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Article

Stable isotope tracers identify sources and transformations of mercury in rice (Oryza sativa L.) growing in a mercury mining area

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ABSTRACT

Methylmercury (MeHg) contaminated rice is a global issue, particularly in mercury-polluted areas, posing a potential threat to human health. The sources and transformations of mercury (Hg) species in rice are critical points that are not yet fully understood. In this study, field experimental pots together with a stable Hg isotope tracing technique were used to provide direct evidence of the sources and transformations of Hg species in rice plants. Enriched inorganic Hg (IHg) isotope ($^{200}Hg(NO_3)_2$) was spiked into paddy soils, and the concentrations of inorganic Hg tracer ($I^{200}Hg$), MeHg tracer ($Me^{200}Hg$), and ambient Hg species (IHg and MeHg) were measured in the tissues of rice plants and their corresponding soil samples during the rice growing season. Here, we show that, in addition to the atmosphere, the soil is an important source of IHg to rice grains and was previously largely underestimated. We also show that MeHg is formed in paddy soil via microbial IHg methylation, absorbed through the rice root, translocated from the root to above-ground parts, and finally accumulated in rice grains. Although in vivo methylation of IHg in rice plants is unlikely to occur during the rice growing season, we observed in vivo demethylation of MeHg in the above-ground parts of rice plants, possibly via photolytic demethylation. Promoting in vivo demethylation of MeHg may be an effective approach to mitigate MeHg accumulation in rice grains.

1. Introduction

Mercury (Hg) has long been a research hotspot in aquatic ecosystems due to the neurotoxicity of methylmercury (MeHg) for fish-eating populations. In recent decades, pioneering work confirmed that the consumption of rice rather than fish is the major MeHg exposure pathway for inland populations of China [1–4]. Since then, biogeochemistry cycling of Hg in the rice paddy ecosystem has emerged as a new topic and receives increasing attention. Submerged rice paddies provide favourable anoxic conditions for the methylation of inorganic Hg (IHg) [5,6], which results in the accumulation of MeHg in rice [7]. MeHg-contaminated rice is not only found in Hg mining areas but also in paddy fields close to chemical plants, coal-fired power plants, smelting plants and chloralkali plants, suggesting that MeHg bioaccumulation in rice is ubiquitous in Hg-contaminated regions [8]. Besides China, MeHg-contaminated rice has also been documented in America [9], India [10], Thailand [11], and Indonesia [12]. Populations who live far from Hg-contaminated areas can also be affected by exposure to MeHg in rice due to international trade and the globalisation of crops [13].

Environmental remediation strategies in mitigating Hg contamination in the rice paddy ecosystem require a robust understanding of Hg sources and transformations in rice plants. According to measurements of Hg species (IHg and MeHg) in rice plants and environmental media (e.g., soil, irrigation water, and ambient air), statistical analysis provides a preliminary understanding that paddy soils are the predominant source of MeHg, and ambient air is the main source for IHg in rice [6,14–17]. However, Wang et al. [18] suggested that 8.5–15.5% MeHg in the above-ground parts of rice plants could be derived from dimethylmercury (DMeHg) in air. Xu et al. [19] observed a decrease of IHg in rice seeds by adding selenium (Se) into the soil, implying that soil could also be a potential source of IHg in rice grains. Through a mass-independent fractionation (MIF) study of Hg, Yin et al. [20] highlighted mixed sources of Hg (i.e., soil and atmosphere) in rice, but could not distinguish between MeHg and IHg using this method. By using a compound-specific Hg stable isotope technique, Qin et al. [21] showed that both soil and the atmosphere contribute to IHg in rice. Recently, Strickman and Mitchell [22] further supported the dual sources of IHg (soil and air) and the unique source of MeHg (soil) in rice through green-

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house experiments; however, it faced uncertainties on the source apportionment of Hg species, especially when the rice plant was exposed to conditions with high atmospheric/soil Hg levels. From the available literature, we can conclude that previous studies provide a reliable and important, yet incomplete, understanding of the sources of Hg species in rice plants.

Methylation of IHg in plants has been documented in aquatic macrophytes [23] and few marvel peas [24]. Tabatchnick et al. [25] speculated that in vivo methylation of Hg from the atmosphere is the source of MeHg in tree foliage according to the coupling of THg and MeHg concentrations in leaves. However, it is unknown the extent to which methylation of IHg in rice plants—which potentially controls the accumulation of MeHg in rice seeds—occurs. If IHg in rice plants can be methylated, the MeHg burden in rice poses a severe issue. This is because the concentration of IHg in rice can be as high as 11 mg kg^{-1} in the Hg-polluted area [15], and even a small fraction of this IHg being methylated will lead to a great risk of MeHg bioaccumulation in rice grains. On the other hand, in vivo demethylation of MeHg has been widely reported in biota [26,27], but remains questionable in rice plants. Li et al. [28] and Xu et al. [29] reported the in vivo demethylation in rice plants by conducting IHg or MeHg-spiked rice culture experiments. However, acquiring direct evidence of this process in vivo remains necessary. Should demethylation of MeHg be observed in rice plants and its mechanism tractable, it may offer a strategy to reduce the bioaccumulation of MeHg in rice grains and therefore mitigate MeHg exposure risk by eating rice. Overall, the methylation of IHg and demethylation of MeHg in rice plants deserve more investigation.

To better understand these research gaps, we used the enriched stable Hg isotope tracing technique and conducted a rice cultivation experiment at a high atmospheric Hg concentration area using background (low THg levels) soils. We attempted to address the following questions: (1) What are the sources of IHg and MeHg in rice and the relative contributions from each source (soil vs. atmosphere)? (2) Can we detect methylation of IHg or demethylation of MeHg in rice plants during the growing period?

2. Materials and method

2.1. Study area

The Xunyang Hg mining area (XMM) was selected as the study site due to the high Hg level in ambient air. As a recently active Hg mine in China, the XMM is located in Xunyang County, Shaanxi Province. The main ore mineral from the XMM is cinnabar (α-HgS) with accessory minerals of antimony (Sb)-rich minerals. Due to ongoing Hg smelting activities, the concentration of gaseous elemental Hg (GEM) in ambient air and the total gaseous Hg (TGM) deposition flux in this area could reach 410 ng m^{-3} (average in 93 ng m^{-3}) and 978 mg m^{-2} y^{-1} (average in 236 mg m^{-2} y^{-1}), respectively [30,31]. A small watershed (Zhutong River) is close to the XMM. Rice (Oryza sativa L.) is the stable crop planted along the watershed.

2.2. Inorganic ^{200}Hg spiking plot experiment

Two simulated rice paddy plots were set-up close to the Hg smelting plant of the XMM (Fig. S1) to investigate the uptake, transport and transformation of Hg across paddy soil – rice plants. Pre-cleaned polyvinyl chloride (PVC) boxes (54 cm × 42 cm × 33 cm) were used, with each box filled with 40 kg soil (pre-sieved at 2 mm, soil depth of ~20 cm). Soils used in the plot experiment were collected from a regional background cropland (non-contaminated, surface layer 1–20 cm; moisture content of 6.7%) in Xunyang County, which is about 30 km away from the Hg smelting plant. The total Hg (THg) concentration in the collected soil was determined to be 69 ± 10 μg kg^{-1}, roughly 2–4 orders of magnitude lower than the soils close to the XMM (5.4–120 mg kg^{-1}, [30]).

Enriched ^{200}Hg (98.2 ± 0.15%) was purchased from ISOFLEX (USA). $^{200}Hg(NO_3)_2$ stock solution was prepared by dissolving ^{200}Hg in nitric acid (≥99.9%, trace metals basis). A spiking solution of $^{200}Hg(NO_3)_2$ was prepared in deionized (DI) water (Milli-Q, Millipore, USA) with a concentration of 12 mg Hg L^{-1} and the pH was adjusted to neutral by NaOH. $^{200}Hg(NO_3)_2$ was spiked at 10 different positions (100 mL in total) evenly distributed in the soil from experimental boxes. After spiking, the experimental box was immediately covered with dark plastic lids, reducing the potential losses of Hg isotopes through the soil-air interface [32]. Soil spiked with $^{200}Hg(NO_3)_2$ was aged for 24 h before rice cultivation.

Rice seeds (hybrid rice), which are widely used in the XMM, were selected in this study. Rice seedlings were pre-cultivated hydroponically in a greenhouse for 30 days. Twenty seedlings (with similar height) were transplanted to the experimental plots with a space of 10 cm × 10 cm (5 × 4). Local tap water was used as irrigation water, and the soil was flooded by 3–5 cm above the soil surface. In total, ~50 L of tap water was irrigated four times into the experimental box during the rice growing seasons. The experimental plots were placed in the field (Fig. S1), and the rice was cultivated for 110 days from June to September 2016. Air temperature during the rice growing period ranged from 19 to 34 °C. Agronomy managements were performed at the same with local farmers.

2.3. Sample collection and pretreatment

Three random rice seedlings and corresponding rhizosphere soils (10–20 cm) were collected 30, 60, and 90 days after being transplanted, while five seedlings were collected 110 days after being transplanted. Soil samples were also collected on day 0 to record initial conditions. Rice plants were washed using tap water in situ and stored in nylon mesh bags. Irrigation water samples were collected at all sampling locations by using pre-cleaned borosilicate glass bottles (200 mL) acidified with 0.5% of HCl (v/v). All the collected samples were stored in coolers with ice packs and transported to the lab within 24 h. Deionized water was used to wash rice plants carefully in the lab to remove soil particles on the root surface. Rice plants were separated into their root, stalk, leaf, and grain parts (rice grain was obtained on day 90 and day 110). Then, rice tissues were freeze-dried (FD-3–85D-MP, FTS, USA) and ground to 150 mesh (IKA-A11 basic, IKA, Germany). The freeze-dried rice grains were further divided into the hull, bran, and polished rice parts using a laboratory huller and polisher (Taizhou Foodstuff Instrument Inc. China). The biomass of all dried rice tissues was recorded. Soil samples were freeze-dried and ground to 200 mesh using an agate mortar and pestle. All the tools used in homogenisation were thoroughly cleaned between samples to avoid cross-contamination.

2.4. Analytical methods

The total Hg ($T^{200}Hg$) and MeHg isotopes ($Me^{202}Hg$) of rice tissues and soil samples were determined using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies Inc., USA) and gas chromatography ICP-MS, respectively. The extraction and digestion methods for THg and MeHg in soil and plant samples are available in the literature [7,14]. More details related to Hg isotope analysis can be found in our previous works [5,32,33]. The total Hg concentration in irrigation water (unfiltered) was measured by cold vapour atomic fluorescence spectrometry (CVAFS, Brooks Rand Model III, Brooks Rand Labs, U.S.A.). Gaseous elemental Hg was measured during each of the sampling campaigns. An in-situ Hg vapour analyser (RA-915+, Lumex, Russia) with Zeeman correction was used to record the average GEM concentration every 10 s and was continuously measured for 1 h at each location.

2.5. Calculation, statistical analysis, and quality control

Ambient Hg refers to the Hg that is naturally present in the environment in which experiments were conducted [32]. Ambient THg and MeHg concentrations were calculated according to the concentrations of $T^{202}Hg$ and $Me^{202}Hg$ measured and the natural abundance of ^{202}Hg (Eq. (1)). Enriched ^{200}Hg refers ^{200}Hg originating from the spiked ^{200}Hg tracer of this study, which was calculated by subtracting the ambient ^{200}Hg from the measured ^{200}Hg (Eq. (2)).

$$^{200}Hg_{ambient} = {}^{202}Hg_{measured}/29.9\% \times 23.1\% \quad \text{(Eq. (1))}$$

$$^{200}Hg_{enriched} = {}^{200}Hg_{measured} - {}^{200}Hg_{ambient} \quad \text{(Eq. (2))}$$

where $^{200}Hg_{ambient}$ and $^{200}Hg_{enriched}$ represent concentrations of ambient ^{200}Hg (THg and MeHg) and enriched ^{200}Hg (THg and MeHg) in samples, respectively; $^{200}Hg_{measured}$ and $^{202}Hg_{measured}$ represent the concentrations of measured ^{200}Hg and ^{202}Hg (THg and MeHg) in samples; 23.1% and 29.9% are the natural abundances of ^{200}Hg and ^{202}Hg, respectively. More details in ambient and enriched Hg calculations are described in Meng et al. [32] and Mao et al. [34].

The concentration of inorganic Hg (IHg) was obtained by subtracting MeHg from THg [7]. The absolute masses of enriched ^{200}Hg (i.e., $I^{200}Hg$, $Me^{200}Hg$, and $T^{200}Hg$) in different tissues of rice plants were calculated according to the enriched ^{200}Hg concentration, biomass of each tissue, and the number of strains for seedlings (Table S1). The contribution percentages of IHg and MeHg from the atmosphere and root transportation were calculated following that described by Mao et al. [34] and introduced in Text S1. Bioaccumulation factors (BAF) were calculated as follows [16]:

$$BAF = {}^{200}Hg_{\ enriched\text{-}tissues} / {}^{200}Hg_{\ enriched\text{-}soil} \quad \text{(Eq. (3))}$$

where $^{200}Hg_{\ enriched\text{-}tissues}$ indicates enriched ^{200}Hg (i.e., IHg and MeHg) in different tissues (i.e., the root, stalk, leaf, hull, bran and polished rice), and $^{200}Hg_{\ enriched\text{-}soil}$ indicates enriched ^{200}Hg (i.e., IHg and MeHg) in soil.

Statistics were performed using SPSS 23.0 (IBM®, USA). Normality of datasets was assessed by the Shapiro-Wilk test. The Wilcoxon test (non-parametric method) and *t*-test (parametric method) were used for a difference test between paired datasets. The Kruskal-Wallis one-way ANOVA test (non-parametric method) and one-way ANOVA with Duncan's post-hoc test (parametric method) were used for independent datasets. The statistical significance (p) was declared at < 0.05 and < 0.01 (2-tailed). Statistics for enriched ^{200}Hg species in rice tissues during the growing period were conducted from day 30 since no enriched ^{200}Hg in rice tissues on day 0 was assumed. Data reported in this study are shown as mean ± 1 standard deviation (sd).

The detection limits (DL, three times the standard deviation of replicate measurements for the blank solution, 3σ) for THg and MeHg isotopes are 0.1 ng L^{-1} and 0.013 ng L^{-1}, respectively. For irrigation water and GEM, the DL are 0.02 ng L^{-1} and 0.3 ng m^{-3}, respectively. Certified reference materials (CRM) including GSS-5 (THg, $n = 6$, recoveries = 107 ± 2.4%), GSB-11 (THg, $n = 10$, recoveries = 102 ± 4.2%), ERMCC580 (MeHg, $n = 10$, recoveries = 92 ± 10%), and TORT-2 (MeHg, $n = 13$, recoveries = 88 ± 10%) were used (Table S2). Blanks, duplicates and CRM were added every 10 samples in each measurement.

3. Results

3.1. Mercury in irrigation water, ambient air and soils

The mean concentration of THg in irrigation water was 5.3 ± 3.7 ng L^{-1} with a range of 1.6–7.4 ng L^{-1} ($n = 8$). The concentration of THg in irrigation water was close to background water levels (Huaxi, Guiyang, [THg] = 7.1 ± 4.0 ng L^{-1}, [6]), and was 1–4 orders of magnitude lower than the THg levels in Hg mine impacted local stream water [30], suggesting no Hg contamination in irrigation water. Consequently, the contribution of Hg in paddy systems from irrigation water was negligible (only 0.01% in the contribution of Hg from the soil). However, the concentration of GEM in the study area was high, with an average of 49 ± 43 ng m^{-3} (varying from 11 to 298 ng m^{-3}, Fig. S2), which was attributed to ongoing Hg smelting activities [30,31]. Concentrations of GEM in ambient air at the sampling site during the rice growing seasons were much lower than that in the artisanal Hg mining area (403 ± 388 ng m^{-3}) [6], but were significantly higher than those in an abandoned Hg mining area (28 ± 13 ng m^{-3}) [6] and remote sites in China (1.6–4.0 ng m^{-3}) [35]. The high GEM and low THg concentrations in irrigation water suggested that atmospheric Hg deposition is the dominant source of Hg in experimental pots in this study.

The concentrations of ambient THg in the soil during the rice growing period ranged from 53 to 90 μg kg^{-1} (69 ± 10 μg kg^{-1}, Fig. 1a). A slight increase of ambient THg concentration (not significant) in soil was observed during the rice growing season, suggesting a contribution of atmospheric Hg deposition. The concentration of enriched $T^{200}Hg$ in soil samples was 97 ± 9.6 μg kg^{-1} during the rice growing period, which is comparable with the concentration of ambient THg. Ambient MeHg concentrations in soil samples ranged from 0.12 to 0.58 μg kg^{-1} during the rice growing period (0.34 ± 0.13 μg kg^{-1}). Concentrations of enriched $Me^{200}Hg$ in soil samples increased during the first 90 days, from 0.03 ± 0.003 μg kg^{-1} to 0.51 ± 0.10 μg kg^{-1}, and then decreased to 0.37 ± 0.05 μg kg^{-1} during the last 20 days of cultivation ($p < 0.05$, Fig. 1b). The enrichment of $Me^{200}Hg$ in soils indicated microbial methylation of enriched $I^{200}Hg$ tracer ($^{200}Hg(NO_3)_2$) from the beginning to day 90.

3.2. Ambient Hg and enriched ^{200}Hg species in rice plants

A steady increase of ambient IHg concentrations in leaves during the rice growing season was observed, with the highest level (1264 ± 323 μg kg^{-1}) observed on day 110 ($p < 0.01$, Fig. 2a). Through paired dataset tests, ambient IHg levels in leaves were significantly higher than those in roots and stalks during the entire growing period ($p < 0.01$), whereas no significant differences were observed between roots and stalks. For mature rice plants, the highest and lowest ambient IHg concentrations were observed in leaves (1264 ± 323 μg kg^{-1}) and polished rice (50.2 ± 9.1 μg kg^{-1}), respectively ($p < 0.05$, Fig. 3a). Relatively high concentrations of enriched $I^{200}Hg$ were found in the root during the rice growing season when compared with those in above-ground parts (e.g. stalk, leaf, bran, and polished rice) ($p < 0.01$) (Fig. 2c). No difference was observed between enriched $I^{200}Hg$ concentrations in the stalks and leaves during the rice growing season ($p > 0.05$, Fig. 2c). Enriched $I^{200}Hg$ concentrations in matured rice plants were as follows in decreasing order: root (322 ± 85.1 μg kg^{-1}) > bran (70.1 ± 12.8 μg kg^{-1}) ≈ polished rice (58.8 ± 5.9 μg kg^{-1}) > leaf (33.2 ± 7.3 μg kg^{-1}) > stalk (22.6 ± 6.5 μg kg^{-1}) > hull (6.6 ± 3.5 μg kg^{-1}) ($p < 0.05$, Fig. 3c).

Ambient MeHg levels in the roots and stalks first increased and then decreased (from day 60 for stalk, and from day 90 for root). During the rice growing, the ambient MeHg levels in the roots, stalks and leaves could reach 42.4 ± 4.1 μg kg^{-1} (at day 90), 27.4 ± 1.4 μg kg^{-1} (at day 60), and 12.0 ± 0.8 μg kg^{-1} (at day 0), respectively ($p < 0.05$, Fig. 2b). When harvested, the highest and lowest ambient MeHg concentrations were found in the bran (59.7 ± 5.0 μg kg^{-1}) and leaves (0.89 ± 0.2 μg kg^{-1}), respectively ($p < 0.05$, Fig. 3b). Temporal variations of enriched $Me^{200}Hg$ in roots, stalks and leaves were similar, all of which increased from the beginning (day 0) up to day 60, and then decreased (Fig. 2d). During the entire growing period, concentrations of enriched $Me^{200}Hg$ in roots and stalks were significantly higher than in leaves ($p < 0.01$); the highest concentrations of the enriched $Me^{200}Hg$ in roots, stalks, and leaves were 66.2 ± 6.6 μg kg^{-1}, 79.2 ± 5.1 μg kg^{-1}, and 17.4 ± 1.6 μg kg^{-1}, respectively ($p < 0.05$, Fig. 2d). In mature rice

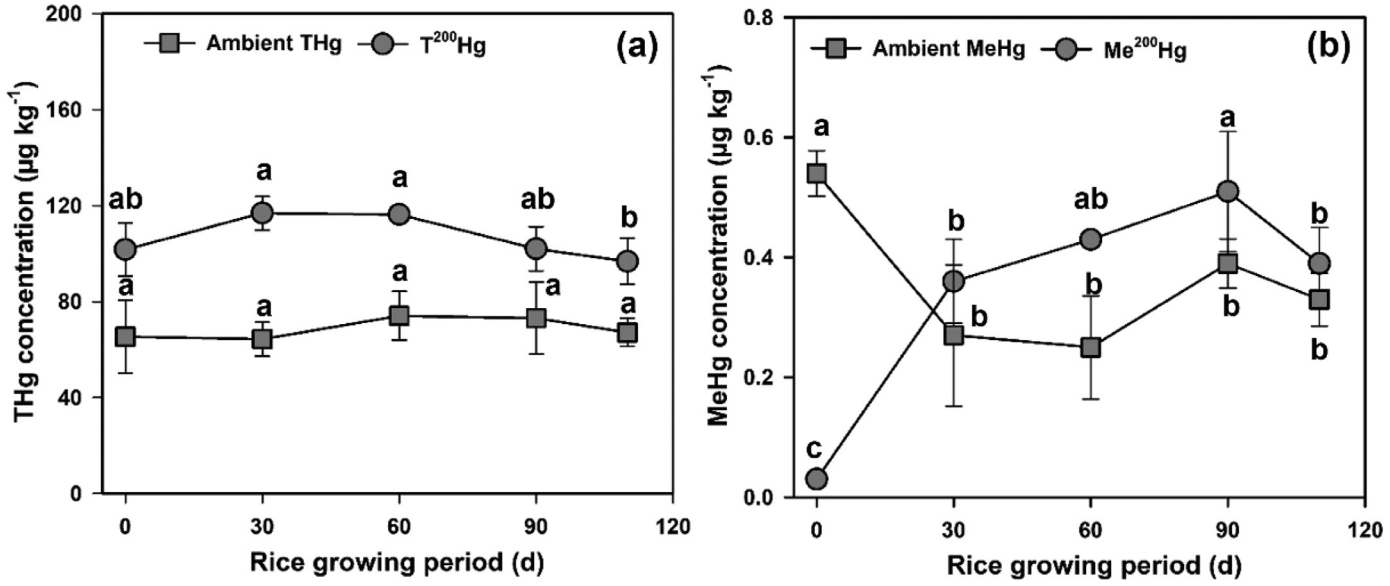

Fig. 1. Concentrations of THg (a) and MeHg (b) in soil during the rice growing period. Different lowercase letters indicate the differences during the growing period are significant ($p < 0.05$). Error bars represent 1 sd of three replicates.

plants, enriched $Me^{200}Hg$ concentrations were as follows in decreasing order: bran (82.7 ± 4.8 μg kg^{-1}) > polished rice (44.3 ± 1.5 μg kg^{-1}) > root (18.9 ± 3.3 μg kg^{-1}) > stalk (11.6 ± 1.7 μg kg^{-1}) > hull (9.0 ± 1.3 μg kg^{-1}) > leaf (1.2 ± 0.2 μg kg^{-1}) ($p < 0.05$, Fig. 3d).

3.3. Relative distribution of the enriched ^{200}Hg and ambient Hg species in rice plants

The mass of the enriched $I^{200}Hg$ in roots slightly increased from 341 ng $plant^{-1}$ on day 30 to 377 ng $plant^{-1}$ on day 60, and then decreased to 145 ng $plant^{-1}$ on day 90 (Fig. 4a). Compared to the roots, the mass of the enriched $I^{200}Hg$ in stalks increased during the period from day 30 to day 60 ($p < 0.05$, Fig. 4a and Table S3). During the period from day 90 to day 110, the $I^{200}Hg$ burden was found to increase in bran and polished rice but decreased in the hull ($p < 0.05$, Fig. 4a and Table S3). Variations in $T^{200}Hg$ burden in rice tissues were similar to enriched $I^{200}Hg$, except for the burden of the enriched $T^{200}Hg$ in stalks and leaves, which were at the same levels between day 90 and day 110 ($p > 0.05$, Fig. 4c). The majority of ambient IHg and THg were distributed in leaves (Fig. S3a and S3c), and the largest mass of ambient IHg in leaves was found on day 110 (1373 ± 142 ng $plant^{-1}$, $p < 0.05$, Table S4).

Lower levels of enriched $Me^{200}Hg$ were found in tissues of rice plants on day 30 when compared to other sampling periods ($p < 0.05$, Fig. 4b). The mass of the enriched $Me^{200}Hg$ decreased during the period from day 60 to the day of harvest (day 110) in roots and stalks, and from day 90 to day 110 in hulls ($p < 0.05$, Fig. 4b and Table S3). Similar to $I^{200}Hg$, a higher enriched $Me^{200}Hg$ mass in bran and polished rice was found on day 110 ($p < 0.05$, Fig. 4b and Table S3). The distribution patterns of ambient MeHg were similar to the enriched $Me^{200}Hg$ (Fig. S3b). Before harvest, stalks held the largest ambient MeHg pool, accounting for 42%–66% of the total ambient MeHg in the rice plant. On day 110, the largest ambient MeHg pool moved to the polished rice (accounted for 61.8%).

To further understand the relative distribution of Hg in tissues of rice plants, the rice plants were further divided into above-ground (stalk, leaf, hull, bran, and polished rice) and below-ground (root) parts (Figs. S4 and S5). During the entire growing period, significant increases of enriched ^{200}Hg (i.e., $I^{200}Hg$, $Me^{200}Hg$, and $T^{200}Hg$) masses were shown in the above-ground parts ($p < 0.05$, Fig. S4), with enriched $Me^{200}Hg$ between days 60 and 90 as an exception. Moreover, extremely low levels of enriched $Me^{200}Hg$ were found in the above-ground parts on day 30, only accounting for 11%–15% of those from day 60 to day 110 (Fig. S4).

4. Discussion

4.1. Source apportionment of Hg in rice plants

Ratios of enriched ^{200}Hg/ $Hg_{ambient}$ (ambient Hg) in soil and rice tissues can be used to distinguish the sources of Hg [22]. Specifically, similar enriched $^{200}Hg/Hg_{ambient}$ in soil and rice tissues suggests that soil is the major source of Hg in rice plants (enriched ^{200}Hg was spiked in soil), whereas other sources will result in different ratios of enriched ^{200}Hg to ambient Hg. For example, a significant decrease of enriched $I^{200}Hg/IHg_{ambient}$ ratio will be observed if ambient air (instead of soil) contributed a large portion of $IHg_{ambient}$. It should be noted that Hg isotope fractionation occurs during methylation/demethylation, rice-uptake and translocation [20,36]. However, the natural isotope fractionations can be ignored in enriched Hg isotope-spiked scenarios [32,34]. Therefore, the ratio of enriched $^{200}Hg/Hg_{ambient}$ is a reliable tool for revealing Hg sources. In this study, the relatively low $I^{200}Hg/IHg_{ambient}$ ratios determined in stalks, leaves, hulls and bran than those in soils suggested that soil is not the only source of IHg to rice plants ($p < 0.05$, Table 1). Taking into account the concentrations (Fig. 3a) and masses (Fig. S3a) of ambient IHg in rice plants, foliar uptake of atmospheric Hg played an important role in the rice-uptake of IHg. This is in line with previous studies from Meng et al. [15], Qin et al. [21] and Strickman and Mitchell [22]. Specific contributions of air and root transport for IHg in the above-ground parts of matured rice (i.e., on day 110) were calculated and are shown in Fig. 5a. Contributions of IHg from the atmosphere to leaves could reach 99.1% under the experimental conditions of this study, and this contribution decreased to 94.6% in the stalk, 84.3% in bran and 58.2% in polished rice ($p < 0.05$).

Alternatively, comparable ratios of $Me^{200}Hg/MeHg_{ambient}$ between the soil and rice tissues (day 110, $p > 0.05$, Table 1) indicate that no other sources than soil contributes to MeHg-uptake in rice. Calculations of contributions from the atmosphere and root transport further support soil uptake by roots and root transportation is the only source of MeHg to above-ground rice plant parts (Fig. 5b). Accordingly, we can conclude that Hg uptake from the soil and the atmosphere are two major sources of IHg in rice, whereas soil uptake is the main source of MeHg in rice. It is noted that dimethylmercury (DMeHg) could be another source of

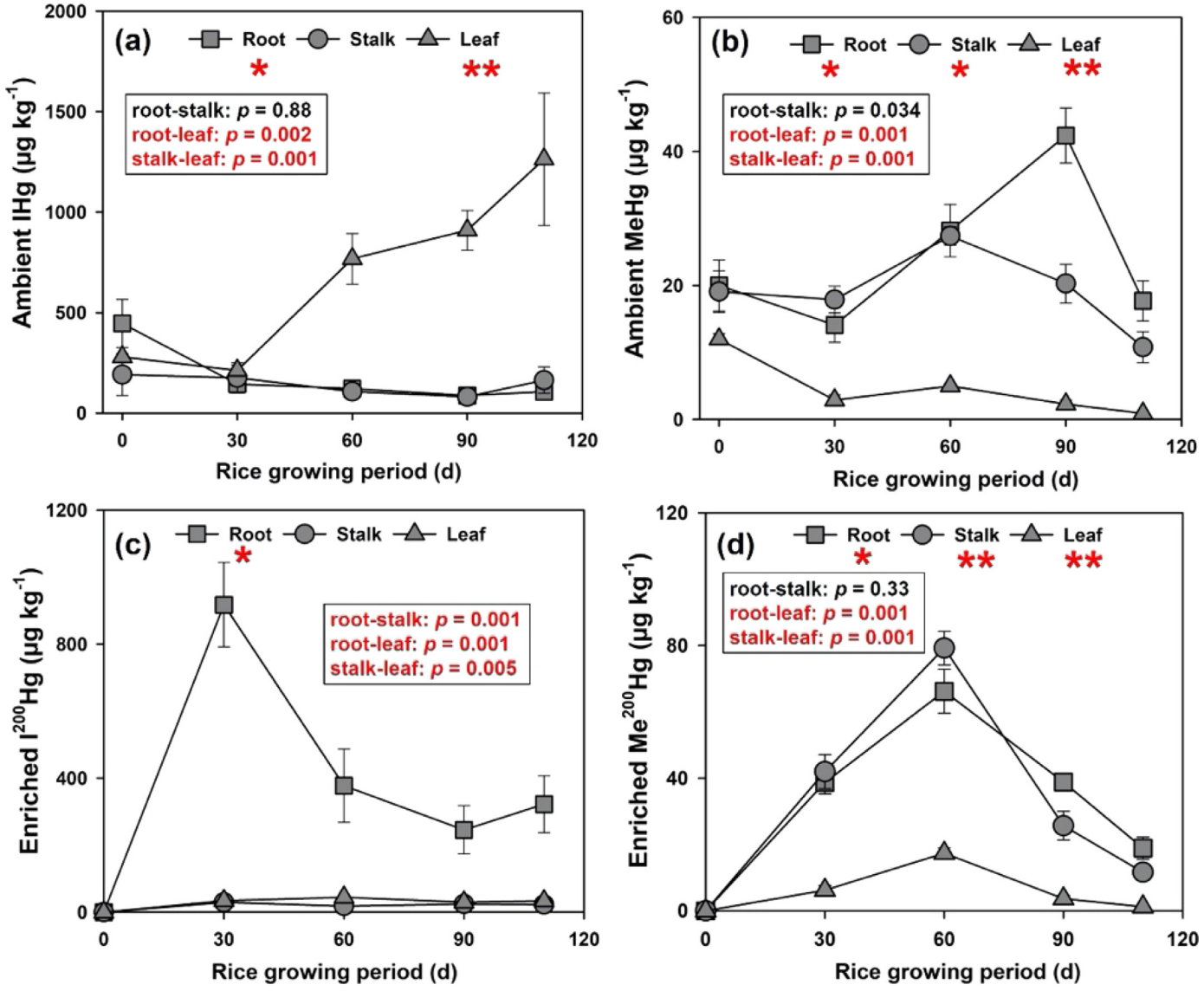

Fig. 2. Concentrations of ambient and enriched IHg and MeHg in the root, stalk and leaf of the rice plant during the growing period. (a) Ambient IHg; (b) ambient MeHg; (c) enriched $I^{200}Hg$; (d) enriched $Me^{200}Hg$. "*" and "**" below the legend indicate the concentration changes of Hg species (both ambient and enriched IHg and MeHg) in rice tissues during the growing period are significant at $p < 0.05$ and $p < 0.01$ through the Kruskal-Wallis one-way ANOVA test, respectively. Information in the frame is the results of the difference test from paired datasets (Wilcoxon test), where p values in red indicate significant difference ($p < 0.05$). Error bars represent 1sd of three replicates (five replicates were included on day 110).

Table 1
Concentration ratios of the enriched ^{200}Hg to ambient Hg in soil and rice plants at the harvest.

	Soil	Root	Stalk	Leaf	Hull	Bran	Polished rice
$I^{200}Hg/\ IHg_{\ ambient}$	1.45±0.13	3.02±0.80	0.15±0.08	0.03±0.01	0.06±0.03	0.45±0.07	1.20±0.21
$Me^{200}Hg/\ MeHg_{\ ambient}$	1.53±0.75	1.07±0.16	1.09±0.13	1.36±0.06	1.47±0.17	1.39±0.10	1.40±0.12

Mean±1sd ($n = 5$).

MeHg in the above-ground parts of rice, especially in Hg polluted paddy soils [18,37].

Different methods have been previously used for source apportionment of IHg and MeHg in rice plants. From concentration and statistical-based studies [7,14,15,17] to Hg isotope fractionation studies [20,21,38], results for IHg and MeHg sources in rice plants are quite consistent. In this study, we used a more direct method (i.e., enriched Hg isotope tracing combined with Hg isotope ratios) and further confirmed the contributions from mixed sources (i.e., soil and atmosphere) of IHg and the sole source (i.e., soil) of MeHg in rice, shedding more light on Hg biogeochemistry in rice paddy systems.

4.2. Absorption and transportation of Hg in rice plant

4.2.1. Inorganic Hg

The root is the main tissue that accumulates $I^{200}Hg$ from soil, and the translocation of $I^{200}Hg$ to above-ground parts of rice occurs via root uptake. On the one hand, in mature rice plants, roots showed the highest concentration of the enriched $I^{200}Hg$, indicating that most of $I^{200}Hg$ is localised in the roots (Fig. 3c); on the other hand, foliar uptake dominated the source of ambient IHg in rice plants since it was mainly distributed in the leaves (Fig. 3a). Iron plaque forming on the root surface and composed of amorphous or crystalline iron (oxyhydr)oxides [39],

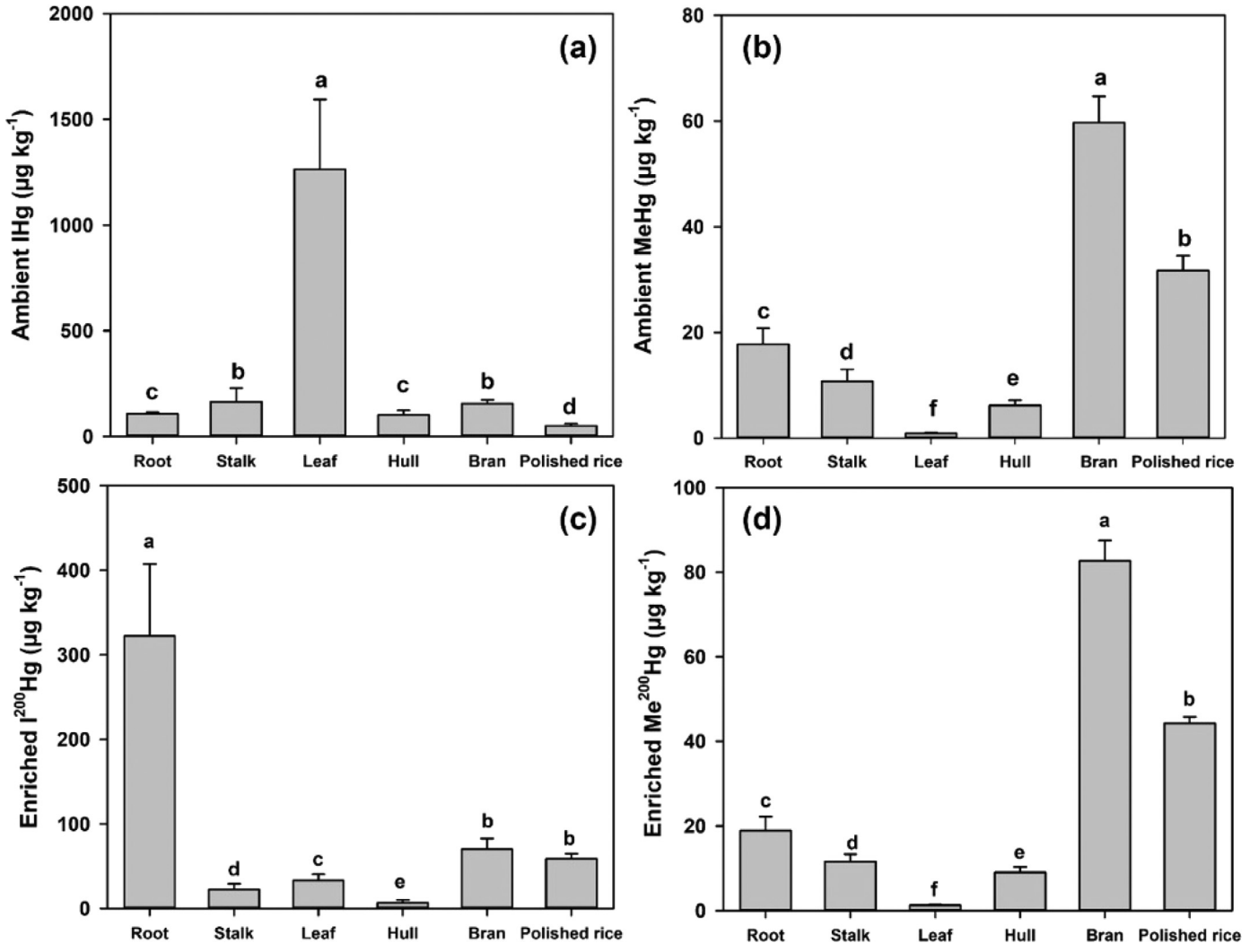

Fig. 3. Concentrations of ambient and enriched IHg and MeHg in different tissues of matured rice plants. (a) Ambient IHg; (b) ambient MeHg; (c) enriched $I^{200}Hg$; (d) enriched $Me^{200}Hg$. Different lowercase letters in each panel indicate the significant differences through the Kruskal-Wallis one-way ANOVA test ($p < 0.05$). Error bars represent 1sd of five replicates.

has been reported as a barrier blocking IHg uptake from roots [28,40]. Moreover, the development of apoplastic barriers in root endodermis also restricts Hg translocation to other tissues [41]. It is worth noting that enriched $I^{200}Hg$ was still observed in the above-ground parts of rice plants (Fig. 3c), suggesting that IHg in soil could permeate the barriers from the root and be distributed evenly to edible parts of the plant. The uptake of IHg by rice roots is likely through transport channels dedicated to other metals (e.g., Ca^{2+}, Zn^{2+} and Cu^{2+}), complexed with phytochelatins (PCs) in the cytosol of roots, and then transported to the vacuole [42–44]. Note, however, that Strickman and Mitchell [22] showed that IHg uptake by roots is in a state of equilibrium with soil IHg concentrations. This is different from the scenario presented here, for which we show variations in the accumulation of enriched $I^{200}Hg$ in roots during the rice growing period before its translocation to the plant (indicated by the increased $I^{200}Hg$ during the first 30 days, followed by a decline, Fig. 2c). If the IHg uptake by the root is a concentration gradient-driven equilibrium process, a coupling of the root and soil IHg concentrations from day 30 to day 110 would be expected, which was not the case in this study ($p > 0.05$, Fig. S6).

We suggest that the translocation of $I^{200}Hg$ into aerial parts of rice from soil could have been previously overlooked. Steady increases of IHg mass in the stalks and leaves were observed during the rice growing period due to atmospheric Hg compensation [15]. In this study, we found that translocation of the enriched $I^{200}Hg$ to leaves is limited, but significant to the stalk, hull, bran and polished rice parts during the growing period (Fig. 4a). The masses of the enriched $I^{200}Hg$ in the above-ground parts of the stalk, hull, bran, and polished rice account for 13.5%, 1.72%, 5.65%, and 43.8%, respectively, of the total mass of $I^{200}Hg$. We recognise that these values could be partially overestimated because of losses of root biomass during sampling, especially in the later period of growth (Table S1), though it is still significantly higher than the concentration of grain $I^{200}Hg$ (0.11 μg kg^{-1}) previously reported by Strickman and Mitchell [22]. Previous studies suggested that the translocation of IHg from the soil to the above-ground parts of rice was dependent on the THg concentration in soils [15,22], and the upward transportation of IHg from soil was mainly reported in Hg-contaminated paddy soils [20]. In this study, we found significant translocation of the enriched $I^{200}Hg$ to grains (i.e., bran and polished rice) planted in paddy soils with low THg concentrations ($[T^{200}Hg] = 97 \pm 9.6$ μg kg^{-1}), suggesting that the translocation of IHg into the above-ground parts of rice cannot be ignored. Food safety guidelines are generally set considering THg concentrations but there is no safe level of MeHg in food [45]. This finding suggested that mitigating atmospheric Hg concentration alone may not be sufficient to lower rice Hg levels. Alternatively, it is possible that the increased mass of the enriched $I^{200}Hg$ in the above-ground parts of rice, especially in the grain, could originate from the demethylation

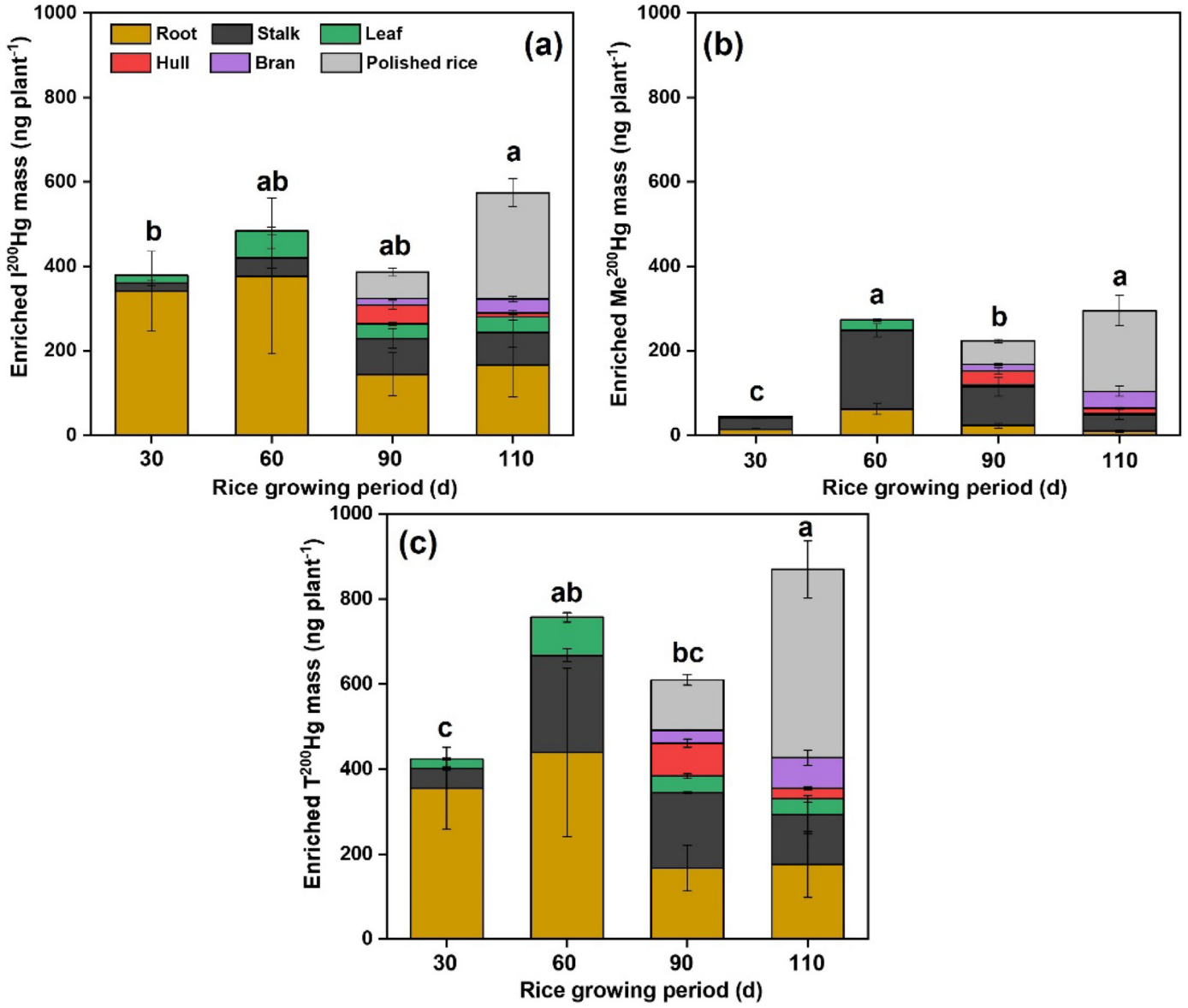

Fig. 4. Distribution of the enriched $I^{200}Hg$ (a), $Me^{200}Hg$ (b), and $T^{200}Hg$ (c) masses in rice tissues during the growing period. Different lowercase letters in each panel suggest the differences of the ^{200}Hg mass per plant (the sum of different tissues) are significant through the one-way ANOVA with Duncan's post-hoc test ($p < 0.05$). Statistics for the differences of each part during rice growing period are shown in Table S3. Error bars represent 1sd of three replicates (five replicates on day 110).

of the accumulated $Me^{200}Hg$ during the harvest period, which will be discussed in the next section.

4.2.2. Methylmercury

Methylmercury in rice was absorbed by the root, then transported to above-ground parts (e.g. stalk and leaf) and finally bioaccumulated into a mature rice grain. Uptake and transport of MeHg from roots to stalks and leaves were clearly shown by the enriched $Me^{200}Hg$ from the beginning (day 0) to day 60 (Fig. 2d) of the experiment. After 60 days, the enriched $Me^{200}Hg$ from other tissues (particularly the stalk and leaf) translocated and bioaccumulated into the grain (both bran and polished rice) (Figs. 3d and 4b) with high bioaccumulation factors (BAF, 230 ± 33.6 for bran and 123 ± 17.5 for polished rice) (Table S5). The uptake and translocation of MeHg explained by the enriched $Me^{200}Hg$ tracer agree well with previous observations [7,14,16,17,43]. The enriched $Me^{200}Hg$ was mainly distributed in the roots (BAF: 110 ± 26.6) and stalks (BAF: 117 ± 11.7) on day 30. From day 0 to day 90, most of the enriched $Me^{200}Hg$ was located in the stalks (reflected by high $Me^{200}Hg$ mass per plant and high BAF, Fig. 4b and Table S5). During the ripening period (day 90–110), translocation of the enriched $Me^{200}Hg$ from other tissues (e.g., stalk, leaf, and root) to the rice grains continued. When harvested, polished rice represented the major sink for the enriched $Me^{200}Hg$, and a relatively high degree of bioaccumulation of enriched $Me^{200}Hg$ was found in the bran (Fig. 4b and Table S5).

Through our previous synchrotron study, we reported that MeHg in bran was primarily bound to cysteine and associated with proteins, which can be actively transported to the endosperm during the ripening period [43]. Transport of MeHg from caryopsis to endosperm was independent of the genotype of rice [46]. Lower $Me^{200}Hg$ mass in rice on day 90 than on day 60 ($p < 0.05$, Fig. 4b) was likely attributed to the losses of rice roots during sample collection. More root biomass is expected to be associated with growing rice plants, which can directly influence the absolute mass of enriched Hg tracers in rice. That being said, we recorded lower biomass on day 90 and day 110 (Table S1). Al-

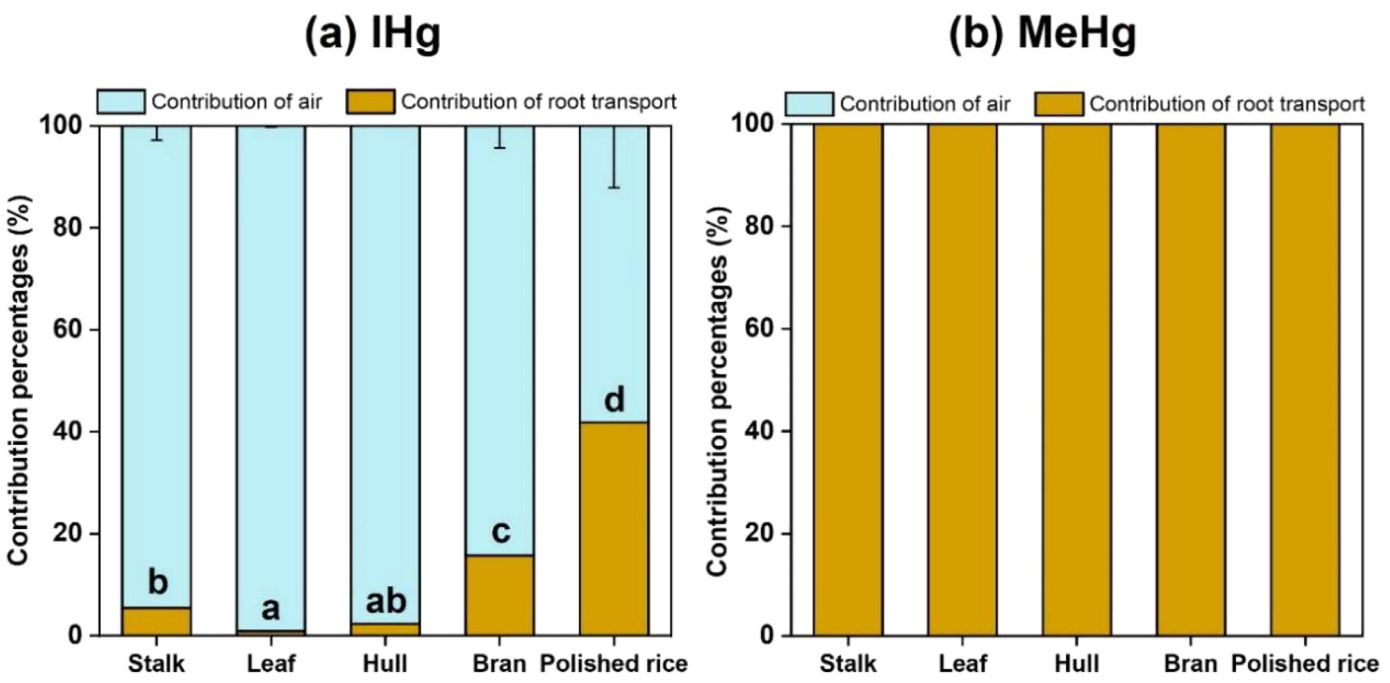

Fig. 5. Relative contribution percentages of air uptake and root transportation for IHg(a) and MeHg (b) in rice tissues at harvest. Different lowercase letters in panel (a) indicate the difference of contributions is significant through the Kruskal-Wallis one-way ANOVA test ($p < 0.05$). Error bars represent 1sd ($n = 5$).

though this variation is acceptable, this requires mentioning since the collection of all root tissues from soil was impossible.

Although MeHg/THg for either ambient or enriched ^{200}Hg were similar in paddy soils, more enriched $Me^{200}Hg$ was found in rice plants than ambient MeHg ($p < 0.05$, Figs. 4b and S3b). When normalising $Me^{200}Hg$ or ambient MeHg masses to $T^{200}Hg$ or ambient THg masses, a higher $Me^{200}Hg/T^{200}Hg$ (34.3 ± 4.65% on day 110) than ambient MeHg/THg (8.47 ± 0.42% on day 110) was observed in rice plants. This difference further suggests the different sources of Hg species (MeHg and IHg) to rice plants, because all ^{200}Hg tracers originated from the soil but air contributed to the majority of ambient THg in the above-ground parts of rice plants. Moreover, the different ratios of MeHg/THg between isotope tracer ($Me^{200}Hg/T^{200}Hg$) and ambient Hg ($MeHg_{ambient}/THg_{ambient}$) may also suggest that the newly formed MeHg (i.e., the enriched $Me^{200}Hg$ in this study) is more bioavailable for rice-uptake, which is also supported by previous studies [22,47]. Given these findings, reducing the phytoavailability of MeHg in paddy soils is a potential strategy to decrease MeHg accumulation in rice.

4.3. Methylation and demethylation of Hg in rice plant

In vivo methylation of Hg in plants was reported in water spinach (Ipomoea aquatica) [23] and peas (Pisum sativum) [24], and hypothesized in tree foliage [25]. Both the plant itself and endophytic bacteria could drive the methylation processes. Amino acid S-adenosylmethionine, as a donor for most metabolic methylations [48], is one candidate as a potential methyl-group donor in plants [23]. In rice plants, methylation of arsenic has been reported [49], whereas the methylation of Hg has not yet been documented. If in vivo methylation of IHg is significant, we would expect a decrease of $Me^{200}Hg/MeHg_{ambient}$ ratios from soil to the above-ground parts of rice. Due to the fact that ambient IHg concentrations were 2–3 orders of magnitude higher than those of enriched $I^{200}Hg$ tracer—especially in the above-ground parts of rice plants (Fig. 3a and 3c)—MeHg production through ambient production from IHg in rice plants would decrease the $Me^{200}Hg/MeHg_{ambient}$ ratio. In this study, however, we observed similar $Me^{200}Hg/MeHg_{ambient}$ ratios between the soil and above-ground parts of rice plants ($p > 0.05$, Table 1), further supporting the fact that in vivo methylation of IHg in rice plants is not an important process during the rice growing seasons.

Both biotic and abiotic demethylations of MeHg were reported in either natural environments or biota [27,50]. In this study, demethylation of MeHg occurs in the above-ground parts of rice plants during rice growing seasons. Once MeHg enters the rice tissues, transportation of the enriched $Me^{200}Hg$ and ambient MeHg should be comparable [34]. The increase of ambient MeHg mass of the above-ground parts was found during the growing period (from 71.6 ± 4.5 ng plant^{-1} on day 60 to 148 ± 17.7 ng plant^{-1} on day 90, $p < 0.05$, Fig. S5b), whereas the total mass of the enriched $Me^{200}Hg$ in the above-ground parts on day 90 (211 ± 15.9 ng plant^{-1}) was found lower than that on day 60 (201 ± 26.3 ng plant^{-1}, $p > 0.05$, Fig. S4b). Currently, no evidence supports a downward transport of MeHg (i.e., from above-ground plant parts to below-ground roots or soils). Therefore, losses of the enriched $Me^{200}Hg$ mass in the above-ground parts of rice are likely to be driven by in vivo demethylation, which was first suggested by Li et al. [28] and Xu et al. [29], and partially addressed by Strickman and Mitchell [22]. Simultaneous increases in the enriched $I^{200}Hg$ mass (Fig. 4a) further supports the in vivo demethylation of MeHg in rice plants. Therefore, our experiments suggest the occurrence of in vivo demethylation of MeHg in the above-ground parts of rice during the rice growing period.

However, whether in vivo demethylation of MeHg occurs in root remains unknown. A decrease in the enriched $Me^{200}Hg$ mass was also found in roots, whereas the upward transport of MeHg would also result in MeHg losses from roots. The mechanism for the in vivo demethylation of MeHg in rice plants remains unclear. So far, endophytic demethylating microorganism mediated demethylation and photolytic demethylation are two possible pathways [22]. Through a natural Hg isotope fractionation study, researchers reported the photoreduction process of Hg in rice plants [20], which partially supported a role for photolytic demethylation as responsible for the in vivo demethylation of MeHg in the above-ground parts of rice plants. However, further work is needed to verify this hypothesis. Accordingly, once the mechanistic basis for in vivo MeHg demethylation is known, promoting in vivo demethylation of MeHg through targeted genetic approaches may be a useful strategy in lowering MeHg exposure risk for rice eating populations.

5. Conclusions

Here, we studied the sources, translocation and transformation of IHg and MeHg in a rice-soil system by using enriched stable Hg isotope tracing techniques. Through the ratio of enriched Hg tracer to ambient Hg, we confirmed that both soil and atmosphere are the sources of IHg in rice, whereas soil is the major source of MeHg in rice. When harvested, the contribution of atmospheric Hg to rice plant IHg can reach 99.1%, 94.6%, 97.7%, 84.3% and 58.2% in leaf, stalk, hull, bran and polished rice, respectively. The root is the major tissue accumulating IHg from the soil, and the soil IHg can be translocated to above-ground parts of the rice plant. No in vivo methylation of IHg was evidenced by similar $Me^{200}Hg/MeHg_{ambient}$ ratios between the soil and rice tissues. MeHg formed from IHg methylation in soil was transported to the above-ground parts of the rice plant during rice growing seasons, and bioaccumulated in the mature rice grain. We observed in vivo demethylation of MeHg in the above-ground parts of rice plants in which photolytic demethylation is likely dominant in this process. This study suggests that reducing the phytoavailability of MeHg in paddy soils and promoting the in vivo demethylation of MeHg may represent important strategies to mitigate MeHg accumulation in rice. However, the mechanism of this demethylation process in rice plants is still unclear; and thus, further work is required.

Declaration of Competing Interest

The authors declare no conflict of interest

CRediT authorship contribution statement

Jiang Liu: Formal analysis, Visualization, Writing – original draft. **Bo Meng:** Funding acquisition, Conceptualization, Methodology, Validation, Writing – review & editing. **Alexandre.J. Poulain:** Methodology, Writing – review & editing. **Qiyi Meng:** Investigation, Data curtion. **Xinbin Feng:** Supervision, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fmre.2021.04.003.

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