displayed in Fig. 1.

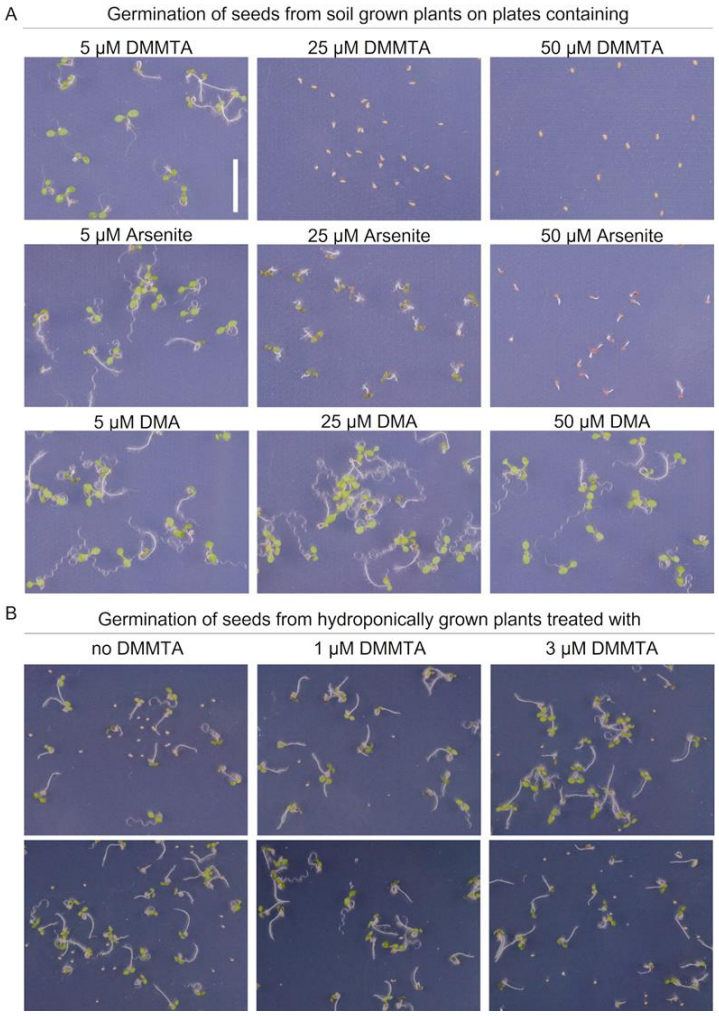


Figure S3. Effects of arsenite, DMMTA and DMA on germination. Seeds were germinated on 1/10 Hoagland medium with 1.5 % Type E Agar (Sigma-Aldrich) containing 5, 25 or 50 µM of DMMTA, arsenite or DMA (A). Germination on plates without arsenic was also tested (data not shown). Stratification was carried out at 4 °C for 2 days and germination took place under long day conditions (16h light, 8 h dark) for 5 days. Order of inhibition was as follows: DMMTA>arsenite>DMA at all tested concentrations. (B) Germination tests of seeds from hydroponically grown plants, that were treated with either 0, 1 or 3 µM of DMMTA during flowering stage. Experiments were performed as described above with seeds from two plants per treatment.

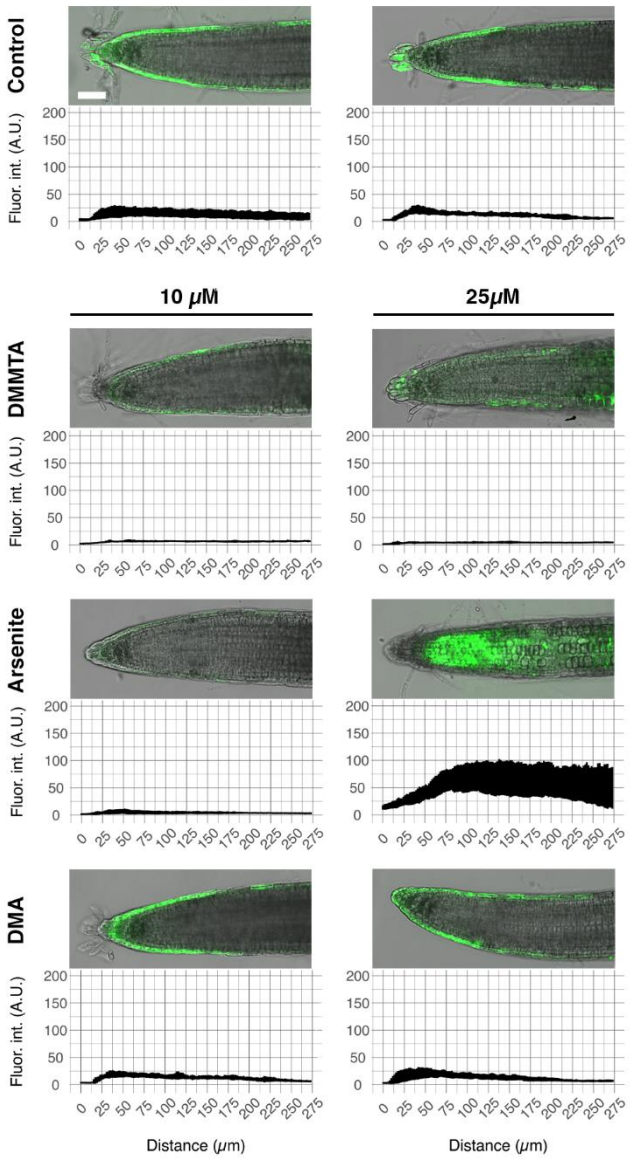


Figure S4. Quantification of ROS accumulation in Col-0 root tips after 24 h of exposure to 10 µM and 25 µM DMMTA, arsenite or DMA. Roots were stained with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Signal strength in confocal microscopy images (9-12 per condition) was quantified along 275 µm of the root tip. Representative images are shown.

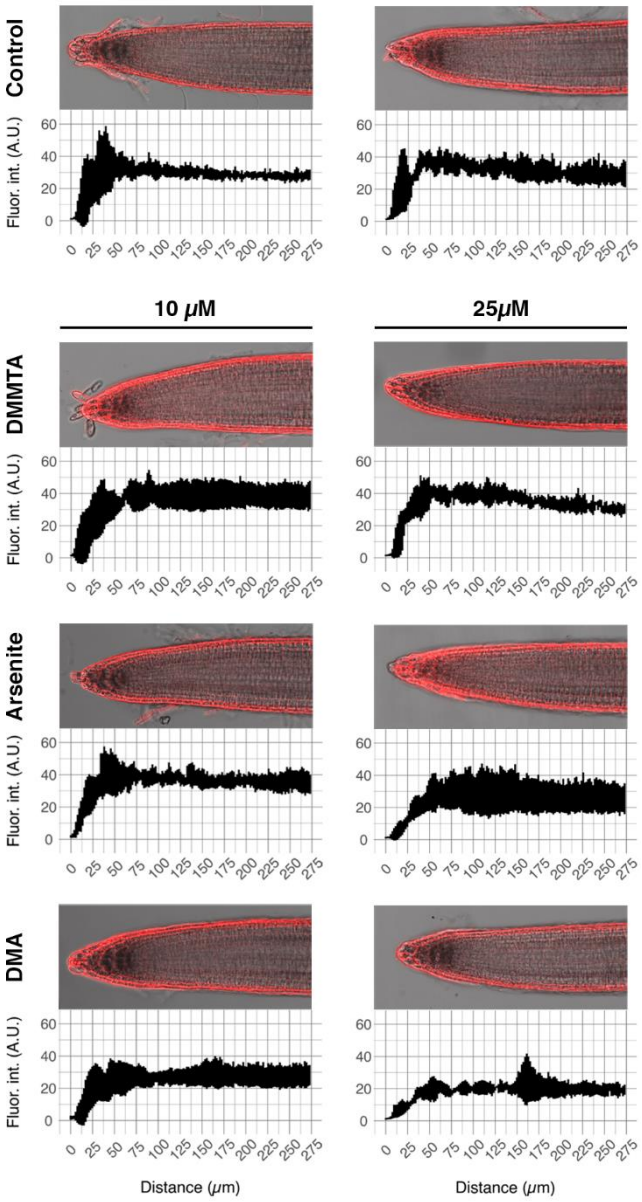


Figure S5. Propidium iodide staining of roots exposed to different As species. Staining was performed after 24 h of exposure to 10 µM and 25 µM DMMTA, arsenite or DMA. Signal strength in confocal microscopy images (9-12 per condition) was quantified along 275 µm of the root tip. Representative images are shown.

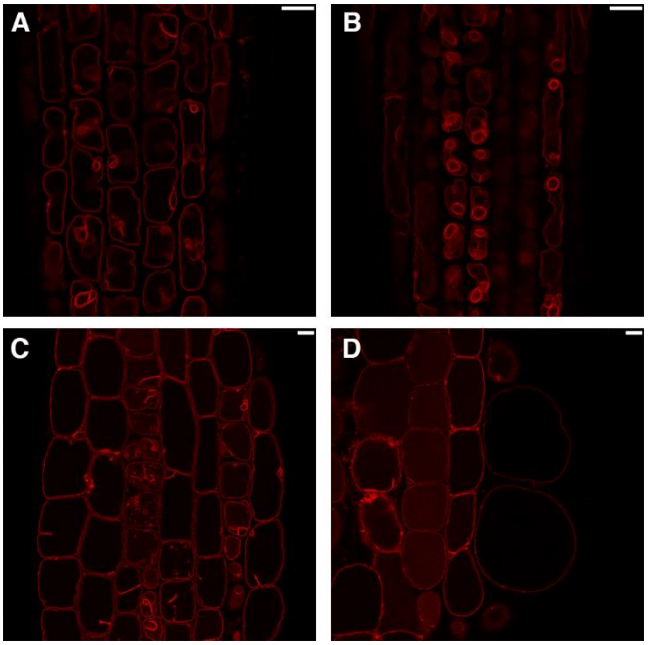


Figure S6. Visualization of the tonoplast in root cells exposed to DMMTA. An RFP::AtSYP51 line was imaged by confocal microscopy under control conditions (A, B) and after 24 h of exposure to 15 µM DMMTA (C, D) at two different magnifications. Scale bar: 10 µm

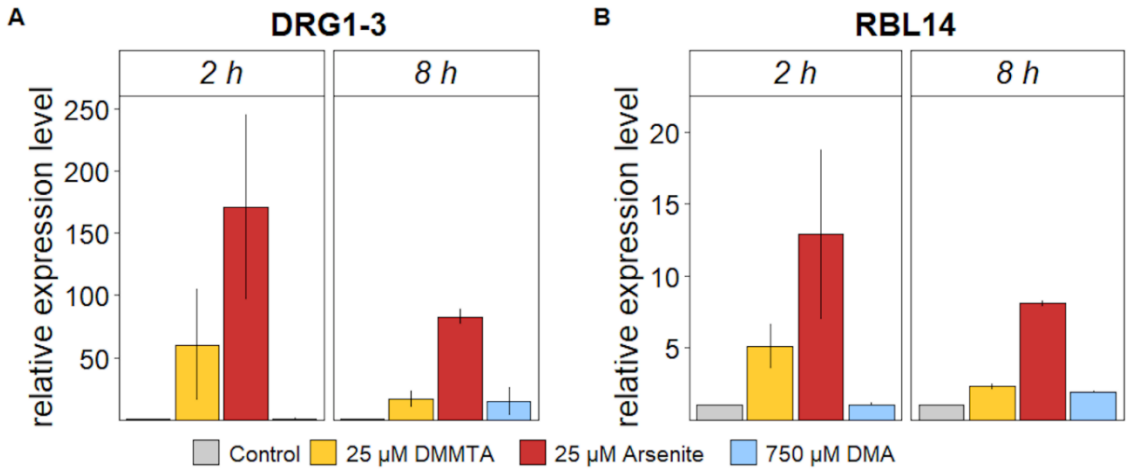


Figure S7. Relative transcript levels of the As-responsive genes *DRG1-3* and *RBL14* in *A. thaliana* Col-0 after exposure to different As species. Six days old seedlings cultivated in a liquid assay system were exposed for 2 and 8 h to either 25 µM DMMTA, 25 µM arsenite, or 750 µM DMA. Transcript levels were determined relative to *UBQ10* and *PP2A*. Data represent mean values +/- standard deviation for 2 replicates of pooled samples of about 35-45 seedlings.

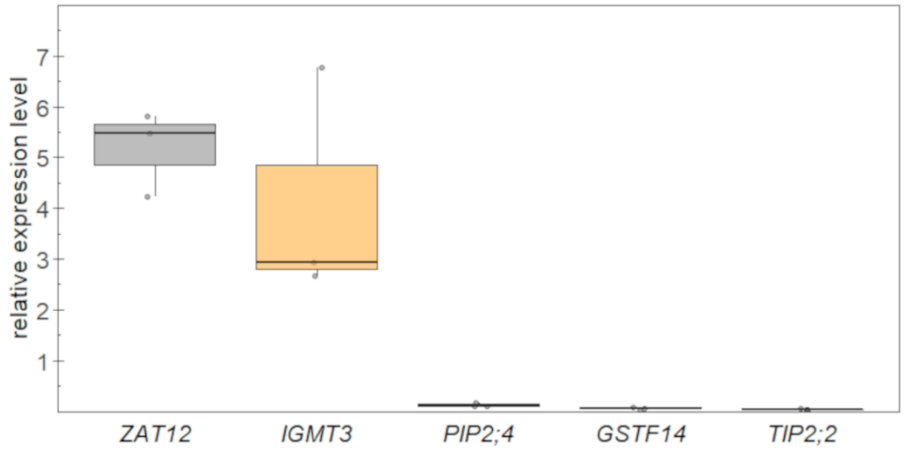


Figure S8. Confirmation of RNA-seq results by qRT-PCR for selected genes. Two strongly up-regulated genes (*ZAT12*, *IGMT3*) and three strongly down-regulated genes (*PIP2;4*, *GSTF14*, *TIP2;2*) were selected and assayed in three independent experiments. Relative expression levels are shown.

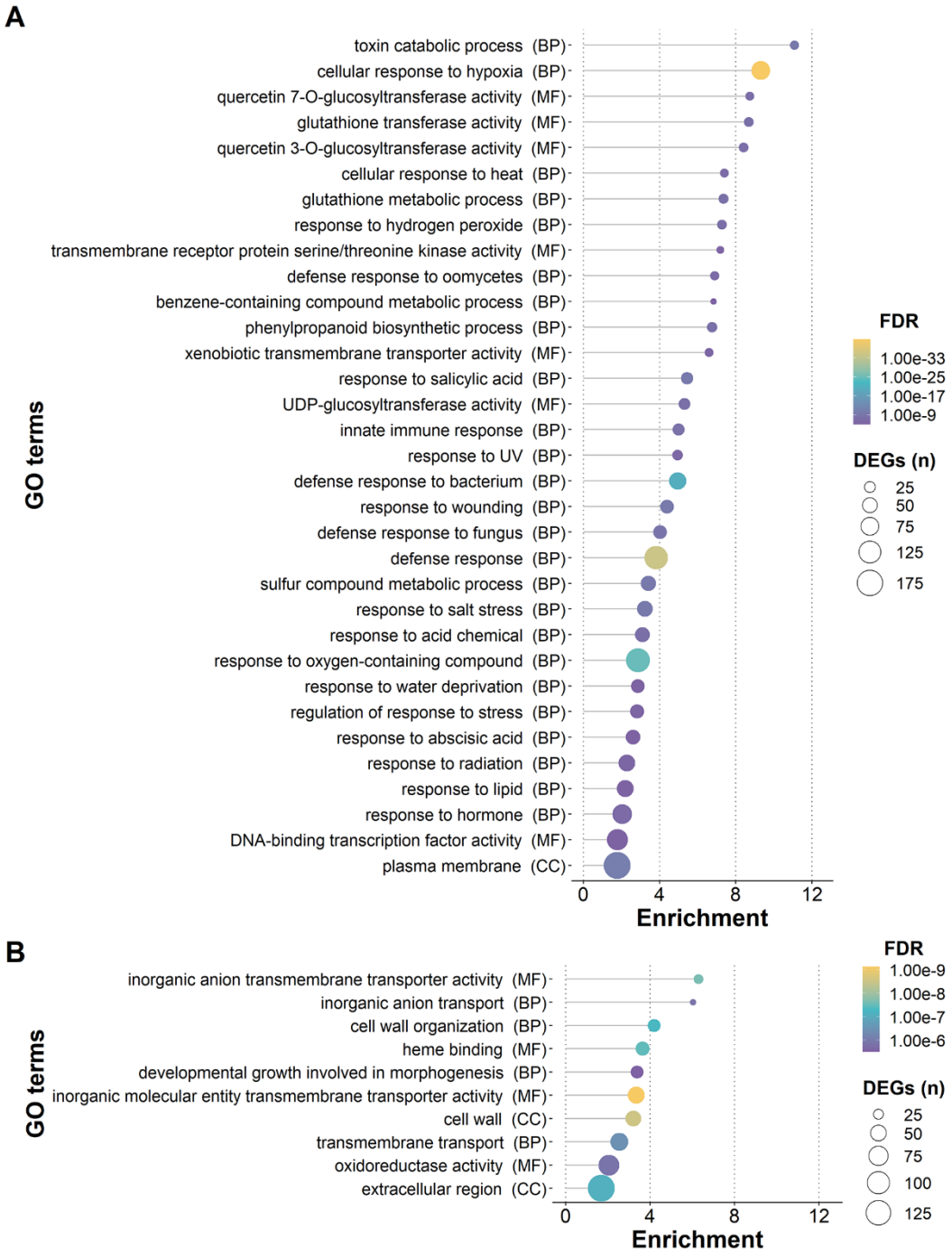


Figure S9. GO term enrichment among DEGs in DMMTA-exposed *A. thaliana* seedlings. GO term enrichment are shown separately for up-regulated (A) and down-regulated (B) DEGs. Seedlings treated with 15 µM DMMTA for 6 h were analyzed by RNA-seq. BP: Biological Process, CC: Cellular Component, MF: Molecular Function.

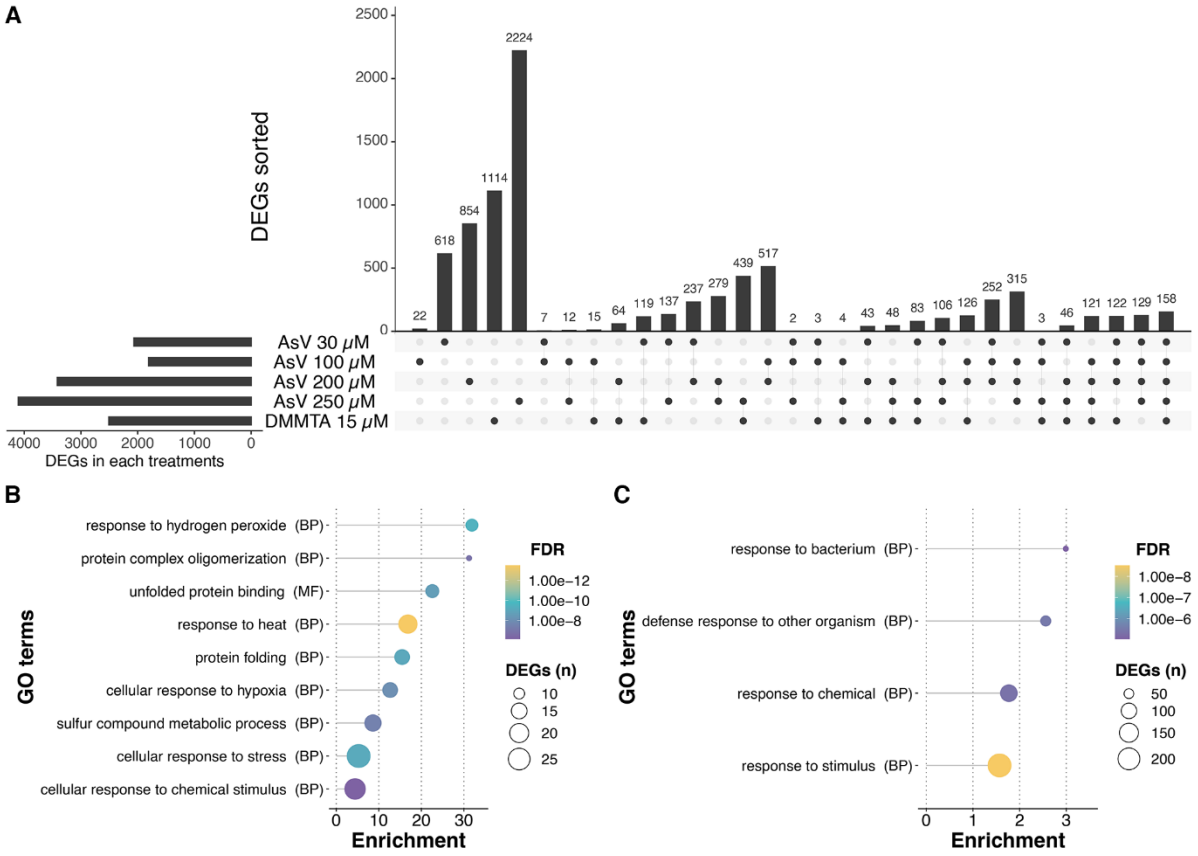


Figure S10. The arsenic core response and genes specifically induced by DMMTA. Available transcriptome data sets were downloaded from the GEO database and analyzed. (A) Venn analysis of DEGs showing each possible sorting group combination. (B) GO term enrichment analysis for the As core response genes that are responsive to DMMTA as well (158 genes, final column in A). (C) GO term enrichment analysis for the genes responsive to DMMTA but not reported as As responsive in published studies (1114 genes, fourth column from the left in A). BP = Biological Process, MF = Molecular Function, CC = Cellular Component.

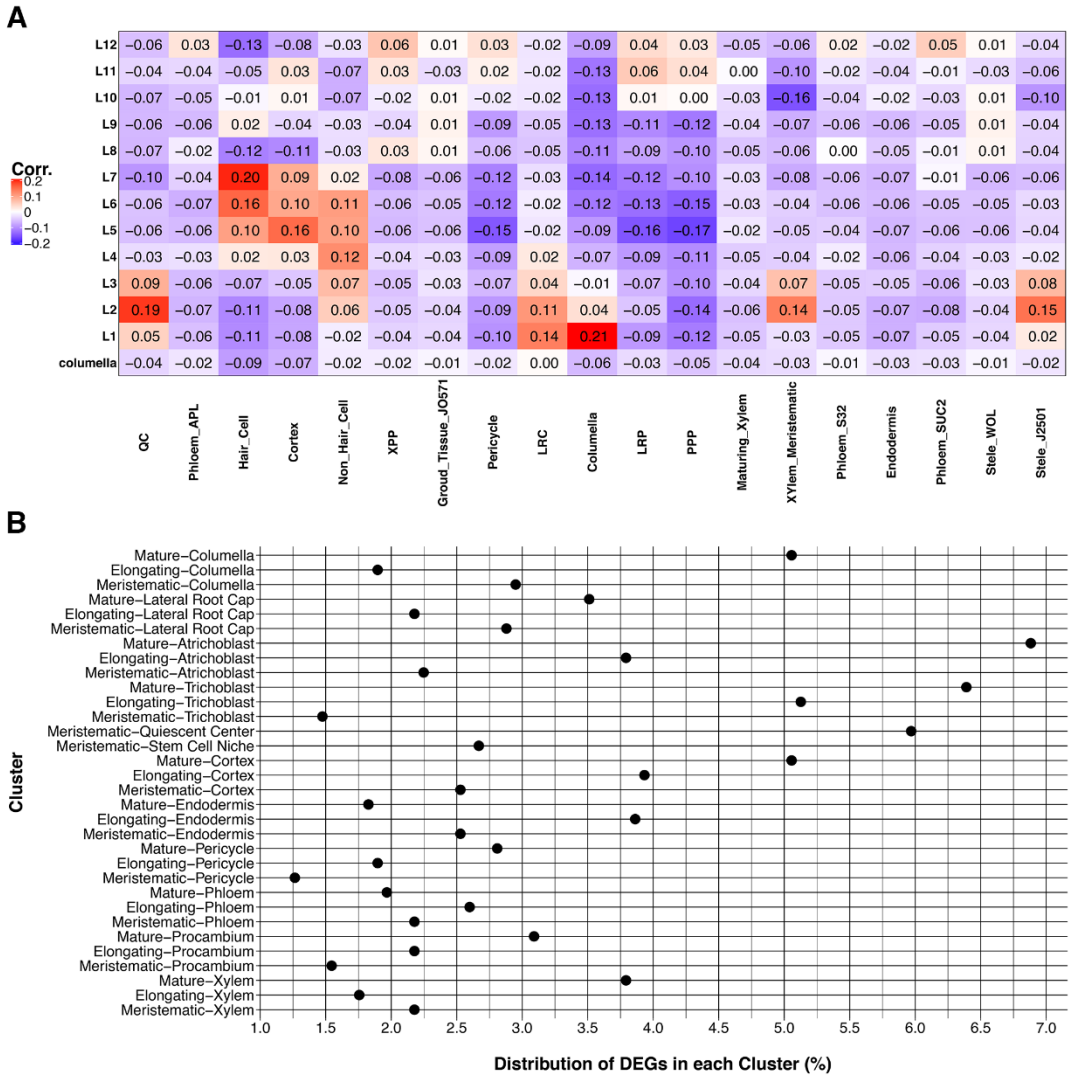
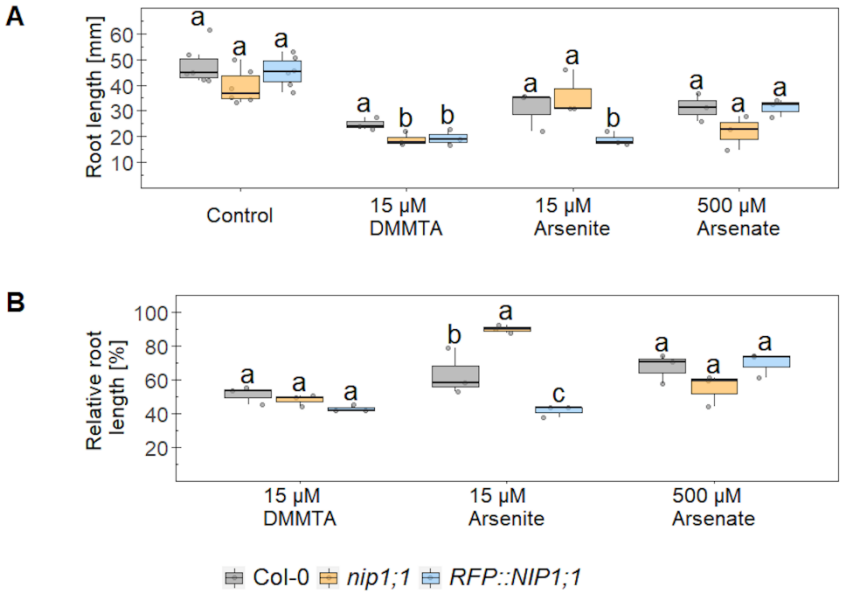


Figure S11. Cellular localization of transcriptional responses to DMMTA in *A. thaliana* roots. DEGs were sorted into clusters according to previously published data. (A) Longitudinal and radial clusters of genes were defined based on gene expression levels on a spatiotemporal map of the *A. thaliana* root published by Brady et al. (2007) and a correlation heatmap was created. (B) DEGs were sorted into clusters based on a single-cell *A. thaliana* root atlas (Shahan et a., 2022). Abundance of DMMTA-responsive DEGs in the different cell-type clusters is indicated.



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105 **Figure S12. The aquaglyceroporin NIP1 does not contribute to DMMTA uptake in *A. thaliana***
106 **roots.** Plants were cultivated for 9 days in the liquid seedling system. DMMTA, arsenite (both 15 µM)
107 or arsenate (500 µM) were added on day 6 for 24 h, followed by a recovery phase. In (A) absolute root
108 lengths are shown with light grey bars representing wild type Col-0, yellow for the loss-of-function
109 mutant *nip1;1* and blue for an overexpressor line of NIP1;1 (*RFP::NIP1;1*). In (B), relative root lengths
110 are shown for Col-0 and both *NIP1;1* mutants. Data of 3-6 replicates with in total 31-86 plants.
111 Statistical analysis by Kruskal Wallis test with Post hoc test and Benjamini-Hochberg correction with
112 $p<0.05$ for each treatment separately; letters show statistically significant differences.

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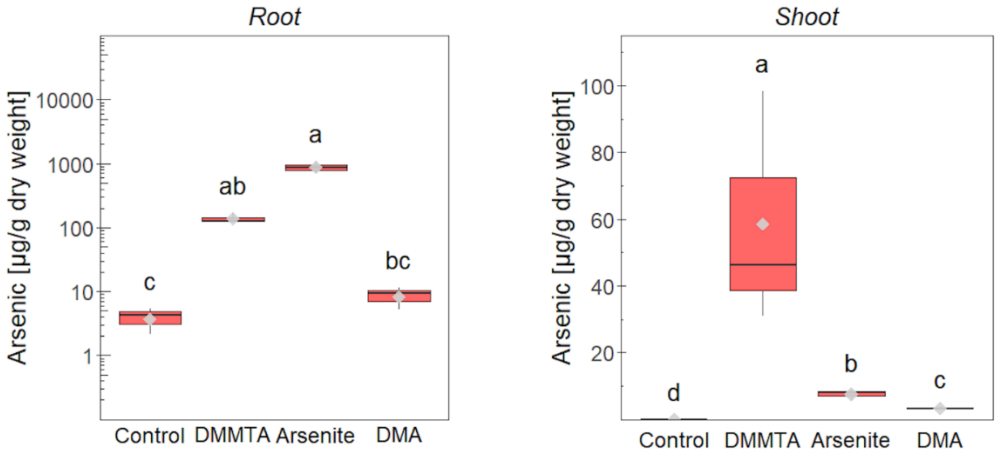

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116 **Figure S13. Arsenic accumulation in *A. thaliana* Col-0 exposed to different As species.** Five- to six-
117 week-old plants grown hydroponically were exposed to 50 µM for one day with 4 days of recovery.
118 Total As was determined separately in roots and shoots by ICP-MS analysis. Data represent 3 replicates
119 with pools of 5 plants each. Mean values for each condition are indicated by a grey rhombus and
120 statistical analysis by Kruskal Wallis test with Post hoc test with $p<0.05$; letters indicate statistically
121 significant differences.

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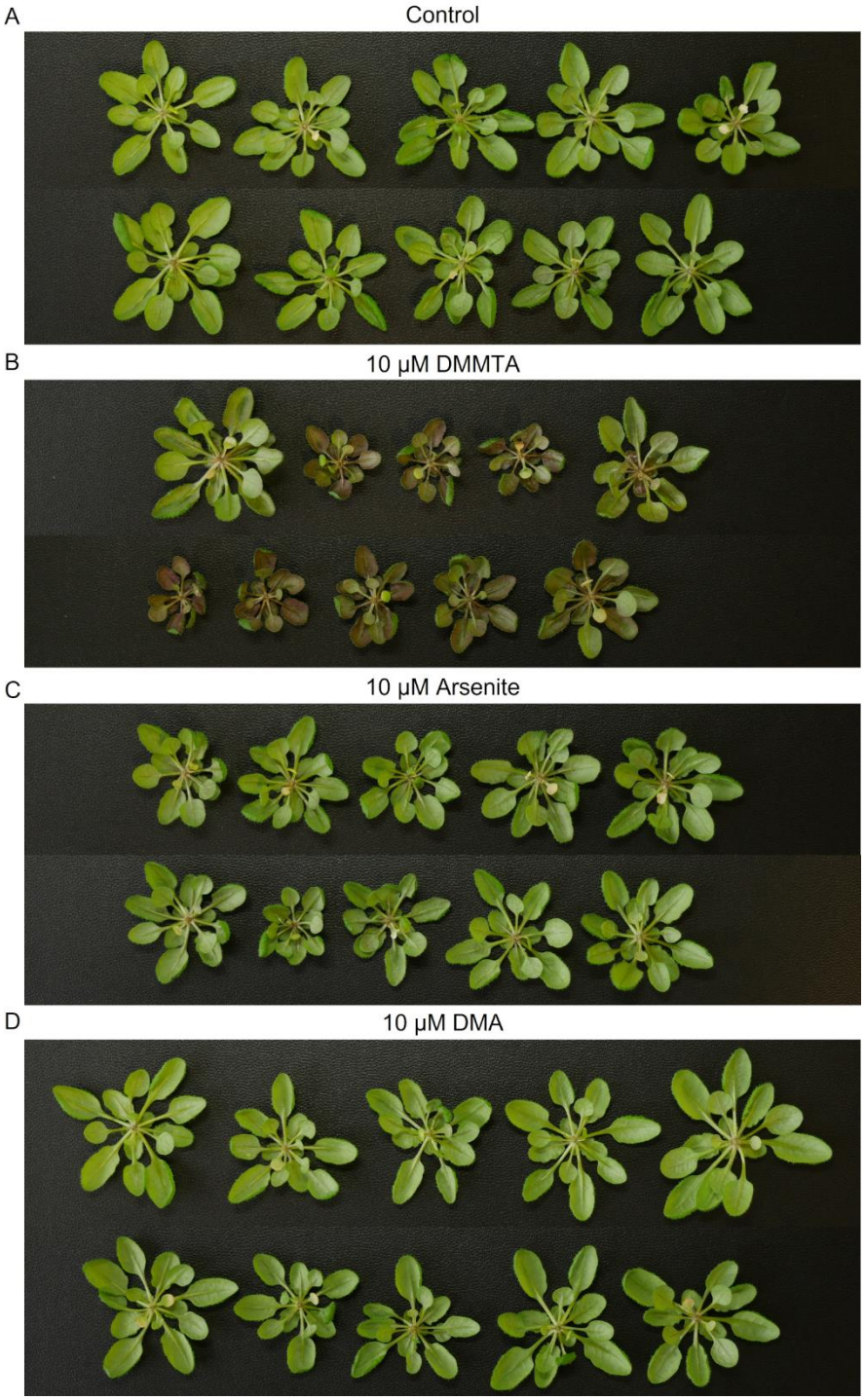

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124 **Figure S14. DMMTA exposure inhibits leaf growth.** Leaf phenotypes of 5-6 week old hydroponically

125 grown Col-0 plants exposed to 10 µM of DMMTA, arsenite or DMA for 5 days.

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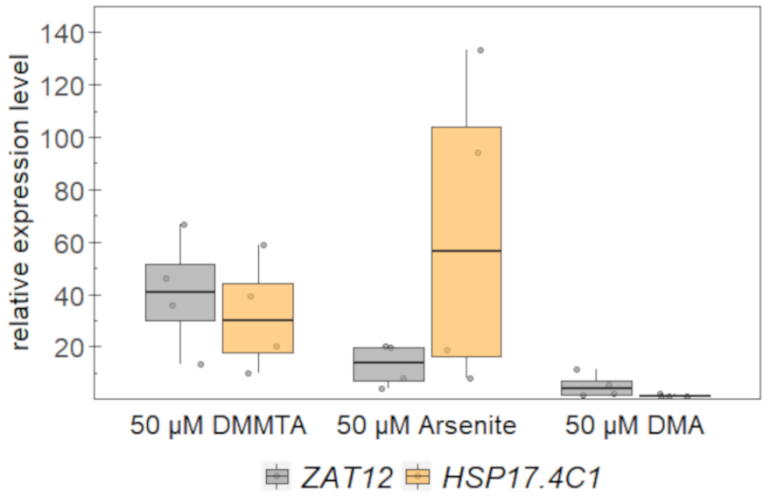

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128 **Figure S15. DMMTA exposure activates stress marker gene expression in leaves.** qRT-PCR
129 analysis of the stress marker genes *ZAT12* and *HSP17.4C1*. Relative expression levels are shown.Data
130 of three independent replicates.

131

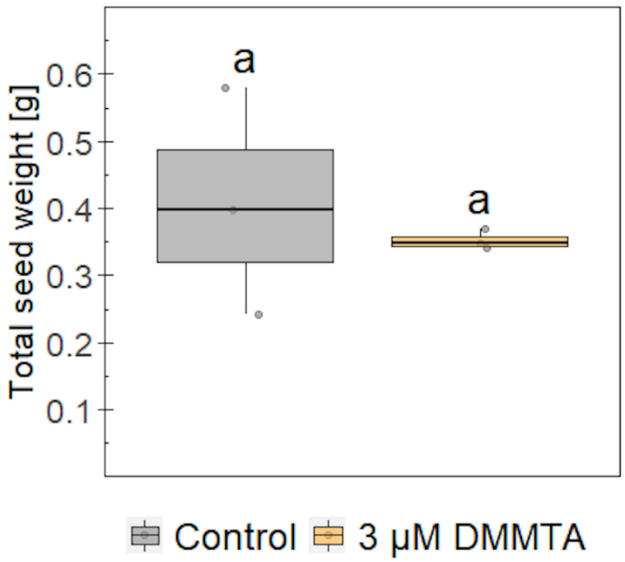

Figure S16. Effect of DMMTA exposure during flowering. Seed weight harvested from hydroponically grown Col-0 under control conditions or treated with 3 µM DMMTA during the flowering stage. Seeds of three plants per condition were weighed.

Table S1. Quality of DMMTA synthesis. Purity analysis by IC-ICP-MS.

Quality of DMMTA Stock Solutions				
	DMMTA [%]	DMA [%]	DMDTA [%]	As species sum [mM]
DMMTA stock 1	91.0	1.6	7.1	10.0
DMMTA stock 2	89.0	2.0	9.0	9.5
	90 ± 1.0	1.8 ± 0.2	8.1 ± 1.3	9.7 ± 0.4

Table S2. Primers used for qPCR analysis.

Primer name	Sequence (5' - 3')	Gene
DRG1-3fw	GCTCGGACGCAGAAGAACAAAGC	*DRG1-3*
DRG1-3rev	ACCATCCCCACCTCCACTAGAGC	
RBL14fw	GGACGGGCTAGAGGAAATGG	*RBL14*
RBL14rev	CTCTCTACTCTTCGACGGCG	
GSTF14fw	TTAGCCACAGACGATACAGCA	*GSTF14*
GSTF14rev	TTCACCAGCCAAGTAAGGAGA	
IGMT3fw	GATGTGGGAGGTGGAGTTGG	*IGMT3*
IGMT3rev	CGGGATAAGTGGGTGCTTGT	
PIP2;4fw	GCAGCAGCGTTTTACCATCAG	*PIP2;4*
PIP2;4rev	CTAAAGGAGCCAAAGGAGCCA	
TIP2;2fw	TTTGCCCTTTTCGTTGGTGTTT	*TIP2;2*
TIP2;2rev	CCGGTTATTACTGTGATGTTGCC	
ZAT12fw	CGTCGCATCCTTGTCCCATA	*ZAT12*
ZAT12rev	CCCACTCTCGTTCCTGTGTC	
HSP17.4C1fw	GTCAAGTGGGAAGTTCATGAGGA	*HSP17.4C1*
HSP17.4C1rev	CCATTCTCCATACTCGCCTTTACT	
UBQ9fw	TCACAATTTCCAAGGTGCTGC	*UBQ9*
UBQ9rev	TCATCTGGGTTTGGATCCGT	
PP2Afw	TAACGTGGCCAAAATGATGC	*PP2A*
PP2Arev	GTTCTCCACAACCGCTTGGT	

149 **Table S3.** Mean values of total As content of hydroponically grown *A. thaliana* Col-0. Root and shoot
150 content of long- and short-term treatments with 10 or 50 µM As, respectively. Ratio of difference
151 between short- and long-term exposure and translocation factor were calculated.

Tissue	Medium	As [µg/g dry weight]		Ratio short/ long term exposure	Translocation factor (Shoot/Root)	
		10 µM long term exposure	50 µM short term exposure		10 µM long term exposure	50 µM short term exposure
Roots	Control	0.00	3.96	-	0	0
Roots	DMMTA	89.51	139.09	1.55	0.39	0.44
Roots	Arsenite	3977.77	886.09	0.22	0.00	0.01
Roots	DMA	8.16	8.72	1.07	0.38	0.41
Shoots	Control	0.00	0.00	-	-	-
Shoots	DMMTA	37.92	58.65	1.55	-	-
Shoots	Arsenite	6.53	7.55	1.16	-	-
Shoots	DMA	2.81	3.27	1.17	-	-

152

Study 4: *In Planta* Arsenic Thiolation in Rice and *Arabidopsis thaliana*

Andrea E. Colina Blanco, Erik Pischke, Alejandra Higa Mori, Carolin F. Kerl, Stephan Clemens, and Britta Planer-Friedrich

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Article

In Planta Arsenic Thiolation in Rice and *Arabidopsis thaliana*

Andrea E. Colina Blanco,[§] Erik Pischke,[§] Alejandra Higa Mori, Carolin F. Kerl, Stephan Clemens, and Britta Planer-Friedrich*

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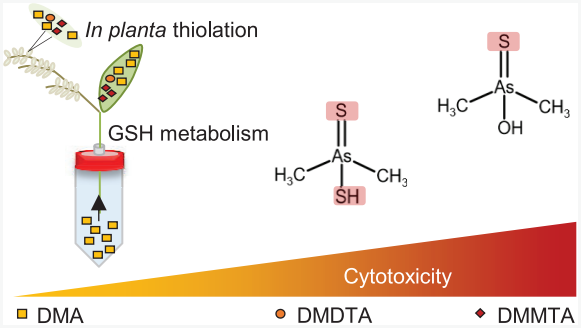
ABSTRACT: Inorganic and methylated thioarsenates have recently been reported to form in paddy soil pore waters and accumulate in rice grains. Among them, dimethylmonothioarsenate (DMMTA) is particularly relevant because of its high cytotoxicity and potential misidentification as nonregulated dimethylarsenate (DMA). Studying DMMTA uptake and flag leaf, grain, and husk accumulation in rice plants during grain filling, substantial dethiolation to DMA was observed with only 8.0 ± 0.1, 9.1 ± 0.6, and 1.4 ± 0.2% DMMTA remaining, respectively. More surprisingly, similar shares of DMMTA were observed in control experiments with DMA, indicating *in planta* DMA thiolation. Exposure of different rice seedling varieties to not only DMA but also to arsenite and monomethylarsenate (MMA) revealed *in planta* thiolation as a common process in rice. Up to 35 ± 7% DMA thiolation was further observed in the shoots and roots of the model plant *Arabidopsis thaliana*. Parameters determining the ratio and kinetics of thiolation versus dethiolation are unknown, yet, but less DMA thiolation in glutathione-deficient mutants compared to wild-type plants suggested glutathione concentration as one potential parameter. Our results demonstrate that pore water is not the only source for thioarsenates in rice grains and that especially the currently nonregulated DMA needs to be monitored as a potential precursor of DMMTA formation inside rice plants.



KEYWORDS: *Oryza sativa, arsenic speciation, DMMTA, DMDTA, glutathione, food safety*

INTRODUCTION

Arsenic (As) mobilization in paddy fields is induced by soil flooding, which is a common agricultural practice for rice cultivation. Under reducing conditions, speciation in pore water is dominated by arsenite and, to a lesser extent, arsenate, monomethylarsenate (MMA), and dimethylarsenate (DMA).[1,2] MMA and DMA are produced by soil microorganisms in reactions catalyzed by the enzyme As(III) S-adenosylmethionine methyltransferase.[3,4] Arsenite, MMA, and DMA enter the rice root cells through nodulin26-like intrinsic channel proteins (NIPs) such as Lsi1 (OsNIP2;1).[5,6] Arsenate enters through phosphate transporters such as OsPht1;1, OsPht1;4, and OsPht1;8.[7] Once inside the root cells, As transport to the shoots is restricted by complexation with thiol ligands such as glutathione (GSH) or phytochelatins (PC). Arsenite is stored as As(III)−PC complexes in the root cell vacuoles. Arsenate and MMA are first reduced in the roots and then complexed by PCs as well.[8] So far, DMA−thiol complexes have not been identified in rice roots.[9] The absence of such complexes explains the high root-to-shoot translocation rates observed for DMA.[10−12] Exposure experiments of rice plants during grain filling have shown that DMA is more mobile in both the xylem and phloem than arsenite[12] and that it is efficiently retranslocated from the flag leaves to the grains.[13] Therefore, the DMA contents in grains can account for up to 90% of total As.[14]

Recent studies have also found inorganic and methylated thioarsenates in rice paddy pore waters.[15,16] Thioarsenate formation is driven by sulfide production from sulfate-reducing bacteria.[15] Inorganic thioarsenates (mono- (MTA), di- (DTA), and trithioarsenate (TTA)) are formed from arsenite, zerovalent sulfur, and sulfide.[17] Methylated thioarsenates (monomethylmonothioarsenate (MMMTA), monomethyldithioarsenate (MMDTA), dimethylmonothioarsenate (DMMTA), and dimethyldithioarsenate (DMDTA)) are formed after the nucleophilic attack of sulfide on MMA or DMA.[18] Little information exists to date on thioarsenate behavior in plant tissues. Thioarsenate uptake by rice roots has been shown by externally applying MTA, MMMTA, and DMMTA to rice seedlings;[19−21] however, the responsible transporters have yet to be identified. Thioarsenate detoxification mechanisms in roots have only been studied in *Arabidopsis thaliana*, where the PC pathway was found to play an important role in MTA detoxification.[22] Transport of

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thioarsenates to the shoots has been confirmed by examining the As speciation in the xylem sap of rice seedlings exposed to MMMTA and DMMTA.[20] Moreover, the analysis of field and commercial rice samples revealed that thioarsenates, namely, MTA, DMMTA, and DMDTA, can accumulate in the rice grains.[23−26]

Our previous studies investigating thioarsenates in rice grains and rice products showed that DMMTA was the predominant thioarsenate species, having the highest contents and occurrence in the samples.[25,27] Moreover, DMMTA has recently been found in rice worldwide in a global market-basket survey.[26] Previous studies have overlooked the presence of DMMTA in grains because routine acid extractions and detection methods transform DMMTA to DMA.[25] Misidentification of DMMTA is problematic because of its high mobility and toxicity in plants[28] and high cytotoxicity in humans.[29−31] Despite its ubiquitous presence in rice and food safety concerns associated with DMMTA, its behavior in rice plants and its path to the grains remain unclear.

Here, we compared the As uptake, accumulation, transport, translocation, and species transformation in rice plants exposed during the grain filling stage to DMMTA or DMA. Surprisingly, the DMA exposure experiments revealed indications for *in planta* thiolation. We, therefore, assessed DMA, arsenite, and MMA thiolation in rice seedlings. Finally, wild-type and GSH-deficient mutants of the model plant *A. thaliana* were used to gain first insights into the mechanisms underlying DMA thiolation.

MATERIALS AND METHODS

Exposure Experiments of Rice Plants during Grain Filling. Rice (*Oryza sativa*) plants were used for pulsing developing grains directly with As, determining uptake/accumulation (Figure S1a) and transport (Figure S1b) from rice panicles to the filling grain as described previously[12] and translocation (Figure S1c) from cut flag leaves to the filling grain as described previously.[13]

All experiments were performed with the rapid life cycle model cultivar Kitaake (ssp. *japonica*). After germination for 7 days on solid media containing nutrient solution[19] (Table S1) and 0.5% agar type E, the plants were transferred into pots with soil (3 parts sowing and pricking soil, 3 parts standard soil clay coconut, and 1 part vermiculite−coarse grit) and were grown for 5 weeks in growth chambers (CLF Plant Climatics AR-66L3) with a long day cycle (16 h light/8 h darkness). Then, the rice plants were transferred to a climate chamber with a 12 h day/night cycle at 27 to 30 °C, a humidity of 70 to 90%, and a light intensity of 600−900 μE. Plants were supplied with nutrients by adding a mixture of basal fertilizers (ENTEC Solub 21, KaraLiva CALCINIT, Agrosil LR, Hakaphos Soft Novell & Hakaphos Green) and Fe-EDTA (5% w/v). The start of anthesis was marked for each panicle by labeling it with a tape containing the respective date. Panicles were harvested individually for As exposure experiments when they reached 10 days post anthesis, which marks the beginning of grain filling.

To study uptake and accumulation (Figure S1a), the rice panicles were cut off below the flag leaf node and were hydroponically exposed for 48 h (the solution was renewed every 24 h) to a nutrient solution containing a pulse of 13.3 μM DMMTA (n = 6) or DMA (n = 6) (including controls without As, n = 6). Arsenic concentrations were chosen the same as in a previous grain pulsing study[12] for better comparability and to ensure sufficient detection limits to capture even minor contributing species with our analytical method.

To study xylem and phloem transport (Figure S1b), the same short time pulse exposure experiments with 13.3 μM DMMTA (n = 6) or DMA (n = 6) (including controls without As, n = 6) were done, but in this case, using rice panicles without and with stem-girdling treatment, i.e., before cutting off the panicles, they were jet-steamed 2 cm below the head for 15 s to interrupt phloem flow. For this test, strontium (Sr) and rubidium (Rb) were added to the nutrient solution (both with a final concentration of 1 mM) as markers of xylem and phloem transport, respectively.[12] To study flag leaf translocation (Figure S1c), the tip of the flag leaf was removed with a sharp blade, and this cut end was inserted into a vial containing 66.6 μM DMMTA (n = 6) or DMA (n = 6), 5 mM MES buffer, 1 mM Sr, and 1 mM Rb (including controls without As, n = 6). The As pulse concentrations applied were 5 times higher than for the panicle exposure experiments to ensure sufficient detection limits for our analytical method at expected lower accumulation via flag leaf feeding.[13] Yet, the concentrations were 2−5 times lower than what was tested in a previous study[13] because at those higher concentrations, toxic effects were already observed for DMMTA exposure. The exposure was conducted for 7 days. Every 24 h, the leaf was recut, and the solution was renewed.

After all of the treatments, the flag leaves were immediately frozen, ground in liquid nitrogen, and stored at −80 °C. Panicles were oven-dried at 60 °C for 7 days, and grains from the top and mid sections were dehusked using a palm husker (Mercer Corporation), milled (Retsch MM 2000, 15 s, 30 Hz), and stored at −20 °C. Husks were frozen with liquid nitrogen, homogenized with pestle and mortar, and stored at −20 °C.

Rice Seedling Exposure Experiments. Rice seedling experiments to further assess DMA *in planta* thiolation were conducted with Kitaake and four of the most widely cultivated Italian varieties (Baldo, Carnaroli, S.Andrea, and Arborio).[32] Growth conditions were described by Kerl et al.[19] Briefly, after the seeds were germinated for 7 days, the resulting seedlings were transferred into tubes containing nutrient solution (Table S1; replaced twice a week) and were grown for 20 days with a long day cycle until exposure. Thiolation in the roots and shoots was measured after the seedlings were exposed *via* the roots to 13.3 μM DMA for 24 h (n = 4 for each rice variety). Additionally, another set of Kitaake seedlings were also exposed to 13.3 μM arsenite (n = 4) and 13.3 μM MMA (n = 4) to establish whether *in planta* thiolation of other relevant As species can occur. Figure S2 summarizes all of the exposure treatments done with rice seedlings. In all treatments, roots were harvested and washed for 10 min in 1 mM KH_2PO_4, 5 mM $Ca(NO_3)_2$, and 5 mM MES buffer.[33] Both root and shoot samples were immediately frozen, ground in liquid nitrogen, and stored at −80 °C.

***A. thaliana* Exposure Experiments.** To gain first insights into the mechanisms underlying DMA thiolation, *A. thaliana* exposure experiments were conducted with DMMTA and DMA. All *A. thaliana* seedling tests were started in a tip box system with surface-sterilized (chlorine gas sterilization) seeds. After stratification for 2 days at 4 °C, the boxes were transferred into short day cycle (8 h light/16 h dark) growth chambers (CLF Plant Climatics AR-66L3). After 3 weeks, hydroponic culture was used to grow the plants in 1/10 Hoagland medium (including microelements without sucrose)[34] for another 10 days until exposure. First, wild-type

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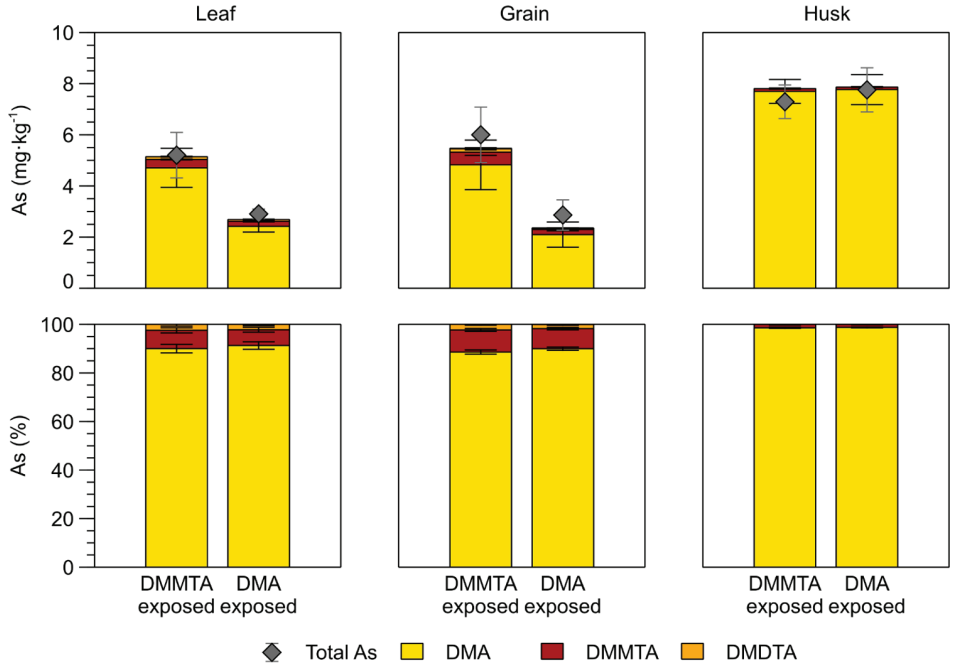

Figure 1. Total As accumulation and ΣAs species in $mg \cdot kg^{-1}$ (top row) and percentage contribution of the As species (bottom row) in flag leaves, grains, and husks of rice panicles exposed for 48 h to 13.3 μM DMMTA or DMA during grain filling. Error bars indicate standard deviation (n = 6). Further quality control data can be found in Figure S6 (showing low As contents in tissues of control plants, <0.030 $mg \cdot kg^{-1}$, all iAs) and Figure S7 (showing no thiolation for DMA in the exposure solution, confirming *in planta* formation of DMMTA and DMDTA) in the Supporting Information.

Col-0 *A. thaliana* seedlings were exposed via the roots for 5 days to 10 μM DMMTA (n = 7) or DMA (n = 8) to assess species uptake, transformation, and *in planta* thiolation in roots and shoots. Then, the kinetics of DMA uptake and thiolation were measured in the roots of Col-0 exposed to 100 μM DMA for 0.5, 1, 2, 3, 6, 24, and 48 h (n = 3 for each time step). Finally, to assess the importance of GSH in DMA thiolation, uptake kinetics were also measured in the roots of GSH-deficient mutants *cad2−1*[35] and *pad2−1*[36] exposed to 100 μM DMA for 3, 6, and 24 h (for each mutant, n = 3 for each time step). Figure S3 summarizes all of the exposure treatments done with *A. thaliana* seedlings. For all of the treatments, roots were washed as previously described. Root and shoot samples were immediately frozen, ground in liquid nitrogen, and stored at −80 °C.

Digestion of Plant Tissues and Determination of Total Concentrations. To determine the total As, Sr, and Rb content, 0.2 g of the sample was digested in H_2O_2 and HNO_3 (5.6 $mol \cdot L^{-1}$ H_2O_2 and 6.4 $mol \cdot L^{-1}$ HNO_3 for grains and husks and 1.9 $mol \cdot L^{-1}$ H_2O_2 and 4.5 $mol \cdot L^{-1}$ HNO_3 for leaves, shoots, and roots) in a CEM Mars5 microwave digestion system (CEM Corp., Matthews, NC). The samples were filtered using 0.2 μm cellulose acetate filters. The rice flour-certified reference material IRMM-804 was used to assess the rice digestion performance (extraction recovery for total As was 96 ± 5%, n = 3). Total As (AsO^+, m/z 91), Sr (m/z 88; standard mode), and Rb (m/z 85; standard mode) concentrations were measured by inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 8900 ICP-QQQ with oxygen as the reaction cell gas) using rhodium (Rh^+, m/z 103) as the internal standard. Arsenic calibration checks were done using the reference material TMDA-62.2 (Environment Canada, recovery was 101 ± 7%, n = 12).

Extraction of Plant Tissues and As Species Determination. To determine As speciation in roots, shoots, and flag leaves, the species-preserving extraction method described by Kerl et al.[19] was used. Since the method was developed only for MTA extraction from rice plant tissues, the stability of the As species of interest in the extracts from rice and *A. thaliana* roots and shoots was tested (Figures S4 and S5). For the extraction, ground plant material (0.01 to 0.08 g), glass beads (0.4 g), and 1.5 mL of phosphatebuffer solution (PBS; 2 mM NaH_2PO_4 + 0.2 mM Na_2-EDTA; pH 6.0) were boiled for 5 min (to denature enzymes and other proteins), cooled in an ice bath for 2 min, and then vortexed for 53 min under anaerobic conditions. After filtering (0.2 μm cellulose acetate filters), As speciation was immediately analyzed. To determine As speciation in rice grain and husks, an enzymatic extraction was performed according to Colina Blanco et al.[25] The rice flour-certified reference material ERM-BC211 was used to evaluate the extraction efficiency (n = 4, recoveries for DMA, iAs, and total As were 102 ± 4, 96 ± 1, and 103 ± 5%, respectively).

For all samples, As speciation was analyzed by ion chromatography (IC) coupled to an Agilent 8900 ICP-QQQ. The IC used was a Dionex ICS-3000 or a Metrohm 940 Professional IC Vario with an AG/AS16 IonPac column (Dionex; 2.5 to 100 $mmol \cdot L^{-1}$ of NaOH with gradient elution, a flow rate of 1.2 $mL \cdot min^{-1}$, and 50 μL injection volume; method capable of preserving inorganic and methylated thioarsenates).[37] The recovery of arsenate (103 ± 5%), arsenite (101 ± 3%), DMA (100 ± 4%), and MMA (102 ± 4%) was evaluated in every analytical run (n = 8) by spiking every 15th sample with 5 and 10 $\mu g \cdot L^{-1}$ of each species and comparing the spike concentration to that of spiked blanks.

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Additionally, a 5 $\mu g \cdot L^{-1}$ calibration standard was measured after every 15th sample to account for instrument signal drift.

Statistical Analysis. Data are presented as the mean ± standard deviation. Differences between treatments were tested for significance using a two-sample *t*-test or one-way analyses of variance (ANOVA). Differences were considered significant when $p < 0.01$.

RESULTS AND DISCUSSION

DMMTA Behavior in Rice Plants during Grain Filling and DMA *In Planta* Thiolation. When comparing the total As accumulation in the rice plant tissues between the DMMTA and DMA treatments, the panicles exposed to DMMTA had 2-fold higher total As contents in leaves ($p < 0.001$) and grains ($p < 0.001$; Figure 1). Noticeably, the same total As accumulation trend (DMMTA-exposed > DMA-exposed) has also been reported in rice seedlings before (roots, shoots, and xylem sap),[20] suggesting a higher mobility of DMMTA than DMA in rice plants, regardless of the growth stage of the plant.

The As speciation results for DMMTA-exposed plants (Figure 1, left bar in each of the six panels) show DMMTA accumulation in the flag leaf, grain, and husk. However, a large share of DMMTA was dethiolated to DMA, with only 8.0 ± 0.1, 9.1 ± 0.6, and 1.4 ± 0.2% remaining DMMTA detected in leaves, grains, and husks, respectively. Detection of DMMTA in the husks already showed that DMMTA was transported during grain filling in the xylem sap of rice plants because actively transpiring tissues, such as the husk, are mainly fed via the xylem.[38,39] Detection of DMMTA in the developing grains after flag leaf feeding of rice plants (Figure S8) showed DMMTA remobilization from the flag leaf into the filling grains, thus indicating DMMTA transport in the phloem as well. To estimate the contribution of xylem versus phloem transport, the content of DMMTA in grains of stem-girdled panicles (where phloem flow is interrupted) and non-stem-girdled panicles was compared (Figure S9). Interruption of phloem flow led to a 76% decrease in DMMTA grain content, indicating a stronger contribution of phloem versus xylem transport for DMMTA. Dethiolation of DMMTA to DMA was also observed in the flag leaf feeding and stem-girdling experiments, which might hamper quantitative statements about DMMTA transport. However, one-way ANOVA revealed that the shares of remaining DMMTA detected in the grain were not significantly different ($p = 0.17$; 6.0 ± 0.3% in the flag leaf feeding experiment, 6.2 ± 0.4% without stem girdling, and 6.9 ± 0.2% with stem girdling), indicating a relatively constant share of dethiolation.

The As speciation results for DMA-exposed plants (Figure 1, right bar in each of the six panels) surprisingly also showed DMMTA accumulation in flag leaf, grain, and husk, indicating *in planta* thiolation. Interestingly, the percentage contribution of As species showed that both the DMA- and DMMTA-exposed panicles have no significantly different percentages of DMMTA in grains (8.3 ± 0.5 and 9.1 ± 0.6%, respectively) ($p = 0.06$), in leaves (6 ± 1 and 8 ± 1%, respectively) ($p = 0.11$), and in husks (1.3 ± 0.2% and 1.4 ± 0.2%, respectively) ($p = 0.35$). Furthermore, also for the DMA-flag leaf feeding (Figure S8) and the DMA stem-girdling experiments (Figure S9), the shares of DMMTA in the grain (6.1 ± 0.5% in the flag leaf feeding experiment, 5.8 ± 0.5% without stem girdling, and 6.7 ± 0.3% with stem girdling) were not significantly different ($p = 0.52$ for the flag leaf experiment, $p = 0.10$ for the experiment without stem girdling, and $p = 0.19$ for the experiment with stem girdling) to what was observed in the respective DMMTA experiments. In all treatments (DMA- and DMMTA-exposed), some further thiolationt to DMDTA was also observed. Plotting DMMTA and DMDTA versus DMA contents in grains shows a strong correlation ($r^2 = 0.82$ to 0.97, Figure S10). A correlation between DMMTA and DMA contents has also been observed before for rice grains from a field survey in China ($n = 103$) and a global basket survey ($n = 140$), but at a higher share of thiolation (DMMTA being 30 ± 8% of DMA).[26] Which parameters determine the extent of thiolation versus dethiolation in different (parts of the) plants and how the ratio might further change after harvest is unknown yet.

To the best of our knowledge, our results are the first clear analytical evidence for *in planta* thiolation for rice. So far, *in planta* thiolation of DMA has only been reported in cabbage, where DMMTA (1.9 $mg \cdot kg^{-1}$ dry weight) and DMMTA-GS (0.2 $mg \cdot kg^{-1}$ dry weight) were detected in the shoots of plants exposed hydroponically to DMA (like in our experiments, only DMA was detected in the solution before and after exposure).[40] Interestingly, Mishra et al.[10] observed an unidentified As species accounting for 2.3% of the total As in the shoots of rice seedlings that were hydroponically exposed to 50 μM DMA for 7 days. Despite reporting an induced production of thiols in roots and shoots after DMA exposure, no DMA−thiol complexes were identified.[10] Based on the analytical conditions applied (shoots were immediately frozen in liquid nitrogen, extracted in MQ, and As species were separated using an AS7 IonPac anion exchange column with a gradient of 0.04 to 50 mM HNO_3), it is, however, possible that the reported unidentified As species was partially preserved DMMTA. Further, there are previous reports where the analysis of As speciation in grains using X-ray absorption near-edge spectroscopy (XANES) revealed that some[39] or most[12,41] of the contained or added pentavalent DMA had transformed. Potential reactions discussed were either demethylation to arsenite (which was ruled out by complementary high-performance liquid chromatography ICP-MS (HPLC-ICP-MS) analyses in two of the studies[39,41]), reduction to trivalent DMA (DMA(III)) or thiolation. Based on the observed absorption edge, formation of DMDTA or a DMA(III)−DMA(V) dimer thiol was speculated to be the main reaction in one study[12] but could not be confirmed due to a lack of standards.

***In Planta* Thiolation in Rice Seedlings.** To test whether DMA thiolation is a general phenomenon in rice, 20-day-old seedlings from Kitaake and common Italian varieties were exposed to DMA for 24 h. Figure 2 shows that DMMTA (up to 15 $\mu g \cdot kg^{-1}$ in roots and 11 $\mu g \cdot kg^{-1}$ in shoots) and DMDTA (up to 2 $\mu g \cdot kg^{-1}$ in roots and 1 $\mu g \cdot kg^{-1}$ in shoots) were detected in all of the varieties. The percentage contribution of As species showed that DMMTA (1.3 ± 0.5% in shoots and 0.9 ± 0.3% in roots) was not significantly different between the five varieties tested (one-way ANOVA: $p = 0.46$ for shoots and $p = 0.07$ for roots). The same was observed for DMDTA (0.2 ± 0.1% in shoots with $p = 0.21$ and 0.15 ± 0.07% in roots with $p = 0.13$). These results demonstrate that DMA thiolation is a common process occurring in rice, even at early growth stages.

Furthermore, Kitaake rice seedlings were exposed to arsenite and MMA to establish whether *in planta* thiolation of other relevant As species is also possible in rice plants. Figure 3 shows that thiolated species were detected in the roots and shoots of both treatments. For the rice seedlings exposed to

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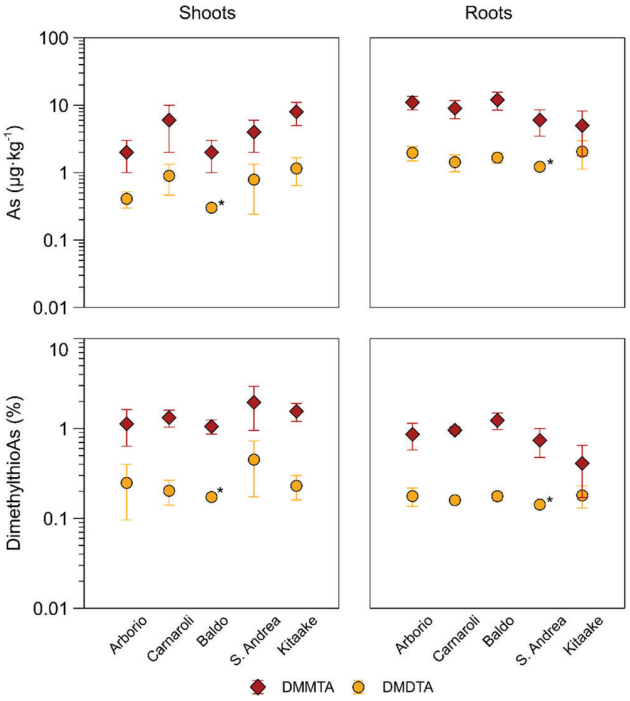

Figure 2. DMMTA and DMDTA in $\mu g \cdot kg^{-1}$ fresh weight (top row) and their percentage contribution to total As (bottom row) in shoots and roots from common Italian varieties and Kitaake exposed for 24 h to 13.3 μM DMA. Error bars indicate the standard deviation (n = 4). In some cases, the standard deviation is smaller than the symbol size, except for symbols with * (in Baldo shoots: DMDTA was detected only in two samples, and in S. Andrea roots: DMDTA was detected only in one sample). Further quality control data can be found in Figure S11in the Supporting Information (showing no thiolation for DMA in exposure solution, confirming *in planta* formation of DMMTA and DMDTA).

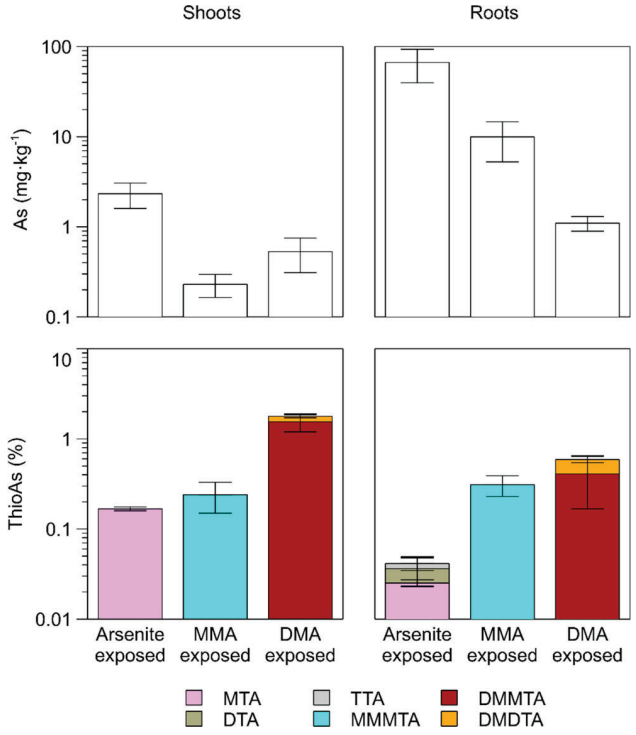

Figure 3. Total As accumulation in $mg \cdot kg^{-1}$ fresh weight (top row) and percentage contribution of the As species (bottom row) in shoots and roots from Kitaake rice seedlings exposed for 24 h to 13.3 μM arsenite, MMA, or DMA. Error bars indicate standard deviation (n = 4). Further quality control data can be found in Figures S4 and S11 in the Supporting Information (showing no thiolation for arsenite, MMA, and DMA during extraction and in the exposure solution, confirming *in planta* thiolation).

arsenite, MTA (up to 24 $\mu g \cdot kg^{-1}$ in roots and 6 $\mu g \cdot kg^{-1}$ in shoots), DTA (up to 18 $\mu g \cdot kg^{-1}$ in roots), and TTA (up to 6 $\mu g \cdot kg^{-1}$ in roots) were detected. For the seedlings exposed to MMA, only MMMTA was detected (up to 42 $\mu g \cdot kg^{-1}$ in roots and 1 $\mu g \cdot kg^{-1}$ in shoots). While the thiolated species accounted for only a small percentage of the total As (in shoots and roots arsenite thiolation <0.2%, MMA thiolation <0.3%, and DMA thiolation <1.5%, Table S2), these results prove that *in planta* thiolation of well-known As species can occur in rice. It is, however, noteworthy that from a toxicological perspective, inorganic thioarsenates and MMMTA do not pose such a hidden health risk as DMMTA does;[31,42] therefore, the focus of the present study remained on DMA thiolation.

***A. thaliana In Planta* Thiolation and Role of Glutathione.** The use of the model plant *A. thaliana* would facilitate future studies of the mechanisms underlying DMA thiolation in plants substantially. We therefore investigated whether *in planta* thiolation also occurs in the dicot *A. thaliana* like in the monocot *O. sativa*. Like in our previous study,[28] *A. thaliana* (Col-0) seedlings exposed to DMMTA showed a 10-fold higher As accumulation in both roots and shoots compared to DMA-exposed seedlings (Figure S12), which is the same trend as observed in rice seedlings[20] and the rice plants during grain filling (Figure 1). The observation supports similar transport and translocation capacities for DMA and DMMTA in monocots and dicots.

Furthermore, similar to what was observed in the present rice experiments, thiolation occurred upon DMA exposure in *A. thaliana* with the formation of DMMTA (73 ± 19 $\mu g \cdot kg^{-1}$ in roots and 23 ± 6 $\mu g \cdot kg^{-1}$ in shoots) and DMDTA (72 ± 17 $\mu g \cdot kg^{-1}$ in roots and 30 ± 15 $\mu g \cdot kg^{-1}$ in shoots). Percentages of DMA thiolation to DMMTA and DMDTA after 5 days of exposure were 35 ± 7% in roots and 35 ± 5% in shoots (Figure S12 and Table S3). Upon DMMTA exposure, dethiolation was observed; however, percentages of remaining thiolated species were still significantly higher (47 ± 6% in roots, $p < 0.01$; and 43 ± 5% in shoots, $p < 0.01$) than those of newly formed thiolated species upon DMA exposure. Whether dethiolation and thiolation percentages will reach the same values upon longer exposure has not been further investigated yet.

Apart from DMA, DMMTA, and DMDTA, substantial percentages of other species were observed for the *A. thaliana* experiments (Table S3). The species separated chromatographically (Figure S13) also contained one unknown species, which could be the complex $DMMTA^{V}$−SG, previously detected in cabbage shoots after exposure to DMA,[40] or its decomposition intermediates DMA^{III}−SG and DMA^{III}−SH.[40,43] In shoots, only iAs was observed upon DMA exposure, which was attributed to demethylation (6 ± 2%). More transformation was observed in roots, with 11 ± 5 and 3 ± 2% reduction to (likely) DMA(III), 9 ± 3 and 3.3 ± 0.6% demethylation to MMA and MMA thiolation, and 10 ± 6 and 1.4 ± 0.7% demethylation to iAs upon DMA and DMMTA exposure, respectively. The presence of MMA in DMA-

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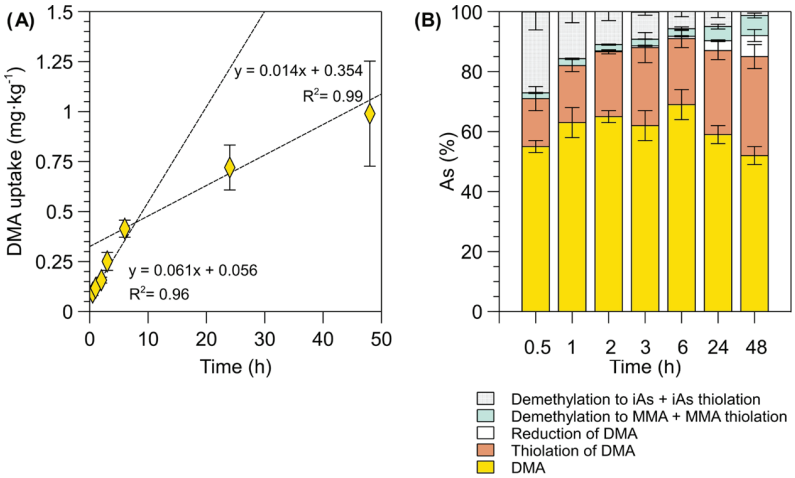

Figure 4. (A) DMA uptake ($mg \cdot kg^{-1}$) kinetics and (B) percentage contribution of grouped species to total As (%) in roots of *A. thaliana* (Col-0) seedlings exposed to 100 μM DMA for 0.5, 1, 2, 3, 6, 24, and 48 h. Error bars indicate standard deviation (n = 3).

exposed plants has been observed before and has been attributed to demethylation in roots.[3,10]

To further follow how quickly thiolation of DMA happens and potentially derive the first indications of it being a purely abiotic or enzymatically catalyzed process, *A. thaliana* was exposed to short-term DMA pulses (0.5 to 48 h). Due to the shorter exposure times, 10 times higher exposure concentrations were chosen, so thiolation percentages may not be directly comparable to the 5 day-exposure treatment. Further, this experiment was only conducted with roots due to the low contents detected in shoots (Figure S12). Figure 4a shows that there was a rapid DMA uptake in the roots. The DMA content tripled within 3 h from 0.051 $mg \cdot kg^{-1}$ to 0.155 $mg \cdot kg^{-1}$ and then showed a less pronounced, but still ongoing, increase for the latest time tested (48 h). Thiolation was already detected at the shortest exposure time tested (0.5 h; Figure 4b). Thiolation percentages increased from 16 ± 4% (0.5 h) to 33 ± 4% (48 h), accompanied by minor increases in DMA(III) (up to 7 ± 2%) and increases in DMA net demethylation to MMA and MMA thiolation (1.9 ± 0.2 to 6.7 ± 0.8%). The share of iAs and thiolated iAs species decreased from 27 ± 6 to 3.3 ± 0.8% (Figure 4b and Table S4).

Based on previously reported rate constants for abiotic thiolation in closed systems with reduced sulfur excess (S/As ≥ 17),[44] the calculated maximum thiolation after 0.5 to 3 h of reaction time would have been 9% for pH 7 (note: pH 7 was chosen assuming thiolation happens in the cytosol, since it contains considerable amounts of reduced S;[45] Table S5). Lower thiolation could be expected, since the root−shoot exposure experiment is an open system, As species transformations occurred, and it is yet unclear whether there is an excess of reduced S for *in planta* thiolation. However, measured thiolation percentages were up to 15 times higher than calculated thiolation percentages after 0.5 h of exposure and still 3 times higher after 3 h of exposure. This rapid thiolation could indicate that *in planta* thiolation is not a purely abiotic process but also enzymatically catalyzed. For longer exposure times, thiolation was less than predicted based on abiotic rates, potentially indicating an increasing contribution of *in planta* dethiolation. Which parameters are determining net thiolation versus dethiolation remains to be investigated.

To gain information about the sulfur-containing compound involved in the thiolation of DMA, *A. thaliana* wild type (Col-0) and two GSH-deficient mutants (*cad2−1* and *pad2−1*) were used to investigate whether thiolation *in planta* is affected by the GSH pool of the plant. GSH-deficient mutants were tested because GSH is the most abundant free thiol compound present in most living cells, and for plants, it significantly contributes to stress response, particularly concerning toxic metal(loids).[35] Previous studies showed that *cad2−1* and *pad2−1* contained approximately 30 and 20% of the wild-type GSH, respectively.[35,36] Figure 5 shows that thiolation of DMA

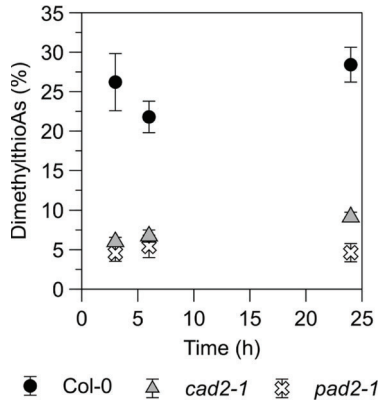

Figure 5. DMA thiolation kinetics in roots of *A. thaliana* wild-type Col-0 and GSH-deficient mutants *cad2−1* and *pad2−1* seedlings exposed to 100 μM DMA for 3, 6, and 24 h. DimethylthioAs (%) refers to the sum of the percentage contributions of DMMTA and DMDTA to total As. Error bars indicate standard deviation (n = 3).

was lower in both GSH-deficient mutants compared to the wild type at the three time points analyzed (for both *cad2−1* and *pad2−1* at 3, 6, and 24 h; $p < 0.01$). After 24 h of exposure, there was a significantly higher ($p < 0.001$) thiolation in the *cad2−1* mutant (9.1 ± 0.6%) compared to the *pad2−1* mutant (5 ± 1%), thus indicating that there is, in fact, lower thiolation when the GSH content is reduced. The abiotic reaction between DMA^V and GSH has been shown to generate DMA^{III} or DMA^{III}−SG.[43] The metabolic conversion of DMA^{III} to DMMTA has been postulated as a possible mechanism to evade the toxicity of DMA^{III} (another highly toxic As species).[43,46] However, at least based on cytotoxicity studies with human cell lines, DMMTA is almost comparable in

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toxicity to DMA^{III}.[31] So, whether DMA thiolation versus reduction could really be a detoxification mechanism in plants remains to be investigated.

Interestingly, a study addressing DMA phytotoxicity in *A. thaliana* determined that inhibition of GSH biosynthesis decreased the plant's sensitivity to DMA.[47] Considering that DMMTA toxicity in *A. thaliana* is far greater than that of DMA,[28] the alleviation in the toxic effect could be due to less production of DMMTA in the roots. From a food security perspective, it would be worth addressing similarly the impact of DMMTA on rice plants. For instance, DMA is commonly associated with straighthead disease in rice, a disorder characterized by sterile spikelets causing reduced grain yield.[48] Considering that (i) our results demonstrate that DMA is a precursor of DMMTA, (ii) DMMTA is more toxic in plants than DMA,[28] and (iii) rice varieties with higher levels of sulfur have been shown to be more susceptible to this disease,[49] addressing the extent of DMA thiolation and DMMTA accumulation in grains of different varieties could provide answers as to why some varieties are more susceptible or resistant to this disease than others.[50]

Implications. Mounting evidence suggests that thiolation could be an underestimated risk for humans related to As exposure in rice.[42] Here, we show that besides previously reported thiolation in pore water,[15,16] *in planta* thiolation of DMA is another source for DMMTA in rice grains. A thorough understanding of where and how thiolation occurs and whether the share of DMMTA is predictable based on DMA occurrence is needed to develop mitigation strategies and/or adapt current regulations.

The present study used hydroponic systems with short-term exposure of plants to pulses of DMA/DMMTA concentrations, typically 1 to 2 orders of magnitude higher than what was reported so far from paddy soil pore waters.[16] Whether absolute concentrations of DMA determine the degree of *in planta* thiolation, either kinetically within an abiotic or enzymatically catalyzed process or also as a dose-related response as part of a detoxification strategy (potentially including even DMA^{III}), is unknown yet. At high pore water iAs concentrations, a limit of accumulation in the grain is often observed, typically explained by activation of increased GSH and PC production for iAs reduction, complexation, and root vacuole storage of As−PC complexes as a detoxification strategy.[8,51] Whether dose-related iAs−S interactions could increase thiolation because of generally higher GSH levels or decrease thiolation due to competition of iAs for GSH is unknown. Production of reactive oxygen species as a response to toxic stress might decrease thiolation.

Further, we cannot yet predict the kinetics of (de)thiolation. Our present experiments were done at the beginning of grain filling with an exposure time of only 48 h. As pointed out earlier,[12] grains are then still developing, are metabolically active, and do not necessarily reflect final speciation. In the field, grain filling typically takes about 3 weeks. The relative importance of translocation of already thiolated species from flag leaves to the grain by phloem transport with potential further (de)thiolation versus potentially continued fresh supply of (thiolated) methylated species from pore water through the roots and the extent of potential (de)thiolation during xylem transport is unknown.

Also, while the present study has shown no significant differences in the percentage of thiolation between the five rice varieties selected (seedlings), more studies are certainly needed to determine whether this holds true for a larger and more diverse panel. Systematic studies using specific mutants of both rice and *A. thaliana* can further help to reveal underlying (de)thiolation mechanisms and to select potentially low-thiolating varieties to limit a DMMTA-associated health risk.

With regard to predicting a percentage of DMMTA in grains based on measured DMA concentrations, further studies will need to show whether that is influenced by both the percentage of thiolation in pore water and within the plant or whether, after uptake, plant-specific (de)thiolation processes completely reset any previous pore water percentage distribution. However, the share of thiolation in pore water will still be important because for DMMTA, a 10-fold higher uptake than for DMA as well as higher toxicity has been shown,[28] which again then possibly closes the loop to a potential dose−response related extent of (de)thiolation in the plant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c06603.

Scheme of exposure treatments; composition of nutrient solution; stability of As species during extraction of rice tissues; stability of As species during extraction of *A. thaliana* tissues; As speciation in control plants; stability of DMA and DMMTA in nutrient solution with rice stem; flag leaf feeding results, stem girdling results; correlation between DMMTA and DMDTA and DMA contents in grains; stability of arsenite and DMA in nutrient solution with rice seedlings; As speciation in *A. thaliana* exposed to DMA or DMMTA; chromatogram of the DMA kinetic root sample; As species of DMA kinetic test in roots of *A. thaliana*; and kinetic calculations using the rate constant for abiotic thiolation (PDF)

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Notes

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Supporting Information

In planta arsenic thiolation in rice and *Arabidopsis thaliana*

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(16 pages, 5 tables, 13 figures)

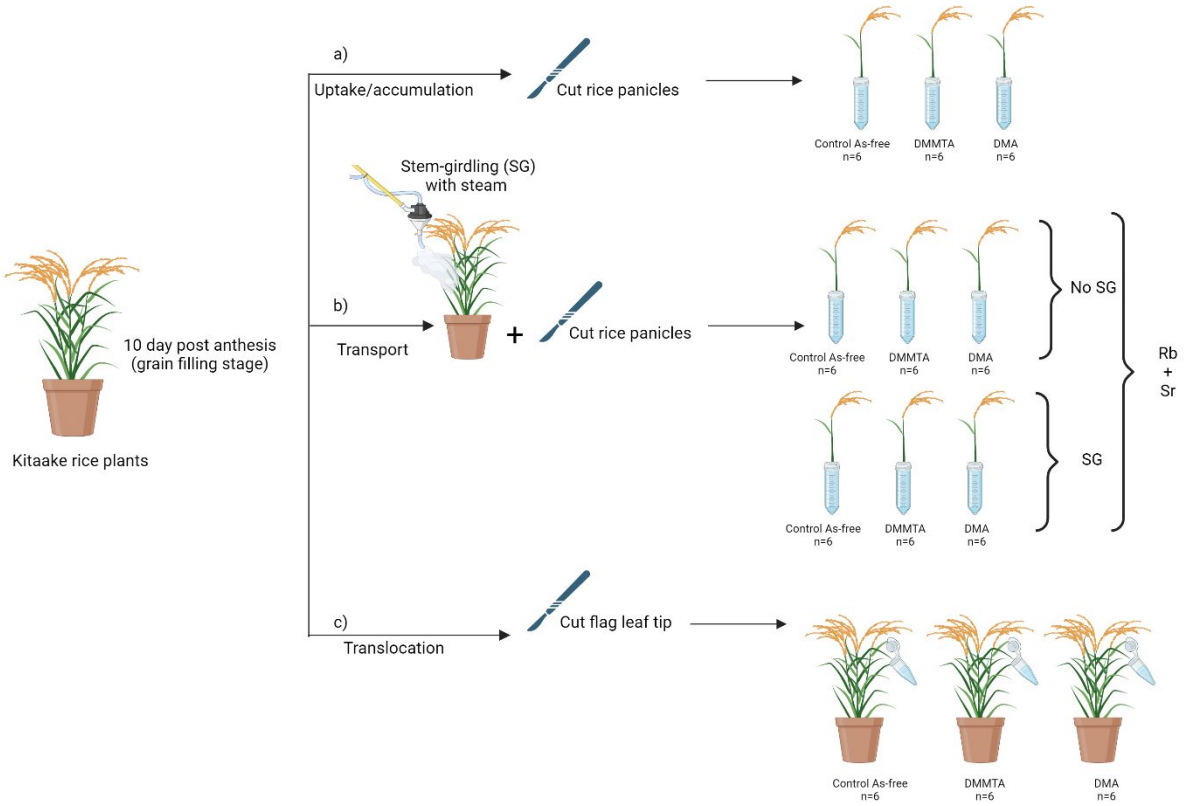

Figure S1. Summary of the exposure treatments done with the Kitaake rice plants during grain filling. Created with BioRender.com

Table S1. Nutrient solution used for rice plant growth.

Macronutrients	Concentration ($mg \cdot L^{-1}$)
$Ca(NO_3)_2$ x 4 H_2O	1000
KCl	120
$MgSO_4$ x 7 H_2O	250
Fe-EDDAH (5.7% Fe)	20
Micronutrients	**Concentration ($\mu g \cdot L^{-1}$)**
KI	27
LiCl	27
$CuSO_4$ x 5 H_2O	55
$ZnSO_4$ x 7 H_2O	111
H_3BO_3	55
$Al_2(SO_4)_3$	55
$MnCl_2$ x 4 H_2O	388
$NiSO_4$ x 7 H_2O	55
$Co(NO_3)_2$ x 6 H_2O	55
KBr	27
$(NH_4)_6Mo_7O_{24}$	55

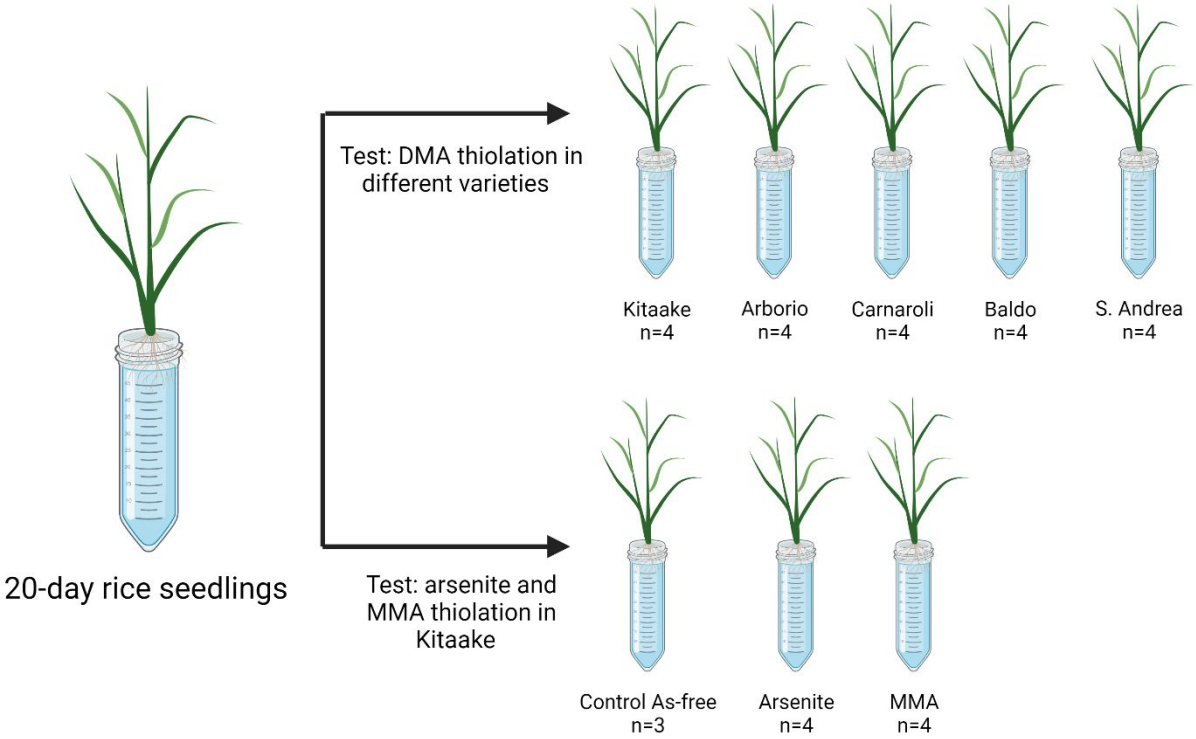

Figure S2. Summary of the exposure treatments done with rice seedlings. Created with BioRender.com

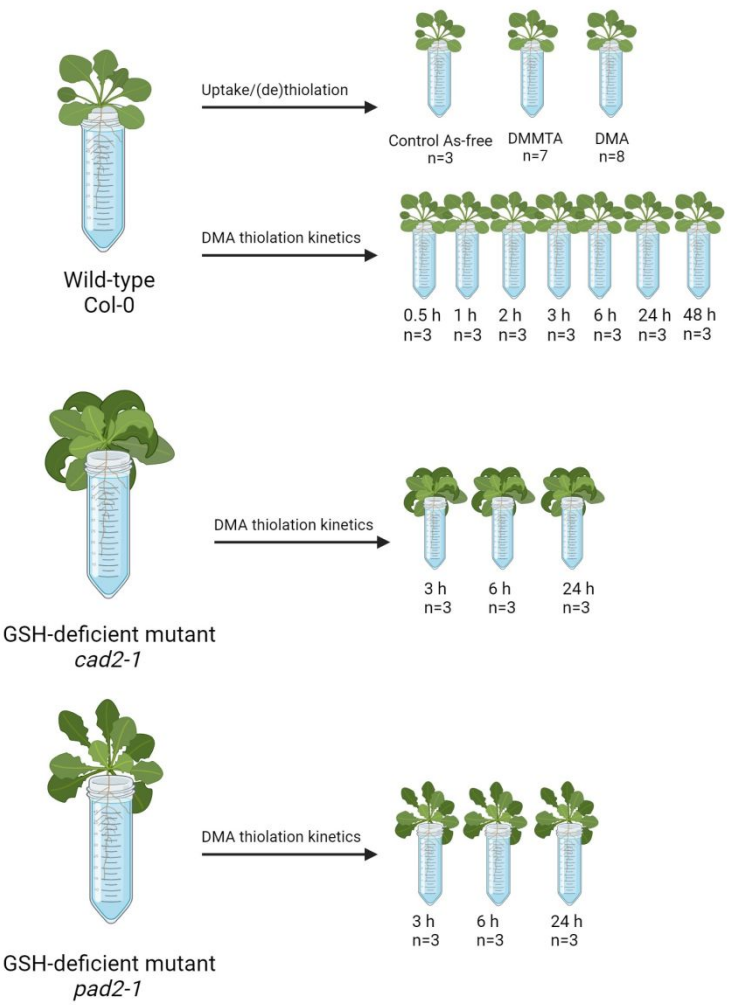

Figure S3. Summary of the exposure treatments done with *A. thaliana* seedlings. Created with BioRender.com

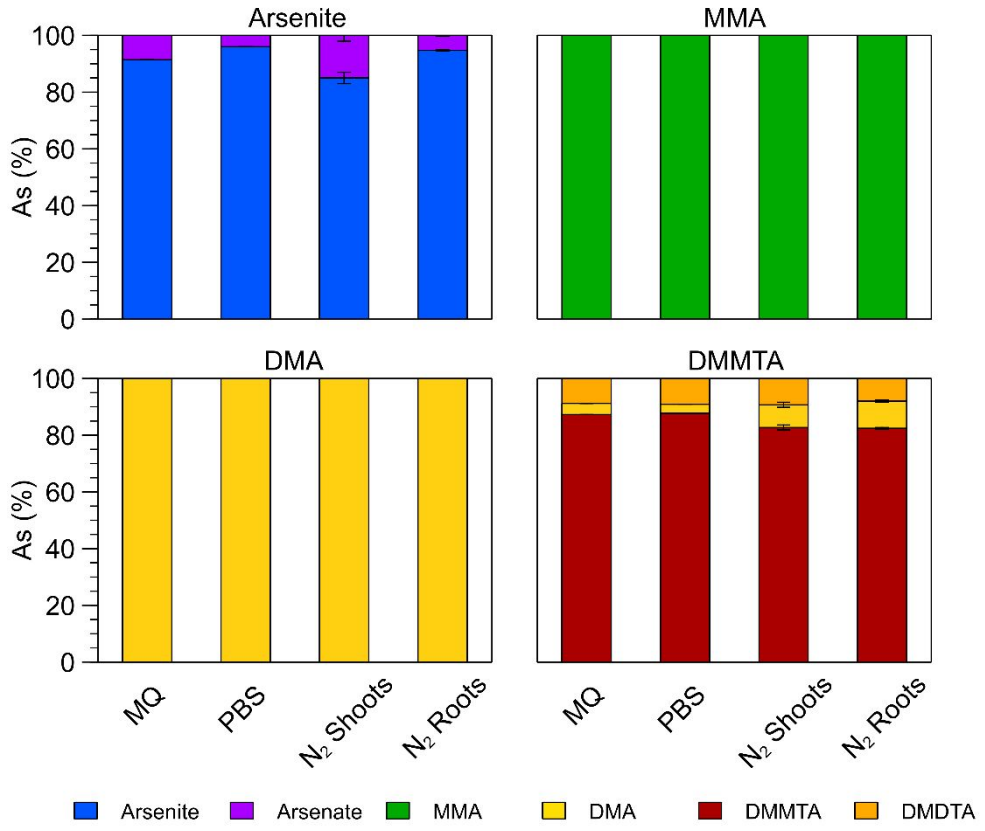

Figure S4. Stability of As species during extraction of rice tissues. For this test, between 0.01 - 0.08 g of shoot or root (As-free & ground in liquid nitrogen (N_2)) were extracted using PBS spiked with 50 ppb of arsenite (n = 3), MMA (n = 3), DMA (n = 3), or DMMTA (n = 3). The test showed that arsenite, MMA, and DMA were not thiolated during the extraction. Furthermore, DMMTA stability during extraction was confirmed (only 5% transformation to DMA in both shoots and roots).

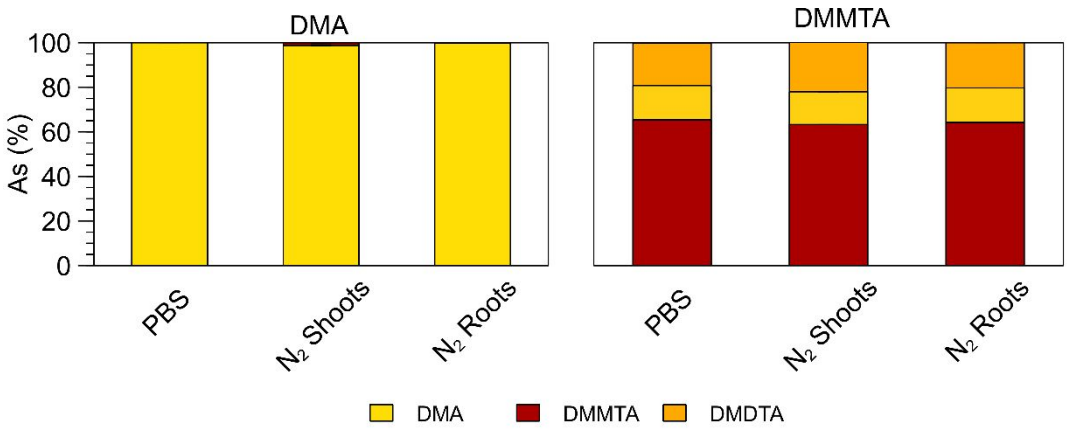

Figure S5. Stability of As species during extraction of *A. thaliana* tissues. For this test, between 0.01- 0.08 g of shoot or root (As-free & ground in liquid nitrogen) were extracted using PBS spiked with 50 ppb DMA (n = 3) or DMMTA (n = 3). The test showed minor thiolation of DMA during the extraction (1% and 0.1% for shoots and roots, respectively) and that DMMTA is stable during extraction. Note: for the rice (Figure S1) and *A. thaliana* method tests, different DMMTA batches were synthesized, explaining the different initial purities (percentages of the DMMTA standards: 87.3% DMMTA, 3.2% DMA, 9.1% DMDTA for rice tests and 65.4% DMMTA, 15.4% DMA, 19.1% DMDTA for *A. thaliana* tests).

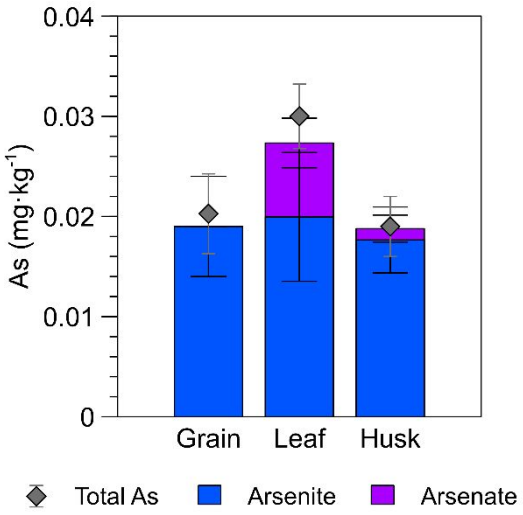

Figure S6. Total As accumulation and ΣAs species in $mg \cdot kg^{-1}$ in rice grains (n = 6), flag leaves (n = 6), and husks (n = 6) from control plants. The soil used to grow the plants contained 1.8 $mg \cdot kg^{-1}$ total As.

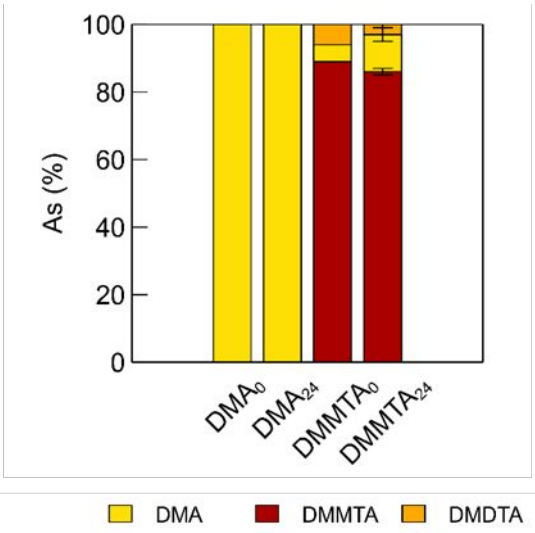

Figure S7. Arsenic speciation of the nutrient solution spiked with 13.3 μM DMA (n = 6) or DMMTA (n = 6) before and after 24 h of contact with the rice panicle stem. For DMA: no transformation. For DMMTA: 3% of DMMTA and 3% of DMDTA transformed to DMA.

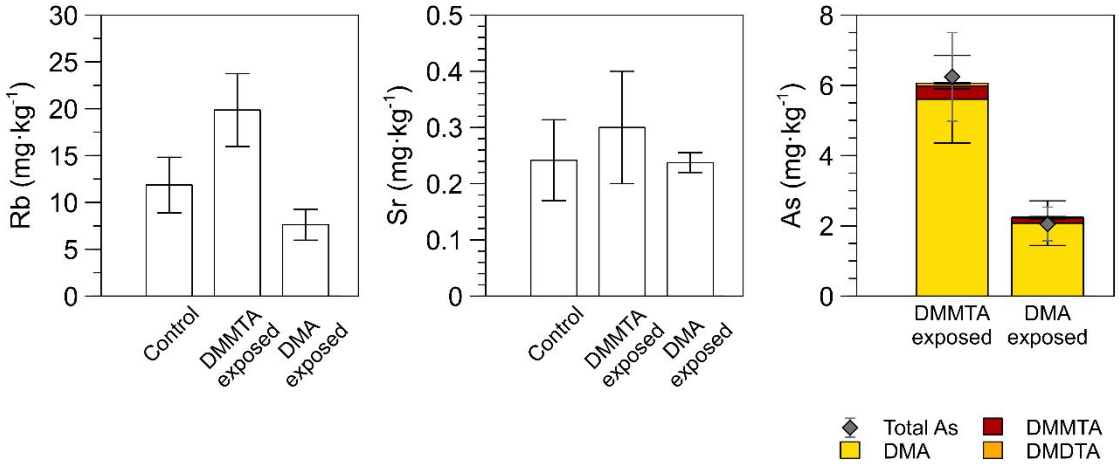

Figure S8. Total Rb, Sr, and As accumulation & ΣAs species in mg·kg^{-1} in grains of control rice plants (As-free media) and plants exposed for 7 days to 66.6 µM DMMTA (n = 6) or DMA (n = 6) during grain filling via the flag leaf. Total As for the control plants was 0.019 ± 0.003 mg·kg^{-1} (arsenite was the only species detected). Grain Rb and Sr results confirmed large phloem and low xylem transport with high Rb and low Sr contents. The total solution uptake was 3.3 ± 0.2 g for the control, 1.8 ± 0.2 g for the DMMTA-exposed plants, and 3.2 ± 0.3 g for the DMA-exposed plants.

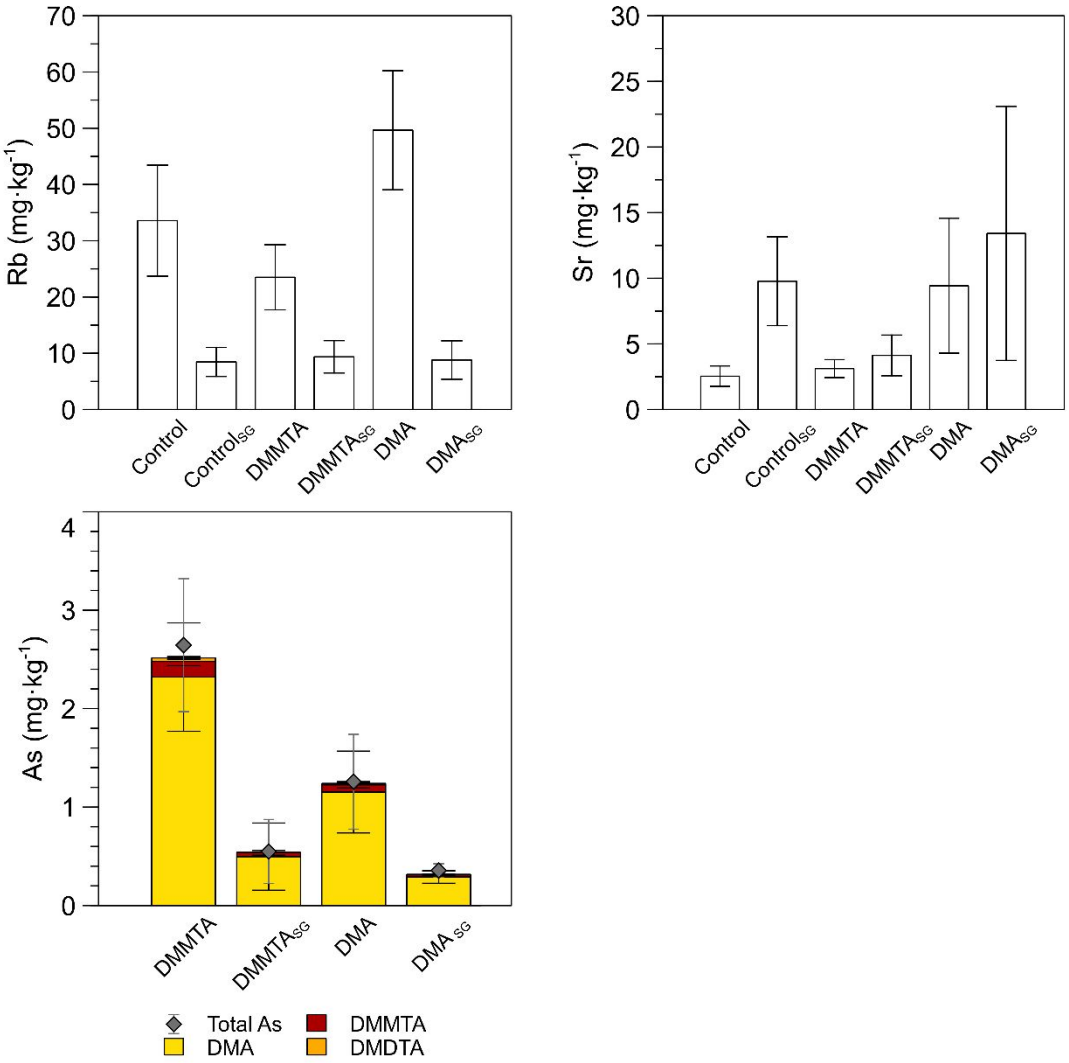

Figure S9. Total Rb, Sr, and As accumulation & ΣAs species in mg·kg^{-1} in grains of rice panicles, without and with stem girdling (SG), from control plants (As-free media) and plants exposed for 48 h to 13.3 μM DMMTA (n = 6) or DMA (n = 6) during grain filling. Total As for the control plants was 0.024 ± 0.007 mg·kg^{-1} and for control plants$_{SG}$ was 0.012 ± 0.004 mg·kg^{-1} (in both treatments, arsenite was the only species detected). Lower contents of Rb in the grains of panicles with SG treatment confirmed the interruption of phloem flow.

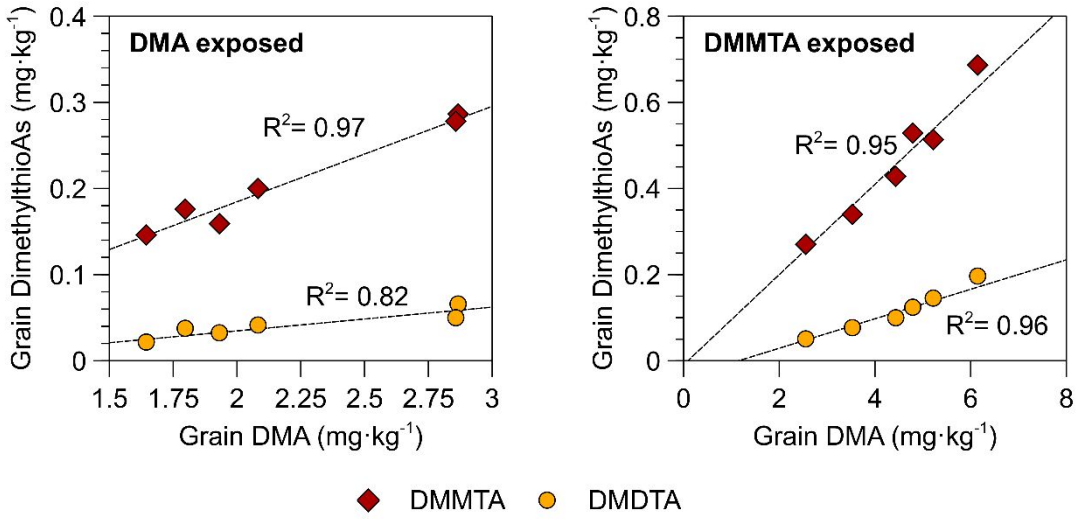

Figure S10. Correlation between DMMTA & DMDTA and DMA contents in rice grains for DMA- and DMMTA- exposed panicles.

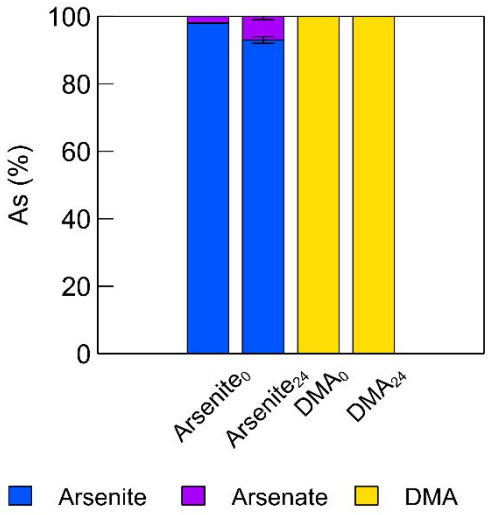

Figure S11. Arsenic speciation of the rice seedlings' nutrient solution spiked with 13.3 µM arsenite (n = 3) or DMA (n = 3) before and after 24 h. For arsenite: no thiolation was observed, only 5% oxidation to arsenate. For DMA: no transformation. The stability of MMA^{V} (10 µM) in the nutrient solution over 24 h with rice seedlings has been shown before by Kerl et.al (Fig SI4)[1], only 2% transformation to MMA^{III} (no thiolation).

(1) Kerl, C. F.; Schindele, R. A.; Brüggenwirth, L.; Colina Blanco, A. E.; Rafferty, C.; Clemens, S.; Planer-Friedrich, B. Methylated Thioarsenates and Monothioarsenate Differ in Uptake, Transformation, and Contribution to Total Arsenic Translocation in Rice Plants. *Environ. Sci. Technol.* **2019**, *53* (10), 5787–5796. https://doi.org/10.1021/acs.est.9b00592.

Table S2. Percentage contribution of the As species in shoots and roots from Kitaake rice seedlings exposed for 24 h to 13.3 µM arsenite, MMA, or DMA. All values are given in %.

	Exposure treatment	MMA	DMA	iAs	Unknown*	MTA	DTA	TTA	MMMTA	DMMTA	DMDTA
Shoots	Arsenite	-	-	99.8	0.02	0.17	-	-	-	-	-
	MMA	70	-	13.6	15.5	-	-	-	0.24	-	-
	DMA	-	96	1.2	0.78	-	-	-	-	1.55	0.23
Roots	Arsenite	-	-	99.9	-	0.03	0.01	0.005	-	-	-
	MMA	74	-	0.99	24.5	-	-	-	0.31	-	-
	DMA	3.7	92	1.67	1.92	-	-	-	-	0.41	0.18

* The same unknown species was observed in the exposure treatments of *A. thaliana*, see Figure S14.

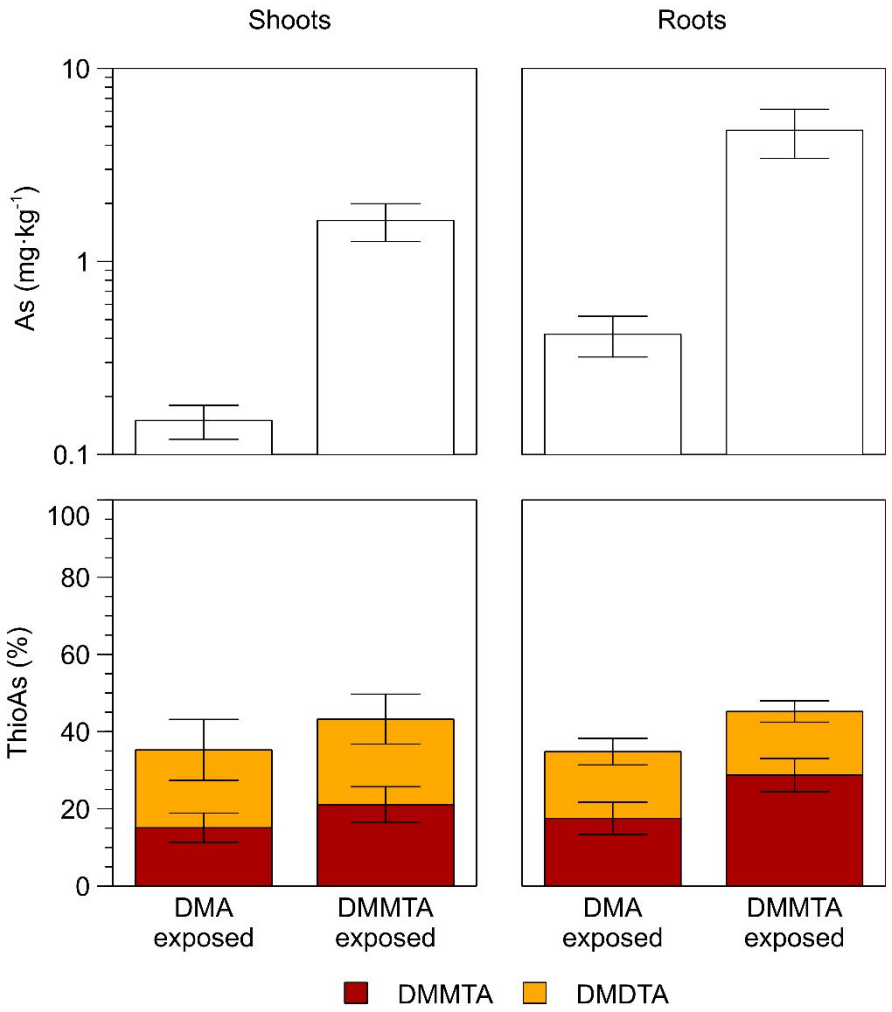

Figure S12. Total As accumulation in mg·kg^{-1} fresh weight (top row) and percentage contribution of DMMTA & DMDTA to total As (bottom row) in shoots and roots of *A. thaliana* (Col-0) exposed for 5 days to 10 µM DMA (n = 8) or DMMTA (n = 7).

Table S3. Data summary of As accumulation in fresh weight ($mg \cdot kg^{-1}$), percentage contribution of individual species to total As (%), and percentage contribution of grouped species (%) in shoots and roots of *A. thaliana* (Col-0) exposed for 5 days to 10 µM DMA (n = 8) or DMMTA (n = 7).

$mg \cdot kg^{-1}$	Unknown	±	DMA	±	Arsenite	±	DMMTA	±	DMDTA	±	MMA	±	Arsenate	±	ΣAs	±
Shoot DMMTA	ND	-	0.9	0.3	0.004	0.001	0.3	0.1	0.35	0.08	0.0023	0.0009	0.009	0.004	1.6	0.4
Root DMMTA	0.15	0.05	2.4	0.6	0.034	0.008	1.6	0.4	0.9	0.2	0.17	0.06	0.04	0.02	5	1
Shoot DMA	ND	-	0.09	0.02	0.0024	0.0006	0.023	0.006	0.03	0.01	0.0005	0.0002	0.006	0.003	0.15	0.03
Root DMA	0.05	0.03	0.15	0.03	0.03	0.03	0.07	0.02	0.07	0.02	0.04	0.01	0.02	0.01	0.4	0.1

%	Unknown	±	DMA	±	Arsenite	±	DMMTA	±	DMDTA	±	MMA	±	Arsenate	±
Shoot DMMTA	-	-	56	5	0.24	0.06	21	5	22	6	0.14	0.03	0.5	0.2
Root DMMTA	3	2	45	6	0.7	0.3	30	4	17	3	3.3	0.6	0.7	0.5
Shoot DMA	-	-	59	6	1.6	0.5	16	4	20	8	0.3	0.1	4	2
Root DMA	11	5	36	3	6	5	18	4	17	3	9	3	4	3

%	DMA	±	Thiolation of DMA	±	Reduction to DMA(III)	±	Demethylation to MMA and MMA thiolation	±	Demethylation to iAs	±
Shoot DMMTA	56	5	43	5	-	-	0.14	0.03	0.8	0.2
Root DMMTA	45	6	47	6	3	2	3.3	0.6	1.4	0.7
Shoot DMA	59	6	35	5	-	-	0.3	0.1	6	2
Root DMA	36	3	35	7	11	5	9	3	10	6

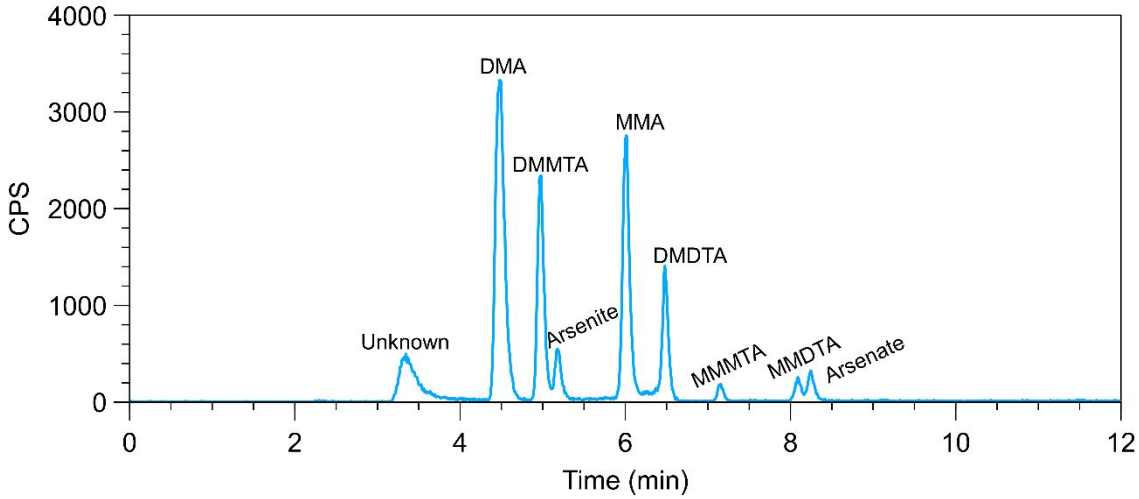

Figure S13. IC-ICP-MS chromatogram of one root extraction of *A. thaliana* (Col-0) seedlings exposed to 100 µM DMA for 48 h. Signal intensity is given in counts per second (cps).

Table S4. Data summary of As accumulation in fresh weight ($mg \cdot kg^{-1}$), percentage contribution of individual species to total As (%), and percentage contribution of grouped species (%) in the roots of *A. thaliana* (Col-0) seedlings exposed to 100 µM DMA for 0.5, 1, 2, 3, 6, 24, and 48 h (n = 3 for each time step).

Time ($mg \cdot kg^{-1}$)	Unknown	+-	DMA	+-	DMMTA	+-	Arsenite	+-	DMDTA	+-	MMA	+-	Arsenate	+-	MMMTA	+-	MMDTA	+-	MTA	+-	ΣAs	+-
0.5	ND	-	0.051	0.007	0.0075	0.0006	0.011	0.004	0.008	0.001	0.0018	0.0001	0.015	0.005	ND	-	ND	-	ND	-	0.09	0.01
1	ND	-	0.074	0.006	0.011	0.001	0.008	0.003	0.011	0.002	0.00267	0.00002	0.011	0.003	ND	-	ND	-	ND	-	0.12	0.01
2	0.0004	0.0004	0.102	0.009	0.018	0.002	0.007	0.002	0.016	0.002	0.0033	0.0005	0.010	0.003	ND	-	ND	-	ND	-	0.16	0.01
3	0.0015	0.0006	0.16	0.03	0.031	0.007	0.0108	0.0008	0.03	0.01	0.005	0.001	0.012	0.002	ND	-	ND	-	ND	-	0.25	0.04
6	0.0031	0.0004	0.29	0.05	0.047	0.003	0.014	0.002	0.042	0.003	0.010	0.002	0.012	0.004	ND	-	ND	-	ND	-	0.41	0.04
24	0.023	0.004	0.42	0.08	0.10	0.02	0.017	0.005	0.10	0.01	0.028	0.009	0.02	0.02	0.002	0.001	0.004	0.002	0.0011	0.0001	0.7	0.1
48	0.06	0.02	0.5	0.2	0.14	0.04	0.016	0.005	0.18	0.04	0.05	0.01	0.012	0.005	0.005	0.002	0.015	0.009	0.005	0.001	1.0	0.3

Time (%)	Unknown	+-	DMA	+-	DMMTA	+-	Arsenite	+-	DMDTA	+-	MMA	+-	Arsenate	+-	MMMTA	+-	MMDTA	+-	MTA	+-
0.5	-	-	55	2	8	2	11	2	8	2	1.9	0.2	16	3	-	-	-	-	-	-
1	-	-	63	5	9	1	6	2	9.3	0.6	2.3	0.2	10	2	-	-	-	-	-	-
2	0.3	0.3	65	2	11	1	5	1	10.3	0.3	2.1	0.2	6	2	-	-	-	-	-	-
3	0.6	0.2	62	5	13	2	4.3	0.5	14	3	2.18	0.09	4.7	0.7	-	-	-	-	-	-
6	0.8	0.2	69	5	12	2	3.3	0.8	10	1	2.4	0.6	3	1	-	-	0.1	0.1	-	-
24	3.3	0.3	59	3	14	1	2.4	0.4	15	2	3.8	0.7	3	3	0.32	0.09	0.6	0.3	0.16	0.03
48	7	2	52	3	15	2	1.6	0.4	19	3	4.7	0.4	1.2	0.5	0.53	0.08	2	1	0.5	0.2

Table continues on next page

Time (%)	DMA	+-	Thiolation of DMA	±	Reduction to DMA(III)	±	Demethylation to MMA and MMA thiolation	±	Demethylation to iAs and iAs thiolation	±
0.5	55	2	16	4	-	-	1.9	0.2	27	6
1	63	5	19	2	-	-	2.3	0.2	16	4
2	65	2	21.6	0.7	0.3	0.3	2.1	0.2	11	3
3	62	5	26	5	0.6	0.2	2.2	0.1	9	1
6	69	5	22	3	0.8	0.2	2.5	0.6	6	2
24	59	3	28	3	3.3	0.3	4.7	0.8	6	3
48	52	3	33	4	7	2	6.7	0.8	3.3	0.8

Table S5. Data summary of measured and calculated distribution between DMA and thiolation of DMA for roots of *A. thaliana* (Col-0) seedlings exposed to 100 μM DMA for 0.5, 1, 2, 3, 6, 24, and 48 h.

Measured distribution between DMA and thiolation of DMA

Time	DMA ($mg \cdot kg^{-1}$)	Thiolation of DMA (DMMTA+DMDTA) ($mg \cdot kg^{-1}$)	DMA+DMMTA+DMDTA ($mg \cdot kg^{-1}$)	% distribution DMA	% distribution thiolation (DMMTA+DMDTA)
0.5	0.051	0.015	0.067	77	23
1	0.074	0.022	0.096	77	23
2	0.102	0.034	0.136	75	25
3	0.155	0.066	0.221	70	30
6	0.286	0.090	0.375	76	24
24	0.422	0.202	0.624	68	32
48	0.505	0.325	0.830	61	39

Calculation distribution between DMA and thiolation of DMA[2]

$k = 8.41 \times 10^{-6}\ s^{-1}$ at pH7

Time	DMA*	Thiolation of DMA (DMMTA+DMDTA) ($mg \cdot kg^{-1}$)**	% distribution DMA	% distribution thiolation (DMMTA+DMDTA)	Thiolation Factor***
0.5	0.07	0.00	98	2	15
1	0.09	0.00	97	3	8
2	0.13	0.01	94	6	4
3	0.20	0.02	91	9	3
6	0.31	0.07	82	18	1
24	0.17	0.45	27	73	0
48	-0.38	1.21	0	100	0

** calculated thiolation of DMA ($mg \cdot kg^{-1}$) = time * 3600 * k * measured DMA+DMMTA+DMDTA ($mg \cdot kg^{-1}$)

* DMA = Measured DMA+DMMTA+DMDTA ($mg \cdot kg^{-1}$) – calculated thiolation of DMA ($mg \cdot kg^{-1}$)

*** Thiolation factor = measured % distribution thiolation / calculated % distribution thiolation

(2) Kim, Y. T.; Lee, H.; Yoon, H. O.; Woo, N. C. Kinetics of Dimethylated Thioarsenicals and the Formation of Highly Toxic Dimethylmonothioarsinic Acid in Environment. *Environ. Sci. Technol.* **2016**, *50* (21), 11637–11645. https://doi.org/10.1021/acs.est.6b02656.

Study 5: Dimethylated Thioarsenates: A Potentially Dangerous Blind Spot in Current Worldwide Regulatory Limits for Arsenic in Rice

Britta Planer-Friedrich, Carolin F. Kerl, Andrea E. Colina Blanco, and Stephan Clemens

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Dimethylated Thioarsenates: A Potentially Dangerous Blind Spot in Current Worldwide Regulatory Limits for Arsenic in Rice

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ABSTRACT: Arsenic (As) occurrence in rice is a serious human health threat. Worldwide, regulations typically limit only carcinogenic inorganic As, but not possibly carcinogenic dimethylated oxyarsenate (DMA). However, there is emerging evidence that "DMA", determined by routine acid-based extraction and analysis, hides a substantial share of dimethylated thioarsenates that have similar or higher cytotoxicities than arsenite. Risk assessments characterizing the in vivo toxicity of rice-derived dimethylated thioarsenates are urgently needed. In the meantime, either more sophisticated methods based on enzymatic extraction and separation of dimethylated oxy- and thioarsenates have to become mandatory or total As should be regulated.

KEYWORDS: *arsenic, thioarsenates, DMMTA, thio-dimethylarsinate (thio-DMA), rice, risk assessment, food safety risk management*

1. INTRODUCTION AND SCOPE

Rice is the main staple food for over half the World's population. Unfortunately, it also has a propensity to accumulate up to 10-fold more arsenic (As) in grains than other cereals[1] and is therefore a major source of human As exposure, especially in many Asian and Latin American countries. In addition, rice is also becoming increasingly popular for alternative diets, for example, gluten-free diet. Moreover, rice and rice-based products contribute substantially to infant diets,[2] even in countries where rice is not a staple food to the general population, for example, within Europe, the USA, or Australia. Since a few years, the topic of "arsenic in rice" has received considerable attention among concerned consumers and in politics. A number of countries have now introduced regulatory limits or are at the stage of risk assessment as a basis for introducing limits. Here we argue that recent reports[3−5] on previously nonaddressed As species in rice, so-called dimethylated thioarsenates, seriously question the effectiveness and appropriateness of current limits, as well as currently applied analytical methods and previous risk assessments. To provide stakeholders with the necessary concise background, we summarize in the following the current state of As regulation in rice and what is known about thioarsenates in rice so far. We explain why thioarsenates were previously overlooked in rice and discuss their relevance in light of what is known about their toxicity. Finally, we derive an estimate on prevalence and type of rice samples that may pose a risk because of hidden thioarsenates, and outline what has to be done now, given the ubiquitous and quantitatively relevant occurrence especially of dimethylated thioarsenates.

2. ARSENIC OCCURRENCE IN FOOD AND CURRENT REGULATIONS IN RICE

Arsenic occurs ubiquitously in the environment and also in many items of human diet. Total As concentrations can range from a few μg/kg to tens of μg/kg in drinking water,[6] hundreds of μg/kg in rice,[7] or up to thousands of μg/kg in seafood.[8] Different food items typically contain different predominant As species of very different toxicities. Drinking water contains mainly the inorganic As (iAs) species, arsenite and arsenate, which are classified as "carcinogenic to humans" (Group 1).[9] Rice can contain, besides iAs, the simple organic As species monomethylated arsenate (MMA) and, especially, dimethylated arsenate (DMA), which are classified as "possibly carcinogenic to humans on the basis of animal studies" (Group 2).[9] Seafood typically contains less inorganic As but more complex organic As species, for example, arsenobetaine in marine fish[10] and oxy- and thio-arsenosugars in marine algae.[10] Besides water-soluble organoarsenicals, marine algae and animals can also contain lipid-soluble organoarsenicals, such as arsenic-containing fatty acids and hydrocarbons, mono/diacyl arsenosugar phospholipids, phytyl 2-*O*-methyl/2-hydroxy dimethylarsinoyl ribosides, and lipids with arsenic-containing fatty acids.[11] Many unknowns remain about formation and degradation pathways of organoarsenicals.[11] As of today, water-soluble organoarsenicals are still regarded as "not classifiable as to their carcinogenicity" (Group 3).[9] Lipid-soluble organoarsenicals are not well characterized toxicologically;[12] however, arsenic-containing hydrocarbons might be both cytotoxic and exert other toxicological effects, e.g., crossing the blood−brain barrier (reviewed in ref 11). Considerations of As in food range from a regulatory limit of 10 μg/kg total As (assuming a predominance of Group 1 As species) for drinking water[13] to no maximum limits for marine

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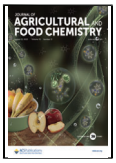

Table 1. Current Limits for Inorganic (iAs) or Total Arsenic (totAs) in Rice and Rice-Based Products Introduced by Different Food Authorities and Countries

country/organization	limit [μg/kg]	type of rice	effective since
FAO/WHO[31]	200 iAs	polished rice	2014
FAO/WHO[17]	350 iAs (drafted)	husked rice	2016
Argentina[24]	300 totAs	rice and rice products (except oil)	2012
Australia/New Zealand[27]	1000 totAs	rice (not further specified)	2020
Brazil[25]	300 totAs	rice and rice products (except oil)	2013
Canada[23]	100 iAs (proposed action level)	rice-based foods intended for infants and young children	2021
Canada[70]	200 iAs	polished rice	2020
	350 iAs	husked rice	
Chile[71]	200 iAs	polished rice	2018
China[72]	350 iAs	paddy rice, brown rice	2020
	200 iAs	rice (flour)	
European Union[21]	100 iAs	rice destined for the production of food for infants and young children	2016
	200 iAs	nonparboiled milled rice (polished or white rice)	
	250 iAs	parboiled rice and husked rice	
	300 iAs	rice waffles, rice wafers, rice crackers and rice cakes	
India[28]	1100 totAs	foods not specified	2011
Paraguay[26]	300 totAs	rice and rice products (except oil)	2019
Singapore[65]	100 totAs	food for babies	2017
	200 iAs	polished rice	2017
	350 iAs	husked rice	2019
South Africa[73]	200 iAs	polished rice	2016
	350 iAs	husked rice	2016
Taiwan[74]	100 iAs	rice destined for the production of food for infants and young children	2017
	200 iAs	polished rice	2017
	350 iAs	husked rice	2017
United States of America[22]	100 iAs (proposed action level; no limit on a federal level)	rice (used for infant cereals)	2020
Uruguay[75]	300 totAs	rice and rice products (except oil)	2013

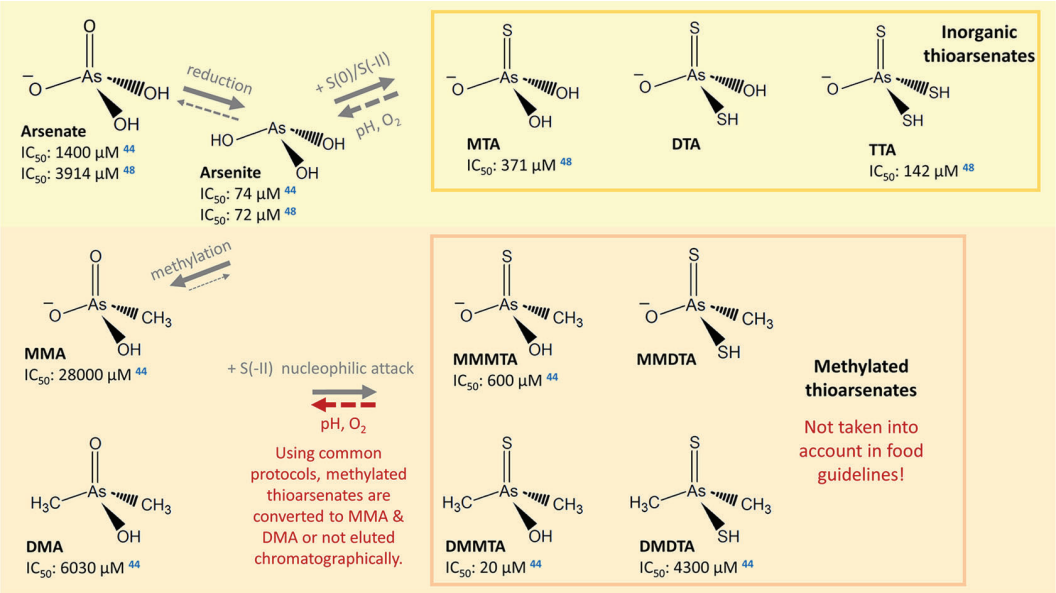

Figure 1. Formation, structure, and cytotoxicity of inorganic and methylated thioarsenates. In the orange box, the potentially dangerous blind spot: methylated thioarsenates that are converted to methylated oxyarsenates during extraction and analysis, therefore escaping regulations of food guidelines despite high cytotoxicity. MTA: monothioarsenate. DTA: dithioarsenate. TTA: trithioarsenate. MMA: monomethylated arsenate. MMMTA: monomethylated monothioarsenate. MMDTA: monomethylated dithioarsenate. DMA: dimethylated arsenate. DMMTA: dimethylated monothioarsenate. DMDTA: dimethylated dithioarsenate. Bold blue numbers correspond to the references mentioned in the text. Two IC_{50} values are given for arsenite and arsenate to compare against inorganic thioarsenates[48] and methylated oxy- and thioarsenates,[44] respectively.

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fish and algae, e.g., in the European Union (assuming a predominance of Group 3 As species).

Rice is challenging with regard to setting regulatory limits because it contains, on the basis of currently established knowledge, iAs (Group 1, carcinogenic), but can also have substantial shares of DMA (Group 2, possibly carcinogenic) or even a clear predominance of DMA over iAs.[14,15] Throughout the world, whether and how As in rice is regulated is handled quite differently (Table 1). Panels in charge of risk management have reviewed risk assessments and derived recommendations that balance necessary food safety and consumer protection versus economic feasibility. Organizations, such as the Food and Agricultural Organization (FAO),[16,17] the United States Food and Drug Administration (US FDA)[18] or the European Food Safety Authority (EFSA),[19,20] have assessed a risk with regard to iAs, which lead to its regulation by risk managing authorities. For DMA, the available toxicological and species-specific occurrence data prevented reaching any conclusion. DMA was classified as a Group 2 possible carcinogen and not included for regulation by the risk managing authorities. The more conservative approach of limiting total As was not adopted because of economic considerations and because it currently is regarded as "considerable overestimation of the health risk related to dietary arsenic exposure".[20] The FAO has drafted a maximum level of 200 μg/kg iAs for polished rice in 2014, and of 350 μg/kg iAs for husked rice in 2016.[17] Countries and unions that have implemented similar species-selective maximum levels are, for example, Canada, Chile, China, the European Union, Singapore, South Africa, and Taiwan (Table 1). The European Union has further implemented a lower level for the most vulnerable population group: rice used for infant food production has to meet a 100 μg/kg iAs limit.[21] The US FDA and Health Canada have proposed the same in 2020[22] and 2021,[23] respectively, but the proposed action level is still undergoing review and therefore not yet legally enforceable. Only a few countries have implemented regulatory limits based on the total As content, and the concentration levels differ substantially, ranging from 300 μg/kg in Argentina,[24] Brazil,[25] Paraguay,[26] and Uruguay[26] to 1000 μg/kg in Australia and New Zealand[27] and 1100 μg/kg in India[28] (Table 1).

3. ARSENIC SPECIATION IN RICE BEYOND INORGANIC ARSENIC AND DMA: QUANTITATIVE IMPORTANCE OF THIOARSENATES

Recent reports,[3−5] however, suggest that iAs and DMA are not the only relevant As species in rice but that also inorganic and methylated thioarsenates (for information on molecular structures and formation pathways see Figure 1) can be found in rice grains and rice products. The database is still comparatively thin. Still, the thiolated structural analogue to DMA, dimethylated monothioarsenate (DMMTA; also termed thio-dimethylarsinate (thio-DMA)), emerged as a major species. DMMTA was detected in 50 out of 54 commercially available rice grain and rice product samples purchased in Costa Rica, Germany, and the United States,[3] in rice from two paddy fields in China with and without sulfur fertilizer and rice straw incorporation,[4] in 103 out of 103 soil-rice grain paired samples collected across the major rice-producing regions in China,[5] and in 138 out of 140 commercial polished rice samples from around the world.[5] DMMTA concentrations in polished rice typically were in the lower tens of μg/kg, equivalent to up to 15%,[3] 21%,[5] and 28% of total As,[4] but some rice products contained up to 250 μg/kg DMMTA (almost 40% of total As).[3] In relation to DMA, Dai et al.[5] reported for both field and commercial samples a surprisingly uniform share of 30 ± 8% DMMTA, whereas in the other two studies the share was more variable (31−57%[4] and 1−176%[3]). Only one out of the three mentioned studies addresses other inorganic and methylated thioarsenates.[3] Besides DMMTA, which was the predominant species in all samples, inorganic monothioarsenate (MTA), dimethylated dithioarsenate (DMDTA), and inorganic dithioarsenate (DTA) plus, rather seldom and in relatively low concentrations, monomethylated mono- and dithioarsenate (MMMTA and MMDTA) were detected.

4. THIOARSENATE TRANSFORMATION DURING ROUTINE EXTRACTION AND SEPARATION METHODS

If thioarsenates occur so abundantly, the question arises why they have been overlooked before. In contrast to drinking water, which is homogeneous and relatively straightforward to analyze, analysis of solid foods poses additional challenges with regard to obtaining correct and representative sample information and, for risk assessment, information about the fraction that is bioaccessible upon ingestion. Different As species typically show a nonhomogeneous distribution within the solid food item, for example, among bran, germ, and endosperm in rice.[29] These species have to be extracted quantitatively, yet in a manner conserving their original redox and coordination state. High-resolution spatial imaging at the cellular level in combination with speciation analysis at low detection limits would be the ideal tool of choice, but even state-of-the-art techniques like micro X-ray absorption near edge spectroscopy (μ-XANES) or nano secondary ionization mass spectrometry (NanoSIMS) lack the spatial resolution or the species information, respectively. For food safety monitoring, most laboratories use simple microwave-assisted acid digestion to determine total As concentrations followed by analysis via atomic absorption or fluorescence spectrometry (AAS and AFS) or inductively coupled plasma atomic emission or mass spectrometry (ICP-AES and ICP-MS). Only when total As exceeds the regulatory limit is iAs typically determined by extraction with milder reagents, for example, 2 M trifluoroacetic acid,[30] 0.28 M nitric acid[31,32] with extraction efficiencies between 80 and 120%,[33] or 0.1 (or 0.2) M nitric acid in 3 (or 6) % H_2O_2 with recoveries of 90−115%,[34] and analysis by hydride-generation (HG)-AAS[35] or high-performance liquid chromatography (HPLC)-ICP-MS.[31,32,34]

To correctly assess how much of the matrix-bound As is bioaccessible, a two-step enzymatic extraction can be used, which mimics biochemical processes in the human gastrointestinal tract (stomach with incubation for 1 h at 37 °C in a pepsin solution at pH 2 and small intestine with incubation for 2 h at 37 °C in a pancreatin solution at pH 6).[36] For rice, most of the As was found to be bioaccessible. In comparison to acid extractions, 83−94% of iAs and 72−91% of DMA were detected after enzymatic extraction and HPLC-ICP-MS analysis.[36] Most monitoring laboratories therefore use the less cost- and time-intensive acid extraction. To the best of our knowledge, currently only the Canadian Food Inspection Agency prescribes in their standard operation manual for arsenic speciation in foods the use of an enzymatic extraction.[37] The choice of enzymatic over acid extraction does, however, have a significant impact on correct species

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identification. Already in 2005, Ackerman et al.[36] reported that the seemingly poor recovery for DMA from enzymatic extraction (for example, 105 μg/kg vs 146 μg/kg from acid extraction) is explained by the presence of an additional species that, upon addition of acid, converts into DMA. The species was characterized as DMMTA, and it was clearly demonstrated not to be an extraction artifact. DMMTA was also observed in a later rice grain survey of the same group using enzymatic extraction.[38]

Until 2021, no further studies addressed the natural occurrence of DMMTA in food. Aside from a lack of awareness on the occurrence of this species and the fact that routinely applied acid extractions would convert DMMTA to DMA, an additional reason for the lack of DMMTA reports could have been an insufficient detection limit when the method of Ackerman et al. was used.[36] We recalculated the detection limit as ca. 1 μg/L in the extract, equaling 16 μg/kg in the original rice sample. Thus, DMMTA concentrations below that level would have remained unrecognized. In contrast, Colina Blanco et al.[3] reported significantly improved detection limits (0.024 μg/L equaling 0.4 μg/kg in the original rice sample). Another reason might have been a lack of the respective DMMTA standard to identify potentially observed unknown peaks.

Detection of other thioarsenates requires even more effort. To separate and quantitatively elute not only DMMTA but also the inorganic thioarsenates, MTA and DTA, as well as the mono- and dimethylated thioarsenates MMMTA, MMDTA, and DMDTA (Figure 1), a highly alkaline IC-eluent (2.5−100 mM NaOH gradient with 2.4% methanol) is required, which in turn requires a separation column and instrument to withstand the high pH (Colina Blanco et al.[3] used a Dionex IonPac AS16 column with ion chromatography (IC) instead of the previously used Hamilton PRP X-100 HPLC column). Especially the inorganic thioarsenates readily transform into their respective oxy species upon acidification, heating, oxidation, or dilution or are lost by precipitation or irreversible binding, for example, when weak eluents were used during species separation. The higher thiolated arsenates only elute under the extreme conditions of pH 13. Furthermore, there is no certified reference material for thioarsenates, yet, and for many of them, there are not even commercial standards (there are, however, now for DMMTA [https://www.trc-canada.com/product-detail/?D471360; accessed on 11.07.2022; CAS#754217-65-7] and DMDTA [https://www.trc-canada.com/product-detail/?D471350; accessed on 11.07.2022; CAS#27318-74-7]. Thus, using routine methods, there is a high potential of overlooking thioarsenates and reporting them as the respective oxyarsenic species instead.

5. THIOARSENATE TOXICITY

An urgent next question then is whether misidentification of thiolated species for their oxylated analogues is problematic with regard to toxicity-based risk assessment. Even though not commonly known from food studies, DMMTA is well-known in As toxicology. It has been identified as a metabolite of arsenite and arsenate after human exposure to drinking water[39,40] and of arsenosugars after human exposure to a seafood-based diet.[41] Though some studies have attributed the carcinogenic effect of As to formation of trivalent methylated species inside the human gastrointestinal tract, Hansen et al. showed in 2004 that previously described DMA(III) in fact was DMMTA.[42] Cell culture tests demonstrate that DMMTA is much more cytotoxic than DMA (Figure 1). In fact, it shows higher cytotoxicity even in comparison to arsenite, for example, a 6.7-fold stronger cytotoxicity for human bladder EJ-1 cells[43] as well as a 3.7-fold[44] to 5-fold stronger cytotoxicity and 2-fold higher cellular bioavailability for A549 lung adenocarcinoma epithelium cells.[45] Further, DMMTA caused a decreased damage-induced cellular poly(ADP-ribosyl)ation at 35,000-fold lower concentrations than arsenite.[46] DMMTA also has genotoxic effects, confirmed in cultured hamster embryo cells by induction of cell-cycle arrest, aneuploidy, chromosome structural aberrations, the apoptotic mode of cell death, and abnormalities of spindle organization and centrosome integrity.[47] Misidentification of DMMTA as the nonregulated DMA is therefore likely a serious underestimation of risk. Despite early calls for full (also in vivo) toxicological characterization for DMMTA as "by far the most toxic human metabolite",[46] to the best of our knowledge, such direly needed data are still lacking.

Even less information exists with regard to other thiolated arsenates. Cytotoxicity of inorganic thioarsenates, however, seems to be lower than that of arsenite. For example, for human hepatocytes (HepG2), cytotoxicity was reported to increase with increasing thiolation; MTA was more cytotoxic than arsenate, TTA is a bit less cytotoxic than arsenite[48] (Figure 1). On the basis of these scarce data, misidentification of inorganic thioarsenates as inorganic (oxy)arsenic species might pose no additional health risk where iAs is already regulated on the basis of the toxicity of arsenite. However, as for DMMTA, no in vivo toxicity data exist. For the methylated thioarsenates, MMMTA was shown to be more cytotoxic than MMA in cell culture tests,[44] but it seems quantitatively less important in rice. The species most critical from a toxicological point of view, besides DMMTA, likely is DMDTA. Its cytotoxicity is slightly higher than that of DMA,[44] but there likely also is potential for transformation to DMMTA during food preparation or digestion.

6. ESTIMATE OF PREVALENCE AND TYPE OF RICE SAMPLES CARRYING HIDDEN DMMTA

Considering how little is known about thioarsenates, yet, and how much additional analytical effort a proper species-selective assessment requires, it would be useful to obtain an estimate on the prevalence and type of samples that may contain a hidden risk because of thioarsenate occurrence, specifically the highly cytotoxic DMMTA. We therefore compiled iAs and total As concentrations in rice from the publicly accessible WHO Global Environmental Monitoring System (GEMS) food database (https://extranet.who.int/gemsfood) and placed these concentrations, as one example, in relation to current regulatory limits within the European Union (Figure 2). The entries in the database comprise domestic and imported samples reported from different countries. They were analyzed between 2004 and 2016, i.e., largely in preregulatory times, reflecting natural variation throughout the world. After data treatment (for details see Supporting Information, Table S1), 3742 samples remained for which information on iAs and total As concentrations was available and which are displayed in Figure 2. From the samples in this database, it becomes obvious that meeting the 100 μg/kg iAs-limit, the "safe" concentrations for the most vulnerable population group, generally is very challenging (dark gray bars in the histogram of Figure 2b). Many of the samples in our data set (59%) had higher concentrations. On the contrary, 87% of all our samples

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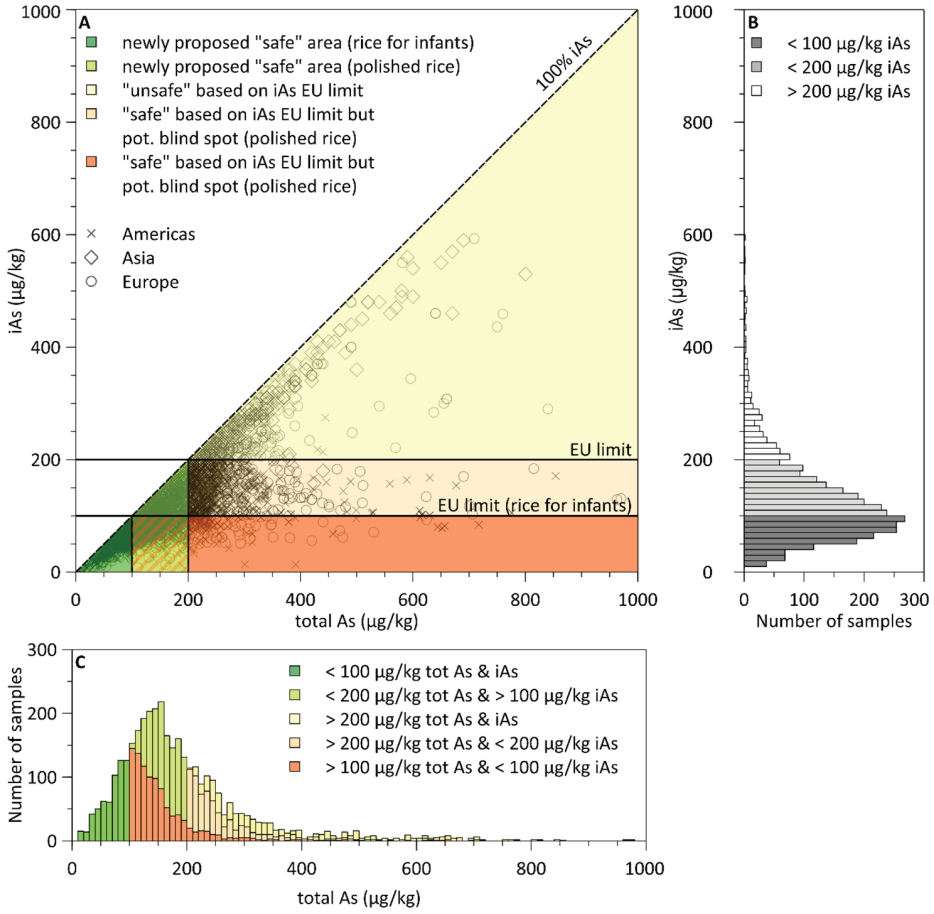

Figure 2. Concentrations of total versus inorganic As in rice in relation to current EU limits and potential blind spots. (A) Arsenic concentrations in commercially available rice samples reported from Asian, American, and European markets between 2004 and 2016 assembled from the WHO Global Environmental Monitoring System (GEMS) food database (for information on sample selection and full data set, see SI Table S1). Light and dark green areas delineate samples "safe" for ingestion, the yellow area encompasses samples above the current threshold that are excluded for trade, and light and dark orange areas delineate potential blind spots of samples that are not classified as "unsafe" on the basis of current regulations, but where high concentrations of DMMTA can pose a hidden risk. Histograms show the number of samples that are categorized in (B) as "safe" or "unsafe" on the basis of iAs and in (C) as "safe", "unsafe", and potentially dangerous on the basis of total As concentrations.

met the 200 μg/kg iAs limit (sum of dark and light gray bars in Figure 2b). Many of the samples surpassing this limit (the whole area marked in yellow in Figure 2a) come from the Asian market.

On the other side, there are also about 25% (dark orange bars in Figure 2c) and 21% of samples (light orange bars in Figure 2c) in our data set, mainly from the European and the American markets, for which iAs is below 100 and 200 μg/kg, respectively (so, considered "safe" based on iAs), whereas total As is, sometimes substantially, above 100 and 200 μg/kg (up to 1980 μg/kg). The difference of total As minus iAs is typically assumed to be DMA. The observation that DMA increases linearly with total As whereas iAs seems to level out at around 100−150 μg/kg has been widely reported before, mostly in samples from Europe and the Americas.[7,14,15,49−52] Such samples were reported to have DMA as major As species, whereas the percentage of iAs can be as low as 10% of total As.[15] The reason for these "geographical differences" is unclear, yet. On the basis of the ubiquitous detection of DMMTA in recent studies,[3−5] it is highly likely that also for other samples, the difference between total arsenic and iAs is not quantitatively attributable to DMA. Instead, given the strong correlation between DMA and DMMTA cited above, it is very likely that most rice contains hidden DMMTA, a highly cytotoxic species, which should be included in future risk assessment and risk management.

In our Figure 2, we have tentatively marked the potential risky blind spot by a line indicating less than 200 μg/kg iAs but more than 200 μg/kg total As (or 100 μg/kg in the case of infant food), which means arsenite and "DMA" would be considered as equally toxic. This thought is based on the rather simplified assumption that a certain share of analyzed "DMA" in fact is DMMTA (here assumed to be one-third of DMA;[5] the share varies and can also be much higher as pointed out before[3,4]) and that the cytotoxicity of DMMTA is at least 3 times higher than that of arsenite.[43,44] More data on DMMTA occurrence and its in vivo toxicity will help to better assess whether such an approach is justified.

7. REQUIRED STEPS FOR IMPLEMENTING THIOARSENATES IN FOOD SAFETY RISK ASSESSMENT

Given their ubiquitous detection at substantial concentrations in different rice samples and previously reported cytotoxicity, thioarsenates, and especially the dimethylated thioarsenates DMMTA and DMDTA, must be considered when As-related

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risks are assessed for the consumption of rice, which is globally the most important staple food and an important ingredient of infant diets. To create a solid database for decision makers, more studies on thioarsenates are needed, tracking their relevance and fate from the field to the plate to the human body.

On the basis of everything we currently know, thioarsenate accumulation in rice grains cannot be avoided during rice cultivation in the field. One main source is the paddy soil pore water. It has been shown before that thioarsenates form under anoxic conditions in flooded paddy soils[53] and that, once formed, they can be taken up by rice plants.[54−56] Management practices can to some extent influence thioarsenate concentrations in paddy soil pore water. Alternate wet−drying will decrease the share of thioarsenates[57] whereas sulfate fertilization will increase it.[53] More investigations on thioarsenate occurrence in paddy soil pore water, uptake and transformation in rice plants, and susceptibility of different rice varieties to accumulation of thioarsenates in rice grains are needed.

To better estimate the overall relevance of thioarsenate accumulation in rice grains, first, monitoring laboratories would need to be able to measure them reliably. In principle, establishing an enzymatic instead of an acid extraction is technically not challenging. However, it is more time-consuming and expensive to run. Activity and storage stability of enzymatic solutions might pose challenges for reproducibility. Interestingly, again, Canada at the moment appears to be the only country to require enzymatic instead of acid extractions.[37] An additional challenge is the chromatographic conditions. To separate all thioarsenates, including inorganic thioarsenates, a pH 13 eluent is required as reported by Colina Blanco et al., which in turn requires the use of an IC instead of the more common HPLCs. A compromise could be to focus only on the dimethylated thioarsenates DMMTA and DMDTA, which can also be separated with more routinely applied eluents and columns (for example, using a C18 column and a 20 mM $NH_4H_2PO_4$ (pH 3) eluent[58]). However, one would need to test whether inorganic thioarsenates elute as arsenite or arsenate under these conditions or remain on the column. As mentioned before, summarizing inorganic thioarsenates with inorganic (oxy)arsenic species might be less critical for risk assessment, given their lower cytotoxicity based on in vitro toxicity studies. However, if inorganic thioarsenates remain on the column, total inorganic As concentrations will be wrongly assessed as too low. Apart from data on thioarsenate speciation in the commercially available rice grain, more data would also be needed on potential thioarsenate transformation or their formation from oxy analogues, during rice processing (parboiling, popping, puffing), storage, and consumer handling (washing, cooking, baking).

In a further step, the fate of thioarsenates in rice upon human ingestion needs to be followed. Because the two-step enzymatic extraction mimics processes in the human gastrointestinal tract,[59] it is assumed that within the real human gastrointestinal tract thioarsenates in rice are equally bioaccessible and released at least partially intact upon rice digestion. Further, in vitro assays with microbiota from mouse cecum[60,61] and from the human colon[62] have shown formation of inorganic and methylated thioarsenates from oxyarsenic precursors. Therefore, risk assessment not only has to include potential transformation of, for example, more toxic DMMTA into less toxic DMA but also has to assess how much of the less toxic DMA, released from rice, can be thiolated to more toxic DMMTA. Results from a Caco-2 cell monolayer model indicated substantially higher passage through the gastrointestinal barrier for a spiked DMMTA standard than for DMA,[63] however, not considering the additional complexity of release from a food matrix first. For a solid assessment of risk upon ingestion of thioarsenate-containing rice, a lot more information is required on toxicity in experimental animals, on toxicokinetics (absorption, distribution, metabolism, excretion) as well as on the toxic mode and mechanism of action in experimental animals and humans, observations in humans (including epidemiological studies, case reports, biomarkers of exposure/effect), and human biomonitoring (blood, serum, urine).

8. REGULATING TOTAL AS IN RICE

Considering current knowledge gaps, it will require considerable time and effort to receive and review all data and even more time to implement them into new regulatory limits. Meanwhile, the more conservative approach would be to limit total As, at which concentration needs to be discussed, of course. Currently, only very few countries limit total As. Australia, New Zealand, and India do so, but at unjustifiably high concentrations (1000[27] and 1100[28] μg/kg), which are extremely seldom exceeded by natural samples (see Figure 2, Table S1). Argentina, Brazil, Paraguay, and Uruguay introduced the limit at 300 μg/kg total As. On the basis of our selected database (Figure 2, Table S1), this limit would let 274 out of 3742 samples with iAs > 200 μg/kg and total As < 300 μg/kg pass as "safe" (i.e., 7%, which, for example, in the European Union would be deemed "unsafe"). However, it would classify 198 samples as "unsafe" with iAs < 200 μg/kg but total As > 300 μg/kg (i.e., 5%, which, for example, in the European Union would be deemed "safe"). A recent national Brazilian survey arrived at even higher numbers, showing that more than 30% of polished rice samples were "unsafe" on the basis of the current total As limit, whereas not even 5% would exceed other countries' iAs-only limit.[64] A review of the total As-based limit was therefore suggested as necessary. Considering the latest knowledge of hidden DMMTA and DMDTA, such a change would likely represent a move in the wrong direction. Especially in regions with high "DMA" concentrations, such as Europe and the Americas, a total As limit could substantially reduce dietary exposure risk. For other regions with very high shares of iAs, like Asia, not much might change. The reasons for high DMA (or DMMTA/DMDTA) concentrations in certain rice samples (the "geographical differences") certainly also warrant further investigation. The currently strictest limit is the 100 μg/kg total As limit set in Singapore for rice used for infant food production in 2017.[65]

9. THIOARSENATES IN OTHER FOOD ITEMS

Last but not least, thioarsenates might also play a role in other food items. There is a previous report of MMMTA occurrence in carrot extracts,[66] of DMDTA occurrence in one sample each of mussel and cuttlefish,[67] and of an unknown As species ("likely a thiolated arsenical") in hijiki (brown algae).[68] Arsenocholine has been proposed to form through degradation of dimethylarsenoribosides, the major arsenosugars in brown algae, where, among others, DMMTA is produced as intermediate.[11] As to whether this DMMTA is partially

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preserved and can accumulate in marine food is currently unknown. Consideration of As limits for seafood is currently very diverse, too, ranging from no limits, e.g., in the European Union (just animal feed and feed ingredients are regulated at 25 mg/kg total As in fish[69] and 40 mg/kg total As in seaweed[69]) to limits considering the share of iAs only (for example, in Australia and New Zealand: 1 mg/kg for seaweed and molluscs, 2 mg/kg for fish and crustacea[27]). None addresses DMA or thiolated As species. Moreover, with >300 naturally occurring organoarsenicals,[11] many of which have not been fully characterized toxicologically, there might be more blind spots in our current understanding about the risk of As exposure through food.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c02425.

Description of data treatment of the publicly accessible WHO GEMS food database and list of data for creating Figure 2, including iAs and total As concentrations in rice and rice origin (PDF)

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Notes

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Supporting Information

Dimethylated thioarsenates - a potentially dangerous blind spot in current worldwide regulatory limits for arsenic in rice

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(107 pages, 1 table)

Data treatment GEMS data base

Data downloaded: 16.09.2021

1. Duplicates removed for data from “WHO European Region” and “European Union”
2. All data without “SerialNumber” removed
3. All data converted to µg/kg (including LOD and LOQ)
4. Total As and iAs joined in one database based on matching sample by “SerialNumber”
5. Rice samples that were identified as “brown”, “red”, or “black” rice by “LocalFoodName” were removed from the data set
6. Values in database checked by logical measures:
    a. iAs ≤ total As: all data deleted that were more than 10 µg/kg too high
    b. iAs < LOD (iAs): all data below LOD deleted; all data deleted when LOD was given as concentration
    c. total As < LOD (total As): all data below LOD deleted; all data deleted were LOD was given as iAs or totAs
7. Samples with iAs max 10 µg/kg > total As are not show in figure 2 and one sample with total As of 1980 µg/kg was excluded for better visibility
8. Regions “Americas”, “Asia”, and “Europe” were grouped according to the “Region Name”
    a. Americas: WHO/PAHO Region of the Americas
    b. Asia: WHO Western Pacific Region, WHO South-East Asia Region
    c. Europe: European Union

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jafc.2c02425.

List of Publications

The following papers have been published during the work on this thesis:

Colina Blanco, A. E.; Kerl, C. F.; Planer-Friedrich, B. Detection of Thioarsenates in Rice Grains and Rice Products. *J. Agric. Food Chem.* **2021**, *69* (7), 2287–2294.

Pischke, E.; Barozzi, F.; Colina Blanco, A. E.; Kerl, C. F.; Planer-Friedrich, B.; Clemens, S. Dimethylmonothioarsenate Is Highly Toxic for Plants and Readily Translocated to Shoots. *Environ. Sci. Technol.* **2022**, *56* (14), 10072–10083.

Planer-Friedrich, B.; Kerl, C. F.; Colina Blanco, A. E.; Clemens, S. Dimethylated Thioarsenates: A Potentially Dangerous Blind Spot in Current Worldwide Regulatory Limits for Arsenic in Rice. *J. Agric. Food Chem.* **2022**, *70* (31), 9610–9618.

Colina Blanco, A.E.; Pischke, E.; Higa Mori, A.; Kerl, C.F.; Clemens, S.; Planer-Friedrich, B. *In Planta* Arsenic Thiolation in Rice and *Arabidopsis thaliana*. *Environ. Sci. Technol.* **2023**, *57* (51), 21846- 21854.

Colina Blanco, A.E.; Higa Mori, A.; Planer-Friedrich, B. Widespread occurrence of dimethylmonothioarsenate (DMMTA) in rice cakes: effects of puffing and storage. *Food Chem.* **2024**, *436* (2024), 137723.

Other publications/manuscripts realized/submitted during the time as PhD student:

León Ninin, J. M.; Colina Blanco, A. E.; Held, A.; Planer-Friedrich, B. Environmental Forensics: Mock Trial of a Chromium Contamination Case as a Tool for Interdisciplinary Teaching and Improvement of Soft Skills. *J. Chem. Educ.* **2022**, *99* (10), 3452–3460.

Kerl, C. F.; Basallote, M. D.; Käberich, M.; Oldani, E.; Cerón Espejo, N. P.; Colina Blanco, A. E.; Cánovas, C. R.; Nieto, J. M.; Planer-Friedrich, B. Consequences of Sea Level Rise for High Metal(Loid) Loads in the Ría of Huelva Estuary Sediments. *Sci. Total Environ.* **2023**, 873 (December 2022).

Fang, X.; Christl, I.; Colina Blanco, A. E.; Planer-Friedrich, B.; Zhao, F. J.; Kretzschmar, R. Decreasing Arsenic in Rice: Interactions of Soil Sulfate Amendment and Water Management. *Environ. Pollut.* **2023**, *322*, 121152.

Fang, X.; Colina Blanco, A. E.; Christl, I.; Le Bars, M.; Straub, D.; Kleindienst, S.; Planer-Friedrich, B.; Zhao, F. J.; Kappler, A.; Kretzschmar, R. Simultaneously decreasing arsenic and cadmium in rice by soil sulfate and limestone amendment. *Submitted to Environmental Pollution*.

Supervised Master Theses

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

Classnitz, J. (2021). Transportation and re-translocation of methylated arsenic species in rice shoots
Results of this project are included in study 4 of this thesis.

Cerón Espejo, N. (2020). Characterization of metal(loid)s stored in the salt marshes of Río Tinto and Río Odiel, Spain
Results of this project are included in co-author publication.

Oldani, E. (2020). Potential mobilization of pollutants from mine-water affected coastal marsh soils with sea level rise: insights from the Río Tinto and Río Odiel estuarine system.
Results of this project are included in co-author publication.

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