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RETROSPECTIVE STUDY OF METHYLMERCURY AND OTHER METAL(LOID)S IN MADAGASCAR UNPOLISHED RICE (*Oryza sativa* L.)

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Abstract

The rice ingestion rate in Madagascar is among the highest globally; however studies concerning metal(loid) concentrations in Madagascar rice are lacking. For Madagascar unpolished rice (n=51 landraces), levels of toxic elements (e.g., total mercury, methylmercury, arsenic and cadmium) as well as essential micronutrients (e.g., zinc and selenium) were uniformly low, indicating potentially both positive and negative health effects. Aside from manganese (Wilcoxon rank sum, $p < 0.01$), no significant differences in concentrations for all trace elements were observed between rice with red bran (n=20) and brown bran (n=31) (Wilcoxon rank sum, $p = 0.06–0.91$). Compared to all elements in rice, rubidium (i.e., tracer for phloem transport) was most positively correlated with methylmercury (Pearson's $r = 0.33$, $p < 0.05$) and total mercury ($r = 0.44$, $p < 0.05$), while strontium (i.e., tracer for xylem transport) was least correlated with total mercury and methylmercury ($r < 0.01$ for both), suggesting inorganic mercury and methylmercury were possibly more mobile in phloem compared to xylem.

Keywords

rice ingestion; methylmercury; metal(loid)s; red bran; phloem/xylem transport

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INTRODUCTION

Asian rice (*Oryza sativa* L.) is a staple food in Madagascar, comprising more than 50% of the daily caloric intake (FAO, 2014). Globally, the average rice consumption rate ranks in the 95th percentile (289 g/day/capita), which is exceeded by 9 countries, including Bangladesh, Lao, Cambodia, Vietnam, Myanmar, Thailand, Indonesia, the Philippines, and Guinea (range: 290–475 g/day/capita, from FAO, 2014). In rural areas of Madagascar, average rice ingestion may be higher, reaching 380 g/day/capita (Alain Ramanantsoanirina, FOFIFA, personal communication). Despite one of the highest rice ingestion rates, studies reporting metal(loid) concentrations in rice grain are lacking, which are necessary to better understand potential deficiencies in the diet and exposure to toxic elements. Additionally, Madagascar is a biodiversity hotspot for rice (Mather et al., 2010; Radaneilina et al., 2013); therefore a survey of metal(loid)s among multiple rice varieties is informative to rice breeders seeking sources of genetic variability for hybrid rice cultivars.

The primary focus of this study is to survey total mercury (THg) and methylmercury (MeHg) concentrations in rice cultivated in Madagascar. Fish consumption is considered the primary human exposure pathway for MeHg, a potent neurotoxin (Clarkson and Magos, 2006); however, MeHg exposure also occurs through rice ingestion (e.g., Feng et al., 2008; Rothenberg et al., 2011a, 2011b, 2013, 2014; Windham-Myers et al., 2014a, 2014b, 2014c). Worldwide approximately 90% of rice is cultivated under standing water (Kirk, 2004), creating anoxic conditions that favor microbial conversion of less toxic inorganic Hg(II) to more toxic MeHg (Rothenberg and Feng, 2012; Windham-Myers et al., 2014b), which is subsequently bioaccumulated in rice grain (Rothenberg et al., 2011a, 2014; Zhang et al., 2010). Despite the importance of rice as a staple food for half the global population (Khush, 2005), there are few studies addressing MeHg exposure through rice ingestion outside Asia (Rothenberg et al., 2014).

Madagascar is a signatory to the Minamata Convention on Mercury (UNEP, 2014), which requires development of a baseline Hg inventory by the Madagascar National Focal Point of Mercury Programme. To meet this obligation, Hg releases to the air, water, and land in Madagascar were estimated using the United Nations Environment Programme Hg Toolkit (Randrianomenjanahary, undated; UNEP, 2011). Madagascar imports many Hg-containing products, including dental amalgam (2368 tons/year), soaps with Hg (8288 tons/year), paint (4291 tons/year), and Hg-containing batteries (22.3 tons/year), and highest environmental Hg releases were related to their disposal or incineration (Randrianomenjanahary, undated; UNEP, 2011). Unlike neighboring South Africa, which depends on coal-fired power plants for energy (e.g., Dabrowski et al., 2008), 95% of Madagascar households rely on wood, charcoal or coal for domestic energy due to the lack of electricity or high costs associated with electricity (UNEP, 2011). Biomass burning competes with coal-fired power plants as one of the most important sources of Hg atmospheric emissions (Streets et al., 2009); biomass burning is also more difficult to regulate than industrial power plants. Like other sub-Saharan African nations (e.g., van Straaten, 2000), artisanal and small-scale gold mining is practiced in Madagascar (UNEP, 2011); however, Hg is banned from this process (Randrianomenjanahary, undated). Additionally, mining of sapphire and rubies replaced

gold mining as a more lucrative source of income (Duffy, 2007). Other potential sources of Hg pollution include production of cement (8000–40,000 tons/year) and lime (2200 tons/year) (Randrianomenjanahary, undated). Calculations from the Hg Toolkit predict a maximum of 98.5 tons Hg are released annually to the environment in Madagascar (UNEP, 2011). When estimated Hg emissions are normalized by population, results suggest a moderate-to-high per capita Hg footprint in Madagascar compared to other nations, where Hg environmental emissions are known (see Table 1). However this table does not weight the magnitude of health impairments associated with each Hg pollution source.

In addition to quantifying THg and MeHg, concentrations of arsenic (As), manganese (Mn), copper (Cu), zinc (Zn), cadmium (Cd), rubidium (Rb) and strontium (Sr) were also analyzed as part of the rice ionome, or collection of mineral nutrients (Salt et al., 2008). Metal(loid)s are often studied in isolation; however, chemical similarities, competition for ligands, and plant-soil interactions may alter rice grain content (e.g., Impa and Johnson-Beebout, 2012; Li et al., 2012; Williams et al., 2009a; Zhang et al., 2012). In addition, some trace elements are nutritious (e.g., Zn and Se) while others are toxic (e.g., As and Cd), making it difficult to assess the nutritional quality of rice grain without considering both.

Madagascar rice samples (n=51) were obtained from the International Rice Genebank (located at the International Rice Research Institute (IRRI) in Los Baños, Philippines), which included rice samples with both red bran and brown bran. This is a retrospective study of archived rice germplasm, and other matrices (e.g., paddy soil or irrigation water) were not available. Other researchers reported a strong positive correlation between rice THg and MeHg concentrations for rice cultivated in China, California, USA and Cambodia (Rothenberg et al., 2014), while an inverse correlation was observed between rice Hg species and Se (Zhang et al., 2012). We hypothesize interactions will be similar among these Madagascar rice varieties. Differences in bran color may signify a source of genetic variability, contributing to the accumulation and assimilation of metal(loid)s (Norton et al., 2009). We hypothesize concentrations of some elements will differ in red rice compared to brown rice. This is the first study to the best of our knowledge to document concentrations of metal(loid)s in rice from this African island nation.

MATERIALS AND METHODS

International Rice Genebank

IRRI houses the International Rice Genebank, which holds in trust the world's most comprehensive collection of rice genetic resources, including more than 117,000 germplasm samples (i.e., accessions) from 124 countries collected since 1962 (IRRI, 2014). The Base Collection is comprised of rice germplasm cultivated in the country of origin, which is stored frozen (−18°C to −20°C), and not typically available for researchers due to limited supplies. An exception was made for these Madagascar rice samples because there was sufficient quantity in the Base Collection.

Fumigation test

The Seed Health Unit at IRRI uses the fumigant, Phostoxin™, i.e., aluminum phosphide, for all samples shipped to the U.S. The effect of fumigation on concentrations of all trace elements was tested using two Philippine rice varieties cultivated in five paddies at IRRI. Philippine rice varieties were used because there were insufficient quantities of rice samples from Madagascar; however, the same fumigation methods were employed and any residue remaining was the same, regardless of the rice variety. Half of each sample was fumigated with Phostoxin™, then all samples were dehulled under pressure between two rollers (Huller, JLGJ4.5, China), and unpolished rice samples were ground to a powder using a stainless steel Wiley mill, which was cleaned with ethanol between samples to prevent carryover. MeHg concentrations were measured on-site at IRRI, and other trace elements were quantified at the University of South Carolina (see methods below). Fumigation residues did not affect trace element concentrations (n=10, including 5 fumigated and 5 non-fumigated; two-sided paired t-test, THg p=0.88; MeHg p=0.22; As p=0.64; Cd p=0.29; Cu p=0.20; Mn p=0.23; Rb p=0.41; Se p=0.35; Sr p=0.81, and Zn p=0.34). This comparison provided more confidence in our analyses of IRRI rice samples from Madagascar, which were fumigated with Phostoxin™ prior to export to the U.S.

Madagascar rice samples

Data for Madagascar rice samples discussed below were made available by IRRI (GL Capilit, IRRI, personal communication) and FOFIFA (A. Ramanantsoanirina, FOFIFA, personal communication). Rice samples were cultivated under lowland, irrigated conditions in Madagascar between July and November/December 2008, averaging 143 days between sowing and harvest (range: 137–154 days). Precise locations (including latitude and longitude) were known for only 15 of 51 varieties (Figure 1), and locations without coordinates were known for 2 additional varieties. The 17 locations included 3 districts (Faratsiho n=2 varieties, Betafo n=9 varieties, and Antsirabe II n=6 varieties), which were further subdivided into 11 communes. Germplasm was deposited to the International Rice Genebank at IRRI in 2009 and maintained in cold storage (range: –18°C to –20°C) as part of the Base Collection. All rice samples were landraces (i.e., traditional cultivars) (IRGCIS, 2014). Although the exact number of rice plants was unknown, the amount of rice germplasm for each variety sent to IRRI averaged 299 g (range: 280–311 g), indicating rice was harvested from a field and not 1–2 rice plants.

In 2011, Madagascar rice germplasm (up to 10 g each variety) was obtained from the International Rice Genebank, including 20 varieties with red bran and 31 with brown bran (see Table S1 for variety names, accession numbers, and bran color). Rice samples were dehulled and ground to a powder as described above, except a stainless steel coffee grinder was used to grind samples in place of the Wiley mill. After rice germplasm was dehulled, there was insufficient rice grain to polish the rice samples. Metal(loid) concentrations in unpolished rice were determined at the University of South Carolina in the Department of Environmental Health Sciences (Rothenberg Mercury Lab) and the Center for Elemental Mass Spectrometry in the Department of Earth and Ocean Sciences (Bizimis Lab).

Determination of Hg species

For Hg analyses, glassware and plasticware were rigorously acid-washed in 1.2 N hydrochloric acid (HCl) and triple-rinsed with double-distilled (DDI) H₂O, air-dried, then stored double-bagged. When the blank for borosilicate glass vials was high (once out of 7 times), a second acid-washing step was added to our washing procedures (0.5% 0.2 N bromine monochloride) (Hammerschmidt et al., 2011), which reduced the blank to background levels.

THg concentrations were quantified according to EPA Method 1631 (USEPA, 2002) as follows: approximately 0.5 g of ground rice were weighed into 50 mL borosilicate glass test tubes with Teflon-lined lids (Corning No. 9826-25), 10 mL of freshly-made 8:2 nitric acid to sulfuric acid were added, and samples were gently refluxed for three hours in a water bath (75°C). Then DDI H₂O was added to 50 mL, and samples were oxidized 12–24 hours with 1% 0.2 N bromine monochloride. THg concentrations were analyzed following reduction of inorganic Hg(II) to Hg(0) using hydroxylamine hydrochloride and tin chloride, purge and trap of Hg(0) onto gold sand traps (part # 03020, Brooks Rand), and detection by cold vapor atomic fluorescence spectrometry (CVAFS) (Brooks Rand Model III, Seattle, WA, USA) (USEPA, 2002).

MeHg concentrations were determined following solvent extraction (rather than distillation) (Liang et al., 1996), which prevented artifact formation of MeHg during sample extraction (see Bloom et al., 1997). Approximately 0.5 g of ground rice was weighed into 50 mL polypropylene tubes, and 2 mL of 25% (w/v) potassium-hydroxide (KOH)-methanol were added and heated for three hours (75 °C). Then 6 mL of dichloromethane (DCM) were added, followed by addition 1.5 mL of concentrated HCl, then samples were shaken for 1 hr, centrifuged (4000 RPM = 3220 × g, 30 minutes), and phases separated (Whatman 1-PS). Then 30–35 mL DDI H₂O were added, and samples were heated in a water bath (60 °C) for 1.5 hr to volatilize DCM. After heating, DDI H₂O was added so the final volume was 40 mL. MeHg concentrations were quantified following EPA Method 1630 (USEPA, 2001a), including ethylation with sodium tetraethylborate, purge and trap onto Tenex traps (part #06020, Brooks Rand), and separation of Hg species using gas chromatography and detection by CVAFS (Brooks Rand, Model III, Seattle, WA, USA).

Determination of other metal(loid)s

Concentrations of other trace elements (As, Cd, Cu, Mn, Rb, Se, Sr, and Zn) were analyzed following EPA Method 3050b (USEPA, 1996), as follows: ~0.5 g rice sample were added to a 70 mL vial, 5 mL nitric acid were added, and samples were gently refluxed in a water bath for one hour (75°C). One ml of hydrogen peroxide was added and heated for an additional hour, and DDI H₂O was added so the final volume was 60 mL, then trace elements determined by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Element 2) (Molkalski et al., 2013, Das et al., 2013). Care was taken to determine each detected isotope at the appropriate resolution mode thereby fully resolving isobaric interferences from polyatomic species, for example, using high resolution mode (DM/M=10,000) for As⁷⁵ (interference from Ar⁴⁰Cl³⁵) and Se⁷⁷ (interference from Ar⁴⁰Cl³⁷).

Quality Assurance/ Quality Control (QA/QC)

Measures for QA/QC included analysis of replicates, spike recoveries, multiple standard reference materials, and daily calibration curves for THg and MeHg quantification (r -squared > 0.99) (Table 2). There was no certified reference material specifically for rice MeHg concentrations; instead, matrix spikes were added to samples before extraction started, and certified reference materials were quantified at the same time. The limits of detection (LOD) for THg and MeHg were based on 1) the region of the calibration curve where there was a significant change in sensitivity (i.e., 10 pg for THg and 1 pg for MeHg) and 2) the average mass of rice sample digested (~0.5 g), establishing the LOD as 0.02 ng/g and 0.002 ng/g for THg and MeHg, respectively. For other elements measured by ICP-MS, detection levels were based on 3 times the standard deviation of procedural blanks and divided by the average mass of rice sample (Table 2). For Cd, half the detection level was imputed for those samples with concentrations below the LOD ($n=13$).

Statistics

Fumigated and non-fumigated rice samples were compared using Student's t-test for paired observations. For Madagascar rice, both Pearson's correlation and Spearman's nonparametric correlation were reported. For Pearson's correlation, a $\log_{10}$ -transformation was applied when the distribution was right-skewed (mean >> median). Differences between rice with red bran and brown bran were compared using Wilcoxon rank-sum test. When appropriate, an alpha level of 0.05 was chosen as a guide for significance. Stata 9.2 (College Station, Texas) and the R-platform were used for all statistics.

RESULTS AND DISCUSSION

Hg species

Despite a record of moderate-to-high Hg contamination in Madagascar through waste disposal and solid-waste incineration (Randrianomenjanahary, undated; UNEP, 2011) (Table 1), THg and MeHg concentrations found in rice in these Madagascar sites represented the lowest concentrations reported to date (Table 3, Figure 2; for global data, see Rothenberg et al., 2014). Compared to the global average for non-polluted sites, concentrations of THg and MeHg in Madagascar rice averaged 7.5 and 21 times lower, respectively. Compared to the global average for polluted sites, concentrations of THg and MeHg in Madagascar rice were 59 and 139 times lower, respectively. Average rice THg levels in Madagascar were also 24 times lower compared to rice cultivated near the Rwamagasa artisanal gold mining area in Tanzania (average: 26 ng/g, from Taylor et al., 2005), the only other site in Africa (to the best of our knowledge) where rice THg concentrations were measured. Percent MeHg (of THg) for Madagascar rice averaged 3 times lower compared to other non-polluted sites, and 2.5 times lower compared to other polluted sites.

Lower concentrations of THg and MeHg and a lower proportion of MeHg (of THg) in Madagascar rice suggested several possible explanations. Paddy soil levels were possibly Hg- depleted, or bioavailability of Hg species was reduced (Marvin-DiPasquale et al., 2014; Rothenberg et al., 2012; Windham-Myers et al., 2014a, 2014b, 2014c). A lower proportion

of MeHg (of THg) in Madagascar rice suggested decreased Hg methylation rates in Madagascar paddy soil, which possibly reflected differences in soil organic carbon, sulfate or iron concentrations, microbial anaerobic activity, exudates from rice roots including oxygen and carbon, density of rice plants, or soil pH (Marvin-DiPasquale et al., 2014; Rothenberg and Feng, 2012; Rothenberg et al., 2014; Windham-Myers et al., 2014a, 2014b, 2014c). Lower accumulation of MeHg from paddy soil may also be due to differences in these rice varieties; genetic variability was previously reported for THg and/or MeHg (Rothenberg et al., 2012; Zhu et al., 2008).

Using average values for the Madagascar rice ingestion rate (289 g rice/day; from FAO, 2014) and body weight (bw) for an African adult (50 kg, from Taylor et al., 2005), the estimated daily MeHg exposure through rice ingestion averaged 0.00069 µg/kg bw (range: 0.000087-0.0064 µg/kg bw), which was 145 times lower than the most conservative international reference dose for MeHg (EPA: 0.1 µg/kg bw; from USEPA, 2001b). However, reference doses for MeHg based on fish consumption may not be sufficiently protective for populations exposed to MeHg through rice ingestion because fish tissue contains beneficial micronutrients (e.g., omega-3 fatty acids), which help modify the adverse impacts due to MeHg exposure (see Rothenberg et al., 2014).

Other metal(loid)s

Nutritional guidelines were compared for concentrations of other metal(loid)s in unpolished Madagascar rice (Zn, Se, As, Cd, Cu, and Mn) (Table 3, Figure 2). Polishing reduces concentrations of some elements (e.g., As, THg, Mn, and Zn) but not others (e.g., Cu, MeHg, and Se) (Lombi et al., 2009; Rothenberg et al., 2011a; Villareal et al., 1991). Thus, exposure to toxic elements (e.g., As) was likely lower than estimated, while exposure to essential micronutrients (e.g., Zn) was likely overestimated. Guidelines for each element were derived as follows.

Zn is an essential micronutrient for all living organisms, and acts as a co-factor for the activity of more than 200 enzymes (Impa and Johnson-Beebout, 2012, and references therein). HarvestPlus (2012) developed breeding targets for polished rice for non-pregnant adult women, assuming 400 g rice ingested daily (breeding target: 24 µg Zn/g). However, the rice ingestion rate in Madagascar is lower (289 g/day, from FAO, 2014); therefore the breeding target needed is higher. Using the same assumptions from HarvestPlus (2012) (e.g., Zn loss during cooking and milling), the breeding target for Madagascar rice is 31 µg Zn/g. Only 22% of these rice varieties exceeded this value. Polishing reduces Zn levels (Villareal et al., 1991); therefore the percentage of rice varieties with sufficient Zn concentrations is likely lower, affirming Zn deficiency for a majority of communities in Madagascar ingesting these rice varieties.

Se is an essential micronutrient and plays a fundamental role in antioxidant and thyroid hormone production, but Se is toxic at high levels (> 200 µg/day) (Williams et al., 2009b, and references therein). For these Madagascar rice varieties, the daily ingestion rate for Se (based on 289 g rice/day, FAO, 2014) ranged from 4.3–23 µg Se, which was far below the minimum daily requirement (55 µg/day, Williams et al., 2009b). Additionally, the average Se concentration for Madagascar rice was 2.4 times lower than the global average (95 ng/g;

from Williams et al., 2009b). Using the minimum daily requirement (55 µg/day) and the rice ingestion rate in Madagascar (289 g rice/day, FAO, 2014), the estimated target level for Se concentrations in Madagascar rice is 190 ng/g, which is 4.9 times higher than the observed average Se concentration.

Inorganic As is a class 1, nonthreshold carcinogen, and rice ingestion is an important exposure pathway (Williams et al., 2005). Assuming inorganic As comprised 80% of total As (Williams et al., 2005), the daily As exposure ranged from 0.34–1.3 µg inorganic As/kg/day, which was below the maximum tolerable daily intake (2.1 µg/kg/day; from WHO, 1993, 2003). However, in 2011 the WHO concluded this 2.1 µg/kg/day level for inorganic As was no longer health protective (WHO, 2011). Although the highest As concentration in Madagascar rice (0.28 µg/g) was comparable to low As concentrations for rice cultivated in Bangladesh (Norton et al., 2009), it's uncertain whether As intake levels were safe.

Chronic exposure to elevated Cd levels is associated with renal dysfunction (Li et al., 2012, and references therein). Rice Cd concentrations ranged from <LOD–6.1 ng/g (Table 3), and were up to 680 times lower than the maximum contaminant level for rice Cd concentrations (400 ng/g, from FAO/WHO, 2006), and up to 340 times lower than the more conservative maximum contaminant level for Chinese rice (200 ng/g, from Li et al, 2012). Therefore, Cd exposure is not likely a concern for communities ingesting these Madagascar rice varieties.

Both Cu and Mn are essential micronutrients; the tolerable daily upper intake level is defined as the highest average intake that will not pose a risk of adverse health effects to anyone in the population (Allen et al., 2006). For Cu and Mn, the upper intake levels for adults (19–70 years) are 10 and 11 mg, respectively (Allen et al., 2006). Using the average daily Madagascar rice ingestion rate (289 g/day, FAO, 2014), the estimated Cu and Mn concentrations in Madagascar rice needed to reach the upper intake levels are 20 µg/g and 38 µg/g, respectively. All values for both Cu and Mn were below the upper intake level; concentrations in polished rice would likely be even lower for Mn but not Cu (Villareal et al., 1991).

To summarize, combined with the previous section for Hg species, concentrations of toxic elements were uniformly low in Madagascar rice (THg, MeHg, As, and Cd), while essential nutrients were also low (Zn and Se), indicating potentially positive and negative health effects for communities ingesting these rice varieties. All results were for brown rice; therefore the risk of deficiency for some essential micronutrients (Mn and Zn) was actually greater, while estimated exposure to toxic elements was similar or lower than estimated (THg, MeHg, As, and Cd).

Correlation of Hg species with other trace elements

Table 4 includes Pearson's and Spearman's correlation matrices for all trace elements in Madagascar rice. Using Pearson's correlation, Rb was most highly correlated with MeHg and THg ($p < 0.05$, when all variables were $\log_{10}$ -transformed), while Sr was least correlated with THg and MeHg ($r < 0.01$ for both, when THg and MeHg were $\log_{10}$ -transformed). Using Spearman's correlation, results for Sr were the same (i.e., Sr was least correlated with MeHg

and THg), while Rb was most highly correlated with THg, Se and Mn ($p < 0.05$), and less so with MeHg.

During long-distance transport in plants, solutes are transferred between xylem and phloem depending on nutritional requirements; the stems or the leaves are the main sites for phloem loading (Marschner, 1995). Sr and Rb are tracers for xylem (apoplast) and phloem (symplast) transport, respectively (Carey et al., 2010, 2011, 2012; Kuppelweiser and Feller, 1991; Martin, 1982). Stem-girdling is used to study the transport of nutrients in phloem and xylem, which is applied shortly after anthesis and involves heat to plug the sieve tubes in phloem prior to excision (Kuppelweiser and Feller, 1991). This method limits transport in phloem without damaging xylem transport, which was verified using Rb and Sr as markers of transport for phloem and xylem, respectively (Carey et al., 2010, 2011, 2012; Kuppelweiser and Feller, 1991). Stem-girdling using Rb and Sr tracers was applied to determine As and Se transport in rice plants; results indicated organo-As and organo-Se species were more efficiently transported in phloem from the panicle leaf to the rice grain compared to inorganic As and Se species (Carey et al., 2010, 2011, 2012).

Higher correlation between concentrations of Hg species and Rb, and no correlation with Sr, may reflect greater mobility of inorganic Hg(II) and possibly MeHg in phloem compared to xylem. Phloem is more enriched in sulfur compounds, both reduced (e.g., cysteine and glutathione) and oxidized (e.g., sulfate) (Kuzuhara et al., 2000; Marschner, 1995), while xylem is more enriched in iron, Zn and Cu (Marschner, 1995). Inorganic Hg(II) and MeHg bind strongly to thiols, including cysteine (CYS^{2-}) (HgCYS pKa: 15.28; MeHgHCYS pKa: 26.07) and glutathione (GS^{3-}) ($\text{HgH}_3(\text{GS})_2^-$ pKa: 57.92; MeHgHGS $^-$ pKa: 26.35) (Zhang et al., 2004); therefore, higher mobility of Hg species in phloem may reflect stronger binding to sulfur species.

An inverse relationship between Hg species and Se was previously observed in rice grain (e.g., Zhang et al., 2012) as well as fish tissue (Peterson et al., 2009), while the trend was inverse but weak for this data set ($p > 0.05$, Table 4). In rice grain, an inverse relationship between Hg species and Se may reflect competition for thiols in phloem. This hypothesis should be further investigated to better understand transport barriers for Hg species within rice plants.

Comparison of rice with red bran versus brown bran

All 51 rice varieties from the Madagascar Rice Research Institute were landraces, including 20 with red bran and 31 with brown bran. Significantly higher rice grain concentrations of Mn were observed for rice with brown bran compared to red bran (i.e., on average 1.5 times higher) (Wilcoxon rank-sum test, $p < 0.01$) and borderline significant for Cd (on average 1.4 times higher) (Wilcoxon rank-sum test, $p = 0.059$), while no differences were observed for all other trace elements, including THg, MeHg and percent MeHg (of THg) (Wilcoxon rank-sum test, $p = 0.20\text{--}0.91$) (Figure 3).

Pigmented rice, including rice with red bran, is associated with increased health benefits (e.g., higher antioxidants) compared to rice with brown bran (Deng et al., 2013; Gunaratne et al., 2013). Conversely, other studies reported greater concentrations of toxic elements in

pigmented rice. For example, As concentrations were higher in unpolished and polished red-bran landraces (n=21) compared to landraces with brown bran (n=12), both cultivated in two field sites in Bangladesh (Norton et al., 2009). Red rice purchased in India contributed the highest amount toward the maximum tolerable daily intake for As (18%) compared to white and brown basmati rice (7–14%) (Williams et al., 2005). Li et al. (2013) observed significantly higher rice MeHg concentrations for rice with pigmented bran (n=4 cultivars), compared to *indica* and *japonica* varieties with brown bran (n=22 cultivars) for rice cultivated in Wanshan, Guizhou province, China, and (non-significant) higher MeHg concentrations at two other Chinese sites. The authors concluded pigmented rice should not be cultivated in fields where soil THg levels were elevated (Li et al., 2013).

Results for Madagascar red rice differed from these studies, with no significant differences for most trace elements, which may reflect differences in rice varieties cultivated in Madagascar. Results suggest the benefits (or risks) of ingesting pigmented rice depends on site-specific environmental factors or rice varieties.

CONCLUSIONS

To the best of our knowledge, this is the first study documenting concentrations of metal(loid)s in rice cultivated in Madagascar sites. Madagascar rice varieties (n=51) had low concentrations of toxic elements (e.g., MeHg, As, and Cd); however, essential micronutrients (e.g., Zn and Se) were also low, indicating both positive and negative human health effects for communities ingesting these rice varieties. Rice with red bran and brown bran differed from other reports in that there were no significant differences in concentrations of all elements (aside from Mn). Uniformly low levels for all rice varieties suggested the geology of these sites differed from other global rice growing regions (i.e., less contaminated), which resulted in lower concentrations of metal(loid)s in paddy soil, or their uptake from paddy soil was limited due to soil characteristics (e.g., organic content). Alternatively, all 51 Madagascar rice varieties were low-accumulators of nutrients from paddy soil. Soil measurements in Madagascar paddy soil (e.g., organic content, pH, sulfate, and iron) are needed to understand why levels of all elements were low, and whether it is possible to enhance uptake of nutrients needed for healthy development (e.g., Zn, Harvest Plus, 2014). Differences between Madagascar and other more contaminated rice-growing regions underscore the importance of characterizing elements in rice from areas where the rice ingestion rate is high, but little research exists.

It is currently unknown how Hg species are transported in rice plants. Hg species, especially inorganic Hg(II), were possibly more mobile in phloem compared to xylem, which was determined by correlation with corresponding tracers, including Rb and Sr (Table 4). Further understanding of transport in xylem and phloem will help explain controls on grain filling, and whether rice grain inorganic Hg and MeHg originate from paddy soil or re-mobilized from leaves during senescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Metal(loid)s were low in 51 rice varieties cultivated throughout Madagascar
- THg and MeHg were 7.5 and 21 times lower than global avg for background sites
- MeHg was 12% of THg, i.e., 3 times lower than global avg for background sites
- Hg species were possibly more mobile in phloem, not xylem
- Except for Mn, there were no differences between rice with red or brown bran

Figure 1.
Location of Madagascar and rice cultivations sites for 15 (of 51) rice varieties.

Figure 2. Boxplots for a) toxic elements [total mercury (THg), methylmercury (MeHg), arsenic (As) and cadmium (Cd)], and b) beneficial elements [zinc (Zn), selenium (Se), copper (Cu) and manganese (Mn)].

Figure 3.

Boxplots comparing Madagascar rice with brown bran (n=31) or red bran (n=20), including a) total mercury (THg), b) methylmercury (MeHg), c) %MeHg (of THg), d) manganese (Mn) and e) cadmium (Cd). Aside from Mn, no statistical differences were observed for all elements (Wilcoxon rank-sum test, $p>0.05$) (for figures not shown, $p=0.26-0.91$).

Table 1

Mercury (Hg) releases to air, water and land for Madagascar and other nations.

	Hg releases (tons/year) ^a	Population ^b	Hg emissions (g/capita)
Yemen	0.80	26,052,966	0.000031
Pakistan	10.8	196,174,380	0.000055
Panama	0.40	3,608,431	0.00011
Dominican Republic	2.1	10,349,741	0.00020
New Zealand	1.4	4,401,916	0.00032
Australia	24.6	22,507,617	0.0011
Ecuador	76.3	15,654,411	0.0049
Burkina Faso	2.60	17,812,961	0.145
Cambodia	14.9	15,205,539	0.980
Madagascar	98.5	22,599,098	4.36
Mexico	1,560	116,220,947	13.4
Philippines	1,670	105,720,644	15.6

^a Maximum annual amount of Hg released to air, water, and land from UNEP (2011)

^b Population statistics from CIA (2014)

Table 2

QA/QC results for analysis of all rice samples, including average percent recovery of standard reference materials [NIST 1568a (rice powder), NRC Tort2 (lobster tissue), and NIST 1515 (apple leaves)]^a and matrix spikes^b, average relative percent difference ($\pm 100 \times$ standard deviation/mean) (RPD) between duplicate digestions, and limits of detection (LOD). The number of samples (n) is provided.

	RPD (%) (n)	LOD	NIST 1568a % recovery (n)	NRC Tort2 % recovery (n)	NIST 1515 % recovery (n)	Matrix Spike % recovery (n)
THg	18 (n=65)	0.020 ng/g	93 (n=20)	83 (n=23)	87 (n=2)	130 (n=8)
MeHg	22 (n=36)	0.0020 ng/g		80 (n=19)		100 (n=9)
As	2.8 (n=10)	0.00025 µg/g	99 (n=2)	83 (n=2)		
Cd	23 (n=10)	0.0014 µg/g	93 (n=2)	83 (n=2)		
Cu	2.9 (n=10)	0.0091 µg/g	92 (n=2)	86 (n=2)		
Mn	3.7 (n=10)	0.0018 µg/g	89 (n=2)	98 (n=2)		
Rb	2.4 (n=10)	0.0056 µg/g	97 (n=2)			
Se	12 (n=10)	0.0062 µg/g	89 (n=2)	81 (n=2)		
Sr	12 (n=10)	0.0065 µg/g		93 (n=2)		
Zn	6.4 (n=10)	0.29 µg/g	83 (n=2)	82 (n=2)		

^a Certified reference values for: NIST 1568a (THg 5.8 ng/g, As 0.29 µg/g, Cd 0.022 µg/g, Cu 2.4 µg/g, Mn 20.0 µg/g, Rb 6.14 µg/g, Se 0.38 µg/g, Zn 19.4 µg/g), TORT2 (THg 270 ng/g, MeHg 152 ng/g, As 21.6 µg/g, Cd 26.7 µg/g, Cu 106 µg/g, Mn 13.6 µg/g, Se 5.63 µg/g, Sr 45.2 µg/g, and Zn 180 µg/g) and NIST 1515 (THg 44 ng/g).

^b Matrix spikes were 500 pg and 25 pg for THg and MeHg, respectively.

Table 3

Summary statistics, including mean, (median) and (min-max) for unpolished rice from Madagascar (n=51). Metals and metalloids included total mercury (THg), methylmercury (MeHg), arsenic (As), cadmium (Cd), copper (Cu), manganese (Mn), rubidium (Rb), selenium (Se), strontium (Sr) and zinc (Zn).

Metal(loid)	Madagascar unpolished rice	Global non-polluted sites^a	Global polluted sites^a
THg (ng/g)	1.1 (0.90) (0.36–3.4)	8.2 (4.1) (1.0–45)	65 (26) (2.3–510)
MeHg (ng/g)	0.12 (0.098) (0.015–1.1)	2.5 (2.2) (0.86–5.8)	16 (11) (1.2–63)
%MeHg (of THg)	12 (9.3) (1.2–43)	36 (36) (17–75)	30 (29) (8.1–56)
Metal(loid)	Madagascar unpolished rice	Metal(loid)	Madagascar unpolished rice
As (µg/g)	0.16 (0.15) (0.074–0.28)	Rb (µg/g)	7.3 (6.0) (2.0–28)
Cd (µg/g)	0.0024 (0.0021) ($<LOD-0.0061$)	Se (µg/g)	0.039 (0.033) (0.015–0.080)
Cu (µg/g)	4.4 (4.4) (0.87–6.5)	Sr (µg/g)	0.17 (0.16) (0.094–0.23)
Mn (µg/g)	20 (19) (10–34)	Zn (µg/g)	28 (27) (20–36)

^a Summary statistics for global non-polluted and polluted sites from 51 studies for THg, MeHg and %MeHg (of THg), including polished and unpolished rice (Rothenberg et al., 2014).

Table 4

Correlation matrix for Madagascar rice (n=51) including total mercury (THg), methylmercury (MeHg), arsenic (As), selenium (Se), manganese (Mn), cadmium (Cd), rubidium (Rb), copper (Cu), strontium (Sr) and zinc (Zn) using a) Pearson's correlation and b) Spearman's correlation. For Pearson's correlation, the log₁₀-transformation was applied to highly skewed variables. Underlined values in bold are discussed further in the text. An asterisk (*) indicates the correlation was significant (p<0.05).

a. Pearson's correlation										
	Log ₁₀ MeHg	Log ₁₀ THg	Log ₁₀ As	Log ₁₀ Se	Log ₁₀ Mn	Log ₁₀ Cd	Log ₁₀ Rb	Cu	Sr	Zn
Log ₁₀ MeHg	1.0									
Log ₁₀ THg	0.29*	1.0								
Log ₁₀ As	0.47*	0.30*	1.0							
Log ₁₀ Se	-0.18	0.042	-0.39*	1.0						
Log ₁₀ Mn	0.058	-0.31*	-0.26	-0.27	1.0					
Log ₁₀ Cd	0.025	-0.042	-0.15	0.14	0.29*	1.0				
Log ₁₀ Rb	0.33*	0.44*	-0.040	0.30*	-0.22	-0.15	1.0			
Cu	-0.29*	-0.32*	-0.34*	0.20	0.34*	0.062	-0.26	1.0		
Sr	<u>-0.0037</u>	<u>-0.0065</u>	-0.28*	0.29*	0.16	-0.056	0.29*	0.21	1.0	
Zn	0.22	0.023	0.33*	-0.095	-0.014	-0.18	0.16	-0.051	0.085	1.0

b. Spearman's correlation										
	MeHg	THg	As	Se	Mn	Cd	Rb	Cu	Sr	Zn
MeHg	1.0									
THg	0.22	1.0								
As	0.43*	0.34*	1.0							
Se	-0.13	0.098	-0.38*	1.0						
Mn	0.12	-0.27	-0.23	-0.34*	1.0					
Cd	-0.0035	-0.017	-0.28*	0.14	0.29*	1.0				
Rb	0.14	0.43*	-0.044	0.36*	-0.28*	-0.22	1.0			
Cu	-0.022	-0.24	-0.43*	0.25	0.28*	0.12	-0.063	1.0		
Sr	0.0030	<u>-0.0090</u>	-0.22	0.27	0.12	-0.053	0.31*	0.24	1.0	

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b. Spearman's correlation										
	MeHg	THg	As	Se	Mn	Cd	Rb	Cu	Sr	Zn
	0.20	0.043	0.36*	-0.13	-0.043	-0.26	0.13	0.036	0.062	1.0