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1 **Elemental distribution in developing rice grains and the effect of flag-leaf arsenate exposure.**

2

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17

18

1 **Abstract**

2 We measured the bulk grain concentrations of arsenic (As), along with rubidium (Rb) and strontium (Sr)  
3 as indicators of phloem and xylem transport respectively, in rice (*Oryza sativa* cv. *Italica Carolina*) pulsed  
4 with arsenate at two exposure levels for 5 day periods at progressively later stages of grain fill, between  
5 anthesis and maturity, through the cut flag leaf. We compared these to unexposed (negative) controls  
6 and positive controls; pulsed with dimethylarsinic acid (DMA). We collected elemental maps of As and  
7 micronutrient elements (Fe, Zn, Mn, Cu and Ni) from developing grains of rice. Exposures were either  
8 25 or 100 µg/ml arsenate (As(V)) at various stages of grain development, compared to 25 µg/ml  
9 dimethylarsinic acid (DMA); the most efficiently transported As species identified in rice. We used the  
10 spatial distribution of arsenic in the grain to infer the presence of As transporters. By exposing grains  
11 through the flag leaf rather than via the roots, we were able to measure arsenic transport into the grain  
12 during filling under controlled conditions. Exposure to 100 µg/ml As(V) resulted in widespread As  
13 localization in both embryo and endosperm, especially in grains exposed to As at later stages of panicle  
14 development. This suggests loss of selective transport, likely to be the result of As toxicity. At 25 µg/ml  
15 As(V), As colocalized with Mn in the ovular vascular trace (OVT). Exposure to either As(V) or DMA  
16 reduced grain Fe, an effect more pronounced when exposure occurred earlier in grain development.  
17 The abundance of Cu and Zn were also reduced by As. Arsenic exposure later in grain development  
18 caused higher grain As concentrations, indicating the existence of As transporters whose efficiency  
19 increases during grain fill. We conclude that localization of As in the grain is a product of both As  
20 species and exposure concentration, and that high As(V) translocation from the flag leaf can result in  
21 high As concentrations in the endosperm.

22

23

## 1 1.0 Introduction

2 Rice (*Oryza sativa* L.) is a dietary staple for half of the global population that accumulates the metalloid  
3 arsenic (As) [1-11] under flooded soil conditions. Arsenic is a Class I, non-threshold known carcinogen  
4 that can cause various cancers (skin, lung, bladder, kidney, liver and prostate) [12], and can affect  
5 cardiovascular, hematological, pulmonary, neurological, immunological, and  
6 reproductive/developmental function [13] at high chronic exposure levels. As exposure from food to  
7 children under the age of three is estimated to be 2–3 fold that of adults [14]. Arsenic exposure from  
8 rice in a population with As-free drinking in water in West Bengal (India) has been linked to genotoxicity  
9 [15]. Rice-based foods are second only to seafood and seaweed as dietary sources of As [14], but unlike  
10 the arsenosugars found in seafood and seaweed [16] (specifically arsenobetaine and arsenocholine),  
11 the inorganic forms of As measured in rice are almost 100% bioavailable [17]. The predominant forms of  
12 As in rice are the inorganic species (arsenate, As(V) and arsenite, As(III)) and the organic species  
13 (dimethylarsinic acid, DMA and monomethylarsonic acid, MMA) [5]; the relative proportions of which  
14 are product of both genes and environment [8]. Inorganic As species are more acutely toxic than  
15 organic forms, with the median lethal dose of inorganic As several orders of magnitude lower than  
16 organic (10–20 µg/ml for As(V) and As(III); 700–2600 for DMA and 700–1600 for MMA)[18]. The order of  
17 acute As toxicity is As(III) > As(V) > MMA ≈ DMA, but human carcinogenicity and genotoxicity in  
18 humans may not follow this order [18].

19 As an element with no essential function in plants, As gains entry via the transport systems of essential  
20 nutrients phosphorus (P) and silicon (Si)—the phenomenon of molecular mimicry, based on similar  
21 chemical properties such as charge and ionic radius[19]. Inorganic As(III) present as arsenous acid  
22 ( $H_3AsO_3$ ) in flooded rice paddies[20] and enters rice root cell symplast via the silicic acid transporter,  
23 Lsi1 (Low Silicon 1) [21, 22], whereas membrane-intrinsic phosphate transport proteins are responsible  
24 for As(V) uptake[23]. Organic As species DMA and MMA can also be transported into the root via

1 *Lsi1*[21]. Arsenite is exported from root cells into the xylem by the effluxer *NIP2;1* (nodulin-26 like  
2 intrinsic channel proteins) [24], which works in tandem with influxer *Lsi1* to shuttle As inwards across  
3 the root towards the vasculature [25]. The reduction of As(V) to As(III) governs the transport of As to  
4 the edible part of the grain, with almost all As(III) being delivered via the phloem [26, 27].

5 Arsenite was previously assumed to be the dominant species of inorganic As inside plant cells; however  
6 a study of rice exposed to As under Si-depleted conditions found a high proportion of As(V) in the grain  
7 [28]. Arsenate continues to use the high affinity P transporters for upward transport into vasculature  
8 and leaves[29]. Arsenic is then complexed with glutathione or phytochelatins and sequestered into  
9 vacuoles by ABCC-type transporters[30]. Inositol transporters *AtINT2* and *AtINT4* expressed in the  
10 companion cells of the phloem are involved in As(III) loading into the developing grain [31].

11 In many rice varieties, organic As species constitute a significant proportion of the As in rice, ranging  
12 from 26% [32] to 50% [5]. Since plants cannot methylate As, organic As species may be a product of soil  
13 microbial activity. Microbial biotransformation of soil As from application of As-rich poultry litter [33]  
14 and seaweed fertilizers [33, 34] could theoretically contribute organic As species to the soil. Castlehouse  
15 *et al.*[34] studied the biotransformation of arsenosugars from seaweed fertilizers commonly used in  
16 organic farming and found that DMA was the predominant arsenic species in soil porewater.

17 Quantifying the amount and speciation of As in tissues of the rice grain is important for food safety  
18 because a wide and increasing number of ingredients are derived from rice, particularly in foods  
19 intended for infants, recently been the primary focus of regulatory considerations [35] and gluten-free  
20 foods in which rice is the primary grain. Rice products formulated from specific grain layers range from  
21 flours and oils to emulsifiers and sweeteners, and can be a major ingredient in a wide range of baked  
22 goods [36], infant snacks [37] and powdered milk formulas. The potential of rice products to be  
23 significant sources of As exposure was demonstrated recently when organic brown rice syrup (OBRS),  
24 used as a sweetener, was found to contain unusually high As concentrations[38]. The accumulation of

1 inorganic As in rice bran [27] means that processed forms of rice bran or whole brown rice may be a  
2 significant dietary source of concentrated inorganic As. DMA is efficiently transported into the  
3 endosperm, but As(III) remains in the maternally-derived outer bran layers [32], in particular the ovular  
4 vascular trace (OVT) a strand of vascular tissue that is the main route of nutrient transport from the  
5 mother plant during grain fill. The extent to which As(V) is transported to the grain and its distribution  
6 is less clear; obscured by speciation changes that occur during uptake and long distance transport  
7 within the plant.

8 The aim of this study was to measured the distribution and abundance of total As in the tissue layers of  
9 rice grains pulsed with As(V) directly through the flag leaf during grain development using synchrotron  
10 X-ray fluorescence microscopy (SXRF). We comparing this to a negative control (no As pulse) and a  
11 positive control consisting of a DMA pulse;the form of As most efficiently transported into the  
12 endosperm of rice. Also following our previous work (REF), we used co-exposure to rubidium (Rb) and  
13 strontium (Sr) as markers of phloem and xylem transport activity. While bulk analysis of As  
14 concentrations in grains is critical, only spatially resolved techniques are able to provide information on  
15 the distribution of As between individual tissue layers. Feeding As via the flag leaf as As(V) results in  
16 higher grain total As concentration than feeding with As(III) [26]. Here, we show that As(V) when  
17 provided to flag leaves at high concentrations can accumulate in the endosperm. Moreover, As  
18 accumulation results in changes in the nutrient concentrations and localization in grains. Our results  
19 indicate that As species, exposure concentration and grain developmental stage are all important for  
20 the final As distribution in grains, with the potential to change nutrient distribution.

21

## 22 **2.0 Materials and Methods**

23

### 24 *2.1 Cultivation of Rice Plants*

1 A quick flowering variety of rice (*Oryza sativa* L.) cv. *Italica Carolina* was cultivated in a controlled  
2 temperature greenhouse until anthesis, as described in Carey *et al.*[26]. Briefly, seeds were sown  
3 directly into trays of John Innes No. 2 potting compost and transferred to 1 L pots after two weeks  
4 under tropical conditions (23–35 °C), with additional lighting supplied at 150  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  via sodium  
5 lamps. Plants were fed with Sangral soluble fertilizer (Sinclair) on a weekly basis, with a N:P:K ratio of  
6 1:1:1 plus 1 MgO diluted 1 in 100 (w/v). Rice panicles were exposed to As(V) or DMA at different stages  
7 of grain development as described in Table 1. Treatment stock solutions were prepared by dissolving  
8 the appropriate salt in Milli-Q deionized water, and the appropriate number of subsamples of each  
9 stock were frozen for subsequent removal and use on the relevant day. Treatment vials were prepared  
10 by making 0.25 mL of the defrosted treatment stock up to 1 mL with Milli-Q deionized water and using  
11 a 0.1 mL aliquot in a weighed Eppendorf vial, together with MES buffer at a final concentration of 5 mM.  
12 Rubidium (Rb) and strontium (Sr) were added as markers for phloem and xylem transport respectively,  
13 with final concentration of 85.5 and 87.6  $\mu\text{g}/\text{ml}$  (1 mM) respectively[27]. Solutions were pH adjusted to  
14 6.4 with NaOH.

15 The tip of the flag leaf was removed with a surgical steel blade and discarded. The cut surface of the  
16 flag leaf was inserted in to the Eppendorf vial, taking care to immerse the cut surface in the treatment  
17 solution. Treatment solutions used were As(V) (25  $\mu\text{g}/\text{ml}$  and 100  $\mu\text{g}/\text{ml}$ ) and DMA (25  $\mu\text{g}/\text{ml}$ ) (Table 1).  
18 The experimental design focused on the response of grain development to As(V). Therefore, we  
19 included 25  $\mu\text{g}/\text{ml}$  DMA treatment at 8 DPA as a qualitative comparison to the As(V) treatments. Our  
20 previous work showed that transport of organic As was so efficient that plants would not survive a  
21 parallel treatment schedule using DMA [26]. Treatment vials were foil-wrapped to limit evaporative  
22 loss and contamination, and the vials were attached using tape. Fresh treatment vials were applied  
23 every 24 h, with the tip of the leaf re-cut to ensure entry. Arsenic treatments were applied at varying  
24 stages of grain development, as described in Table 1, using the onset of anthesis as a starting point.

1 Grains exposed to 25 or 100 µg/ml As(V) were pulsed immediately after anthesis (0 DPA), or at 5, 10 and  
2 15 DPA, for a total of 5 days, and then harvested at maturity, 20 DPA. Grains exposed to DMA were  
3 pulsed at 8 DPA. Treatment vials were frozen on removal from the flag leaf, and three replicate panicles  
4 were used.

5

## 6 *2.2 ICP-MS Analysis*

7 Treated panicles were analyzed for total As, Rb and Sr via inductively coupled plasma mass  
8 spectroscopy (ICP-MS) using an Agilent 7500 instrument (Agilent Technologies, Santa Clara, CA, USA).  
9 Grains were selected from the top and middle regions of the panicle and husks were removed manually.  
10 Oven dried samples were subject to microwave-assisted digestion and analyzed for total As  
11 concentration via ICP-MS as described in Sun *et al.* [39]. Quality control procedures were as described in  
12 Sun *et al.* [39].

13

## 14 *2.3 Synchrotron X-ray Fluorescence Mapping*

15 One fresh grain from one replicate of each treatment was randomly selected for SXRF imaging. Fresh,  
16 leaf-fed As-treated grains were analyzed via SXRF mapping at Beamline X26A of the National  
17 Synchrotron Light Source (NSLS) of the Brookhaven National Laboratory (Upton, NY, USA). Details of  
18 Beamline configuration are as described in Carey *et al.* [26]. The storage ring at NSLS operated with low  
19 emittance lattice at 300 mA, 2.8 GeV. The X-ray microprobe at X26A used a focused, monochromatic  
20 beam with Kirkpatrick-Baez focusing optics capable of focusing the beam down to approximately 5–10  
21 µm. Photon flux at 18 keV is approximately  $1 \times 10^9$  photons/second. The experimental set up used a  
22 Canberra 9-element HPGe Array detector and two Radiant Vortex-EX Silicon Drift Diode detectors. The  
23 sample stage sat at 45° to the incident beam, allowing an optical microscope with a gigabit Ethernet  
24 CCD attachment to be mounted horizontally for viewing the sample at normal incidence. Analysis was



1 conducted at an incident energy of 12.5 keV to be above the As absorption edge. Elemental mapping of  
2 unsectioned rice grains included the bottom half of the grain from endosperm to embryo to maximize  
3 efficient use of synchrotron time, because distributions appear consistent throughout the endosperm  
4 for our elements of interest [40]. Scanning resolution was standardized for all grains at 12  $\mu\text{m}$  step size  
5 and a 0.25 s dwell time.

6 Element concentrations were calculated for the fluorescence maps as described in McNear *et al*[41]  
7 using measurements of the standard reference materials SRM1832/1833 (National Institute of  
8 Standards and Testing) thin-film standards, an assuming an object density of 1.2  $\text{g}/\text{cm}^3$ . SXRF  
9 microtomography was also conducted at NSLS beamline X26A on a grain exposed to 25  $\mu\text{g}/\text{ml}$  DMA as  
10 described in Kim *et al*. [42], using 12  $\mu\text{m}$  step size, 250 millisecond dwell times, an incident beam energy  
11 of 12.5 keV measuring  $7 \times 10 \mu\text{m}$  (horizontal  $\times$  vertical), following techniques described in Carey *et*  
12 *al*. [26].

13

#### 14 *2.4 Region of Interest Analysis and Descriptive Statistics*

15 We used the beamline-specific X26A\_plot general user interface data analysis software (controlled by  
16 the IDL programming language (Excelis Visual Information Solutions, Inc., Boulder, CO)) to trace the  
17 borders of the OVT, embryo and endosperm using elements that localized to these areas as markers.

18 We collected the descriptive statistical output for the regions of interest, specifically the total  
19 abundances of elements, expressed as normalized fluorescence counts.

20

### 21 **3.0 Results and Discussion**

#### 22 *3.1 ICP-MS Analysis*

1 Bulk concentrations of As in grains exposed via the cut flag leaf at varying time points prior to anthesis  
 2 are shown in Table 1. Data are shown for As concentrations in unexposed grains (negative control) and  
 3 following a 5-day exposure to DMA (positive control).

4  
 5 **Table 1.** Concentration of As, Rb (a phloem marker) and Sr (a xylem marker) in rice grains pulsed with  
 6 arsenate for 5 days during grain fill and harvested at maturity (20 DPA), compared to negative and  
 7 positive controls. Data are mean ( $\pm$  SD), where N=3. <sup>a</sup>N=2, <sup>b</sup>N=6

As Species	As (mg·L <sup>-1</sup> )	Timing of 5-day pulse (DPA)	$\mu\text{g}\cdot\text{g}^{-1}$		
			As	Rb	Sr
Negative <sup>b</sup>	0		0.3 (0.2)	2.8 (1.4)	0.7 (0.5)
Positive	25	8	47.2 (2.0)	9.42 (1.9)	1.27 (0.1)
As V	25	0 <sup>a</sup>	5.5 (2.4)	287.3 (142.6)	116.3 (15.8)
	25	5	4.0 (0.7)	76.5 (28.2)	34.1 (10.2)
	25	10	9.2 (5.0)	60.0 (24.1)	9.3 (4.4)
	25	15 <sup>a</sup>	8.2 (2.7)	20.5 (0.6)	1.4 (0.5)
As V	100	0	7.7 (1)	62.3 (8.0)	7.6 (2.7)
	100	5	4.7 (2.2)	13.2 (3.4)	1.6 (0.9)
	100	10	22.7 (0.5)	36.0 (6.8)	2.7 (1.0)
	100	15 <sup>a</sup>	17.0 (8.50)	20.2 (8.4)	1.0 (0.3)

8  
 9  
 10 A 5-day pulse of either 25 or 100  $\mu\text{g}/\text{ml}$  As(V) at 10 DPA resulted in higher grain arsenic concentrations.  
 11 Exposure at 15 DPA resulted in toxicity, which hindered grain development, resulting in lower mean  
 12 grain arsenic accumulation. As expected, exposure to 100  $\mu\text{g}/\text{ml}$  As(V) resulted in higher As  
 13 accumulation (Table 1, Figure 1). Inclusion of 1 mM Rb and Sr in the pulse solution acted as markers of  
 14 phloem and xylem transport (respectively), where greater grain concentrations indicate a lack of  
 15 competitive inhibition as a result of transport of other ions, and lower concentrations indicating

1 competitive inhibition. In As(V)-pulsed grains, both phloem and xylem transport declined with  
2 increasing As(V) concentrations as grain fill progressed, suggesting that As(V) transport into the grain  
3 involved both vascular systems. The magnitude of phloem transport was comparatively greater than  
4 xylem transport throughout, and xylem transport declined to a lower final level, suggesting that As(V)  
5 predominantly entered the grain via the phloem. Suppressed Rb grain concentrations in the DMA  
6 positive control confirmed earlier findings [27] that DMA uses phloem tissue to enter the grain.

7

8 Overall, we observed elevated phloem and xylem transport corresponding with lower As levels. These  
9 data suggest that As(V)-responsive transport is most efficient in the phloem during the later stages of  
10 grain development, but also uses the xylem in the early stages of grain fill. Toxicity of As(V) was an  
11 issue at these exposure concentrations, resulting in disruption of transport systems and As being  
12 detected in the embryo and other layers of the grain. The distribution of As and essential  
13 micronutrients in tissues of developing rice grains exposed to As(V) was influenced by As species,  
14 concentration and timing of exposure. The higher abundances of As detected in grain tissues exposed  
15 to 25 µg/ml DMA at 8 DPA compared to 100 µg/ml As(V) at 0 DPA agree with previous studies on the  
16 more efficient transport of DMA in the grain as compared to As(V)[26]. While much of the As(V)  
17 reaching higher grain tissues via root uptake is reduced to As(III)[43], studies have detected varying  
18 amounts of As(V) in the grain as a minor component ranging from 6.6% of total grain As[44] to almost  
19 18%[45]. The role of genetic variation in As uptake is well-established [[46-48]]. Trans-membrane  
20 transport and chemical species changes of As in plants occur at various points along the route from soil  
21 to grain, both of which are under genetic control. Because reduction of As(V) in the plant affects  
22 delivery of As(V) to the grain, and is itself a variable trait, this study intentionally controlled for As  
23 species delivery to the grain, to examine for As(V) and DMA responsive transporters during grain fill.

24

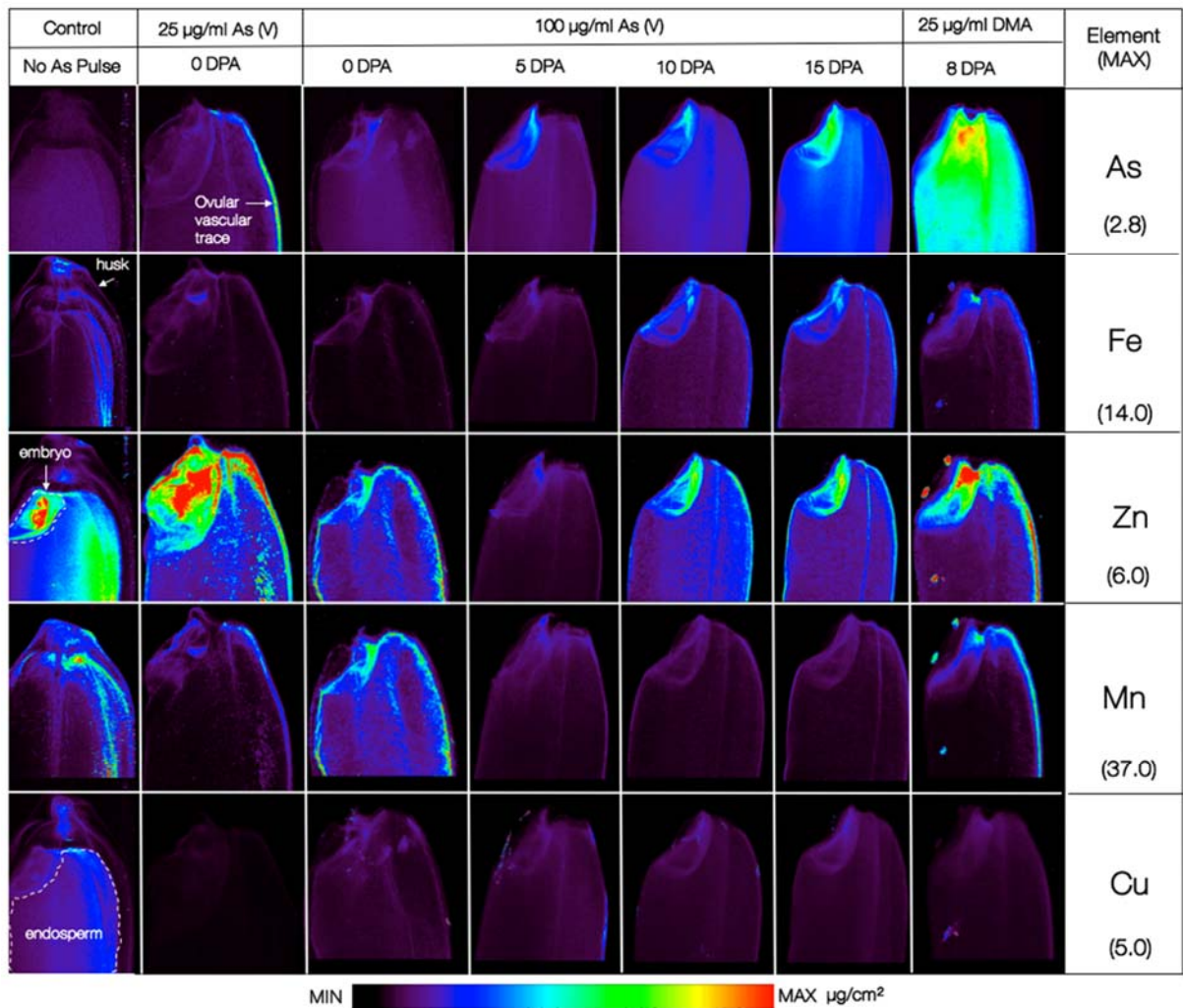
## 1 3.2 Elemental Distribution and Abundance

### 2 3.2.1 Elemental Maps

3 Synchrotron X-Ray Fluorescence elemental maps of As, Fe, Zn, Mn and Cu in grains exposed to As(V)  
4 together with positive and negative controls are shown in Figure 1. Elemental maps of low-atomic  
5 number elements phosphorus and sulphur were not collected from this dataset due to the use of a  
6 higher incident energy needed to reach the As K $\alpha$  fluorescence line. We did not include calcium or  
7 potassium in our analytes of interest because of their high abundance in the husk tissue through which  
8 our images were collected. We analyzed the 100  $\mu\text{g}/\text{ml}$  treatment only for the As(V) time course  
9 because this imparted higher concentrations and more pronounced differences in grain As levels, and  
10 because of the limitations on synchrotron beam time. For 2D imaging at 10  $\mu\text{m}$  spatial resolution, the  
11 bran layers (pericarp, aleurone and tegument) are not distinguishable. Further, distinguishing the bran  
12 layer from the endosperm is also not unequivocal in 2D mapping compared to tomography, therefore  
13 we use caution in interpreting two-dimensional map of unsectioned specimens.

14

15 **Figure 1.** Synchrotron X-ray fluorescence elemental maps of As, Fe, Zn, Mn and Cu in unpolished grains  
16 of *Oryza sativa* cv. *Italica Carolina* pulsed with As(V) or DMA for 5 days during grain fill. DPA: Days post  
17 anthesis. Data are expressed as  $\mu\text{g}/\text{cm}^2$ , using a unified scale for each element. Examples of anatomical  
18 features (husk, embryo, endosperm and ovular vascular trace) are indicated.



1  
2  
3 Elemental maps showed that grains exposed to 25  $\mu\text{g/ml}$  of As(V) at 0 DPA accumulated As primarily in  
4 the OVT, whereas those exposed to 100  $\mu\text{g/ml}$  of As(V) at the same growth stage did not. Bulk grain As  
5 concentrations doubled in 100  $\mu\text{g/ml}$  arsenate at 0 DPA compared to 25  $\mu\text{g/ml}$ . Elemental maps  
6 indicate that As is diffusely distributed throughout the grain.  
7 Phytotoxicity was a factor in As distribution at 100  $\mu\text{g/ml}$  As(V). Exposure to 100  $\mu\text{g/ml}$  As(V) at all  
8 tested timepoints resulted in As accumulation in the embryo, endosperm or bran layer, rather than  
9 solely the OVT. Exposure at later stages of grain development resulted in higher As accumulation  
10 (Figure 1). Arsenic distribution in the DMA positive control confirmed previous findings that as DMA, As

1 can access the embryo and endosperm or bran layer of the grain, and bypass the OVT, where As(III) is  
2 usually retained [26].

3 We infer that transporters for As(V) exist throughout the grain, and that their steady state levels change  
4 during development and their capacity for transport is modified by As toxicity. Higher abundances of  
5 elements in tissue layers may indicate inefficient transport (where elements are not being transported  
6 beyond that point). Lower concentrations may indicate efficient transport or sequestration in tissues  
7 closer to the source, such as the flag leaf. In rice grains, the vascular traces, particularly the OVT, move  
8 mineral nutrients from the flag leaf of the mother plant toward their respective end points in the  
9 embryo and bran layers. Results of grain exposure to 25 µg/ml As(V) at the beginning of grain  
10 development in comparison with 100 µg/ml As(V) later in development may indicate that accumulation  
11 of As(V) in rice grain hinges upon efficient transport by the OVT, which contained the highest As  
12 abundances following early treatment with 25 µg/ml As(V) treatment. This also suggests that transport  
13 beyond this point to the embryo and endosperm is inefficient. This agrees with previous studies that  
14 have focused on inorganic As(III) remobilization during grain fill[26]. When As(V) was introduced later,  
15 even at four times the concentration, accumulation in the OVT was not observed, embryo abundances  
16 were higher and endosperm As levels are approximately equal.

17 Grains pulsed with As(V) later in grain development had higher relative abundances of Fe in the embryo  
18 and lateral vascular trace than those exposed earlier in development, as well as control grains. Given  
19 the toxicity of this treatment, it is likely that variations in Fe stem from disruptions in nutrient transport.  
20 Zinc abundances were consistent between grains from different exposure scenarios, distributed  
21 primarily in the embryo and tissues of the OVT. Manganese (Mn) and Zn distribution and abundance  
22 were disrupted by As exposure; compared to with negative control grains. Grain exposed to 100 µg/ml  
23 of As(V) early in grain development contained comparable Mn levels to controls with indications of  
24 aleurone-wide distribution but grains exposed to 100 µg/ml As(V) later on in grain development showed

1 lower concentrations of Mn. Negative control grain indicated Mn in the aleurone and in the OVT,  
2 consistent with previous studies[26], whereas positive control DMA-exposed grains had strong Mn  
3 localization in the OVT, and comparatively less aleurone-associated Mn. This may indicate that  
4 preferential transport of DMA inhibits centripetal Mn transport out of the OVT.

5 The abundance of Cu was low and distribution was diffuse throughout the endosperm. Although grain-  
6 to-grain variation cannot be discounted, grains pulsed with 25 µg/ml As(V) at 0 DPA contained Cu that  
7 fell below SXRF detection limits.

8 These findings suggest that at sub-toxic concentrations, As(V) is transported via the OVT, in common  
9 with As(III). The higher concentration of As(V) used in the experiment is suspected to have  
10 overwhelmed the transporters in the OVT, disrupting normal membrane transport selectivity and  
11 allowing As to enter the endosperm and embryo.

12

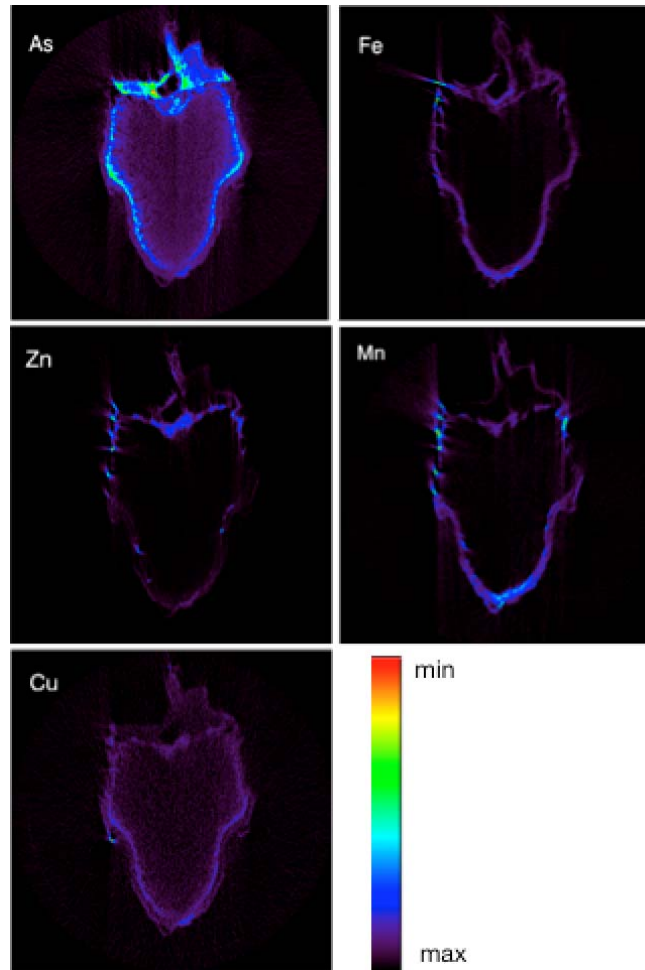
### 13 3.2.2 Tomography

14 SXRF microtomography of grains exposed to DMA are shown in Figure 2. Arsenic was distributed both  
15 in the bran layer and endosperm. The highest abundances of As were in the bran layer, and then  
16 progressively lower As abundances were observed with increasing distance from the bran layer,  
17 consistent with the centripetal movement of mineral nutrients from the bran layer into the  
18 endosperm[49]. Other elements (Fe, Zn, and Mn) were present only in the bran layer, and showed no  
19 indication of movement beyond this layer.

20

21 **Figure 2.** SXRF tomograms of rice grains exposed to 25 µg/ml of DMA at 8 DPA. Data are normalized  
22 fluorescence counts.

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11

### 3.2.3 User-defined Region-of-Interest Analysis

Total fluorescence counts of As, Fe, Zn, Mn and Cu in the embryo, endosperm and OVT are shown in Figures 3. Grains exposed to 25 µg/ml of As(V) at 0 DPA had the highest abundances of As in the OVT; approximately four times higher than treatment with 25 µg/ml DMA at 8 DPA. Exposing grains to 100 µg/ml As(V) later in grain development resulted in greater abundances of As in the OVT compared to earlier exposure, although levels did not reach those observed in exposure to 25 µg/ml of As(V). The OVT contained comparatively higher abundances of Fe, Mn and Zn and lower abundances of Cu. Treatment with As (V) during grain development reduced the abundance of Fe and Mn in the OVT, but did not appear to suppress Zn or Cu. Mean Zn abundances in the OVT were relatively consistent across



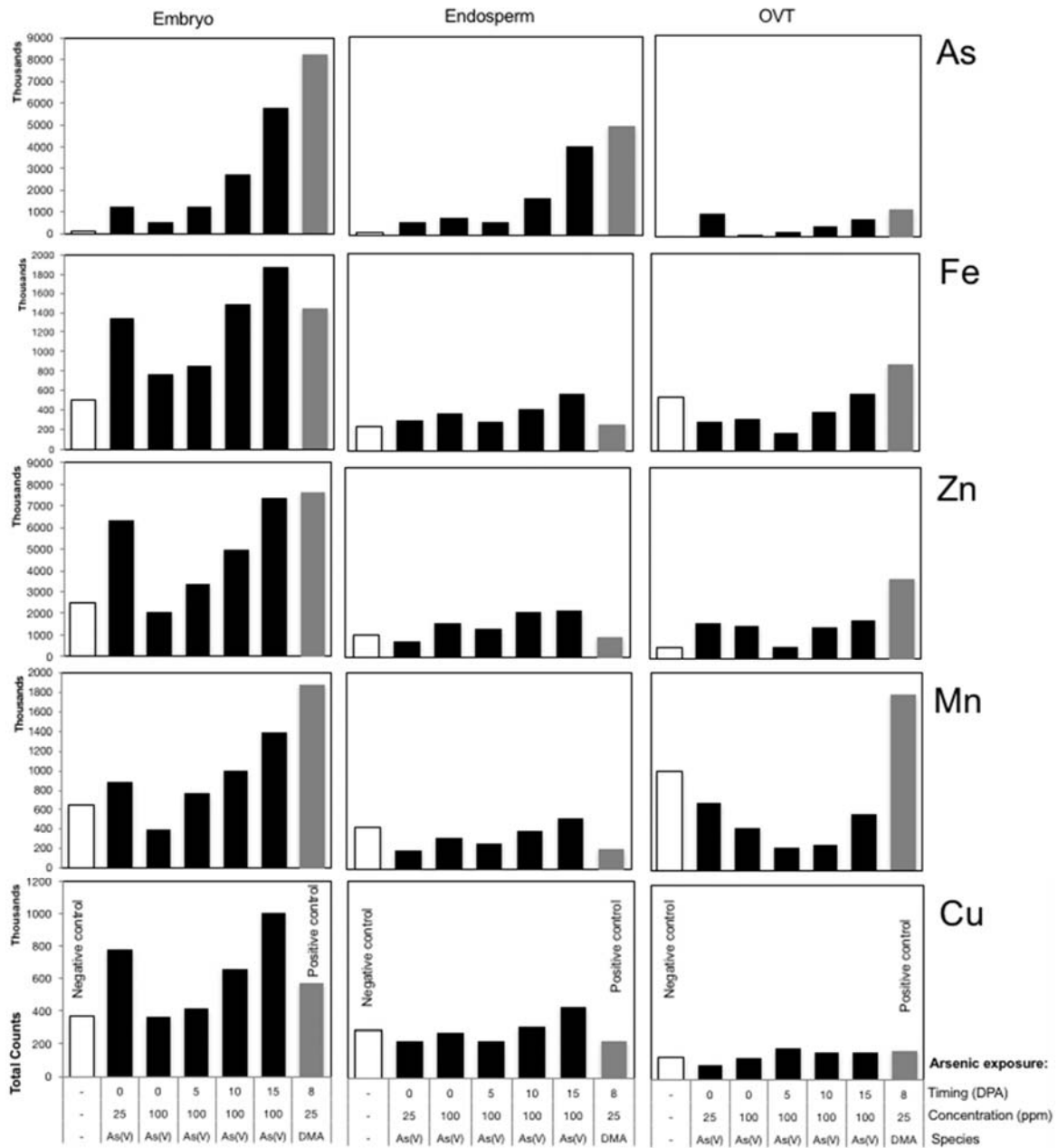
1 treatments, with the exception of early treatment with 25 µg/ml As(V), where OVT Zn abundances  
2 were higher.

3 For all elements including As, the highest total counts were observed in the embryo of the grain.  
4 Treatment with 100 µg/ml As(V) progressively later in development resulted in progressively greater As  
5 abundances in the embryo. This same trend of increasing abundances with later As treatment was  
6 observed for Fe, Zn Mn, and Cu. In each case, treatment with 25 µg/ml As(V) at 0 DPA results in an  
7 elevated abundance relative to treatment with 100 µg/ml, and the later the treatment, the higher the  
8 abundance. These observations are likely due to increasing toxicity in response to the higher  
9 concentration of As(V). Exposure to the lower As concentration earlier in grain fill caused less damage  
10 to translocation system function between leaf and grain, seen in elevated concentrations of metals.  
11 Xylem transport declines as grain fill progresses (Table 1), but phloem transport continues to convey  
12 elements to the grain, where As toxicity affects grain development, and transport systems that convey  
13 essential nutrients to the outer layer of the endosperm are disrupted. Following DMA treatment,  
14 abundances of Fe, but not Zn and Mn were lower.

15 Arsenic was detected in the endosperm as a result of treatment both with As(V) and DMA (Figure 3),  
16 but total counts were lower in the endosperm than in the embryo. In 2D maps, elemental accumulation  
17 in the bran layers cannot be distinguished beyond doubt from the inner endosperm. However,  
18 abundances confirm the trend observed in other tissue areas: treatment with 25 µg/ml As(V) early in  
19 grain fill results in higher As abundances; treatment with 100 µg/ml As(V) progressively later in grain fill  
20 result in progressively higher abundances, and that exposure to 25 µg/ml DMA results in higher  
21 abundances than As(V). Abundances of Fe, Mn and Zn in the starchy endosperm are typically low, and  
22 any of the elements detected are likely to arise from the peripheral bran layers. We observed a positive  
23 trend with Fe (mean abundances increased with progressively later treatment with 100 µg/ml As(V) and  
24 with 25 µg/ml DMA), but observed a negative trend with Zn, Mn, Cu and Ni.

1

2 **Figure 3.** Total fluorescence counts in user-defined region of interest analysis of As, Fe, Zn, Mn, and Cu  
 3 in the embryo, endosperm and OVT of rice grains exposed to As(V) or DMA



4

5 **4.0 Conclusions**

1 In this study, we used presence of an element in a specific grain tissue layer as evidence for metal  
2 transport. Expression of metal transporter genes and XRF-based metal localization in rice seeds has  
3 also been used to identify putative transporters involved in metal movement during initial phases of  
4 germination [50]. Our findings are that As(V) transport into the endosperm is less efficient than DMA,  
5 but efficiency increases during grain fill at the expense of Fe and Mn transport. Zheng *et al.* [51] also  
6 found similar temporal variation in As species in the various tissues of rice grain, with increasing  
7 inorganic As species as development progressed.

8

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18 M.C. carried out the rice growth and exposure experiments and A.A.M. is the Principal Investigator of  
19 the study.

20

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22

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