The relative importance of mercury methylation and demethylation in rice paddy soil varies depending on the presence of rice plants

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ABSTRACT

Neurotoxic methylmercury (MeHg) accumulates in rice grain from paddy soil, where its concentration is controlled by microbial mercury methylation and demethylation. Both up- and down-regulation of methylation is known to occur in the presence of rice plants in comparison to non-vegetated paddy soils; the influence of rice plant presence/absence on demethylation is unknown. To assess the concurrent influence of rice plant presence/absence on methylation and demethylation, and to determine which process was more dominant in controlling soil MeHg concentrations, we maintained six rhizoboxes of paddy soil with and without rice plants. At the peak of plant growth, we simultaneously measured ambient MeHg, ambient IHg, and potential rate constants of methylation and demethylation (K_{meth} and K_{demeth}) in soil using stable isotope tracers and ID-IC-ICPMS. We also measured organic matter content, elemental S, and water-extractable sulfate. MeHg concentrations were differentially controlled by MeHg production and degradation processes, depending on whether plants were present. In non-vegetated boxes, MeHg concentration was controlled by K_{meth}, as evidenced by a strong and positive correlation, while K_{demeth} had no relation to MeHg concentration. These results indicate methylation was the dominant driver of MeHg concentration in non-vegetated soil. In vegetated boxes, K_{demeth} strongly and negatively predicted MeHg concentration, indicating that demethylation was the dominant control in soil with plants. MeHg concentration, K_{meth}, and % MeHg all had significantly less variance in vegetated than in non-vegetated soils due to a consistent elimination of greater values. This pattern suggests that reduced MeHg production capacity was a secondary control on MeHg concentrations in vegetated soils. Importantly, we observed no difference in the magnitude or variance of K_{demeth} between treatments, suggesting that demethylation was robust to soil chemical conditions influenced by the plant, perhaps because of a wider taxonomic diversity of demethylators. Our results

suggest that methylation and demethylation processes could both be leveraged to alter MeHg concentrations in rice paddy soil.

INTRODUCTION

Methylmercury (MeHg) is an organic, bioavailable form of mercury (Hg) which bioaccumulates in a manner more characteristic of an organic than a metallic pollutant (Harris et al., 2003), reaching levels that threaten the health of human consumers in foods that originate from aquatic environments (Driscoll et al., 2013; Rothenberg et al., 2014). Rice represents an important route of MeHg consumption worldwide (M. Liu et al., 2019), and is the major exposure route of MeHg for some rice-dependent populations (Aslam et al., 2020; Bose-O'Reilly et al., 2016; M. Liu et al., 2019; Rothenberg et al., 2021; Wang et al., 2020). Given that rice is the staple food of more than half the world's population (Prasad et al., 2017), MeHg-contaminated rice is a significant emerging public health threat (M. Liu et al., 2019; Rothenberg et al., 2014).

The MeHg in rice grain is absorbed from the soil by the plant during vegetative growth and subsequently translocated to the developing grain (Meng et al., 2011; Strickman and Mitchell, 2017; Tang et al., 2020). Soil MeHg concentrations are thus a major driver of rice MeHg burdens (Meng et al., 2011, 2010; Strickman and Mitchell, 2017), and reducing the MeHg concentration of the rice paddy soil itself is an attractive potential mitigation strategy (Rothenberg et al., 2014; Tang et al., 2019b). In aquatic soil systems MeHg concentration is governed primarily by the creation (methylation) and degradation (demethylation) of MeHg (Beckers and Rinklebe, 2017; Lin et al., 2012; J. Liu et al., 2019; Meng et al., 2011), both of which are primarily biotic processes in rice paddy soils (Zhao et al., 2016; Zhou et al., 2020). Both are influenced by multiple biogeochemical factors including microbial community structure and activity, redox conditions, supply of electron donors and acceptors, quantity and quality of organic carbon, temperature, pH, and bioavailability of divalent mercury (Hg (II)) for methylation (Beckers and Rinklebe, 2017; Ullrich et al., 2001).

Existing management approaches are based on an understanding of the MeHg cycle that was derived primarily from studies of natural freshwater wetlands (Paranjape and Hall, 2017)**.** An unusual feature of rice paddies, in comparison to natural wetlands, is the cyclical planting and removal of vegetation during the cultivation process (Vergara, 1992). Plant presence and absence has the potential to markedly alter MeHg concentrations in soil. A major route of this alteration is the leakage of organic carbon compounds from roots, a process known as root exudation (Kuzyakov and Domanski, 2000). This organic carbon fuels the activity of mercury methylators, leading to higher rates of MeHg formation and MeHg levels in natural freshwater wetlands (Sun et al., 2011; Yin, 2020). Oxygen leakage from plant roots, known as radial oxygen loss (ROL) regenerates the redox-sensitive electron acceptors of microbial sulfate- and iron-reducing microbial groups (Li and Wang, 2013), both of which contain species that are known to methylate Hg in the environment (Compeau and Bartha, 1985; Fleming et al., 2006; Kerin et al., 2006; Yu et al., 2012), including rice paddies (Liu et al., 2014, 2013; Su et al., 2016; Zhao et al., 2020, 2016). In addition, vegetated soils may have more active sulfur and iron cycles due to transpiration-driven movement of oxygenated surface water into the soil profile (Bachand et al., 2014; Ma et al., 2019; Rothenberg and Feng, 2012). Oxygen availability, however, can also reduce the habitat available for freshwater Hg methylators, which are currently believed to consist of taxa that are obligate anaerobes (Gilmour et al., 2013; Podar et al., 2015); demethylators include both aerobic and anaerobic taxa (Li and Cai, 2012; Marvin-DiPasquale et al., 2000). Non-vegetated soil lacks these influences and is characterized by lower organic carbon supply, less dynamic redox conditions, and more complete anoxia (Reddy and DeLaune, 2008).

Despite the potential of rice plants to affect methylation and demethylation, however, knowledge gaps remain about the degree of influence of rice plants on both processes. Existing work has focused on MeHg formation. A comparison of vegetated and de-vegetated paddy treatments found that plots with rice plants had 5—660% greater MeHg concentrations in soil as well as elevated K_{meth} (Ma et al., 2019; Windham-Myers et al., 2009, 2014b). Ma et al. 2019 found that rice rhizosphere soil (soil strongly influenced by plant roots) had 5—50% more MeHg than bulk soil (soil beyond the influence of plant roots) at some field sites. Rothenberg & Feng (2012) observed 70% greater MeHg concentrations in porewater of vegetated paddy versus fallow fields. However, this pattern is not consistent between all studies. Zhao et al (2018) observed no significant differences between soil with and without rice plants in some treatments. Other authors have found a significant decrease in soil MeHg concentration (Ma et al., 2019) or the percent of mercury present as MeHg in soil with plants (%-MeHg; (Strickman and Mitchell, 2017).

Importantly, none of these studies investigated the potential role of demethylation in modulating the plant influence on soil MeHg. This is an important omission, since the final MeHg concentration in soil depends on both methylation and demethylation; without measuring both processes in the same environment, it is impossible to know whether MeHg formation or degradation is a more dominant influence on MeHg concentrations. Demethylation does occur in rice paddy soils, where it appears to be an important control on MeHg concentrations (Zhao et al., 2016), and is facilitated by several taxonomic groups with unknown ecology, or ecology that differs greatly from that of methylators (Zhou et al., 2020). Demethylation does respond to the species identity of plants growing in natural wetlands (Tjerngren et al., 2011) and in agricultural vs. natural wetlands (Windham-Myers et al., 2014a), although neither of these studies compared soil with plants to soil without plants. The response of demethylators to the presence vs. absence of plants may differ from that of methylators, but no evidence on this topic currently exists. This subject is an important gap in our basic understanding of mercury cycling in freshwater wetlands, as well as in the specific environment of rice paddy soils. To date, no study has simultaneously compared methylation and demethylation in response to plant presence in freshwater environments.

Here, we add knowledge about methylation and demethylation in paddy soil with and without rice plants. We compared MeHg dynamics in California paddy soil microcosms (rhizoboxes) with rice plants to identical rhizoboxes without plants. At tillering (the peak of vegetative growth), we collected naturally present (ambient) MeHg and inorganic Hg (IHg; total Hg minus MeHg) concentrations from soil, ancillary soil biogeochemical parameters, and assessed methylation and demethylation potential rates using stable isotope tracers in order to a) provide the first simultaneous characterization of methylation and demethylation in soil with and without rice plants, and b) to explore the influence of rice plants themselves on these two processes.

MATERIALS AND METHODS

SOIL COLLECTION AND RICE GERMINATION

Six vegetated and six non-vegetated rhizoboxes were maintained alongside one another in growth chambers at the Center for Urban Horticulture at the University of Washington. Soil was collected in

October 2017 from a rice paddy field at the University of California Davis, Sacramento, California, in the Yolo Bypass rice cultivation area. Soil storage and preparation are described in Supplementary Text 1.

A short season California rice cultivar, M-206, was selected because it had been used in previous investigations of metal behavior in the rice paddy system (Farhat et al., 2021; Muehe et al., 2019). Rice seeds were disinfected in a dilute (2%) hydrogen peroxide and distilled water solution, then transferred to an aerated, warmed container of tap water for five days. Live seeds, identified by the presence of a mesocotyl, were transferred to sterile 2% agar in autoclaved growth boxes, and maintained at 100% humidity for three weeks until plants were c. 20 cm high.

RHIZOBOX CONSTRUCTION, PLANTING, AND PLANT MAINTENANCE

Rice seedlings were transferred to soil-filled rhizoboxes approximately twenty days after germination. Six replicates of each treatment (vegetated and non-vegetated) were prepared in broad, flat, watertight boxes (rhizoboxes) fitted with removable facings to allow access to the soil. Rhizoboxes (25 x 5 x 50 cm inner dimensions) were constructed from rigid PVC boards; facings were constructed of either opaque PVC board, which excluded light, or of transparent polycarbonate facings to allow visual inspection of root colonization. Light was excluded from the boxes fitted with polycarbonate facings using opaque fabric covers. Before use, rhizoboxes and facings were soaked in dilute (10%) bleach ($NaClO$) for 48 hours, rinsed with tap water, and air dried. The dimensions of the rhizoboxes were chosen in order to ensure that the entire soil volume was colonized with roots, while still allowing rice plants enough soil volume to meet their metabolic and water needs. Replicate number was selected based on estimation of the variability between replicates to be expected when working in these specific growth chambers (Rho et al., 2020).

Vegetated and non-vegetated rhizoboxes were assembled in a random order working over two consecutive days. The assembly of each rhizobox took approximately 20 minutes, minimizing stress to the rice seedlings; to further reduce transplant shock, all components were warmed to c. 20° C in the days before planting. Each rhizobox was filled with soil slurry up to the 30 cm mark, levelled, and tapped vigorously to eliminate air bubbles. For vegetated replicates, eight rice seedlings were carefully freed from the agar and laid atop the soil surface. A 2 mm film of gas-tight vinyl (TAP Plastics, Seattle, Washington) was then laid over the surface of the soil, which reduced the intrusion of oxygen during isotope injections (Supplementary Text 2). The edges of this plastic film were secured to the rhizobox with low-VOC silicone and allowed to dry. A generous strip of plumber's putty was then placed on the plastic film to create a watertight seal with the polycarbonate or PVC facings, which were screwed into the body of the rhizobox (Figure 1a, Supplementary Text 1). To maintain anaerobic soil conditions and supply nutrients to the growing plants, all rhizoboxes were watered with dilute 10% Hoagland solution prepared using DI water (Hoagland and Arnon, 1950) until a 2 cm layer of solution covered the soil surface. Immediately after assembly, all rhizoboxes were transferred to the growth chambers, where they were maintained in the following conditions: relative humidity of at least 80%, 16 hours light/8 hour dark cycle with PAR (photosynthetically active radiation) of at least 500 $\mu mol/m^2$, and temperature 28° C day/25° C night.

After planting, rhizoboxes were equilibrated for two weeks, allowing rice plants to establish and extend their leaves above the rim of the rhizobox. At this point, the overlying water level was raised to flood the space between the vinyl film and the rigid facings, creating a water seal, and rhizoboxes were inclined forward at a 45° angle to encourage roots to grow against the vinyl surface. From this point forward, additions of dilute 10% Hoaglands solutions were made twice weekly to maintain continuous fully flooded conditions. After three weeks, seedlings were thinned to six per replicate. The rhizoboxes were then maintained until the rice plant roots had fully colonized the soil profile, approximately two months after planting. By this point, all plants had reached tillering, a growth stage characterized by the formation of lateral shoots and representing the peak of vegetative growth (Vergara, 1992).

METHYLATION AND DEMETHYLATION ASSAYS

Fifty-four days after planting, methylation (K_{meth}) and demethylation (K_{demeth}) potential rate constant measurements were determined. Both of these assays were made using the method of Hintelmann et al. 2000. In brief, an aqueous injectate enriched with a Hg isotope in the appropriate species was added to the soil with the minimum of disturbance. ^{200}Hg(II) (enriched to 88.07% ^{200}Hg) was used for K_{meth} assays, while 96.17% enriched Me^{201}Hg was used for K_{demeth} assays. To prepare the injectate solution, both isotope stock solutions were diluted and mixed in overlying rhizobox water filtered through a 0.2 µM glass filter, and equilibrated at room temperature for an hour to allow Hg speciation to approximate the native chemistry (Mitchell and Gilmour, 2008). To access the soil, the overlying water was drained, the rigid facings removed, and each rhizobox was laid flat to expose the vinyl-covered soil surface. Each injection of isotope was then made with a gas-tight syringe through a single central aperture in the vinyl, and delivered approximately 47 ng/g ^{200}Hg (II) and 2.5—3.5 ng/g of Me^{201}Hg to the soil. The needle was manipulated in a circular motion to disperse the isotopes evenly around a 3.2 cm diameter, 1 cm thick cylinder of soil. After the injection of isotopes, the rhizobox was then maintained for a carefully-measured duration of exactly 8 hours, allowing the native microbiome to methylate or demethylate the newly-added tracer isotope. To ensure that measurements were not impacted by oxygen from the former soil-water interface, K_{meth} and K_{demeth} assays were conducted in the strip of soil that was between 5 and 15 cm from the top of the soil profile. In vegetated rhizoboxes, this soil compartment was densely colonized with roots. A schematic of the process is presented in Figure 1b and additional information on measures taken to prevent oxygen intrusion during isotope placement and incubation is provided in Supplementary Text 3. All boxes were maintained at the same temperature (approximately 22° C), and vegetated rhizoboxes were illuminated with fluorescent lights positioned directly over the lidded boxes in order to support continued photosynthetic activity by the plants during incubations; these lights were on a 16-hour day 8-hour night cycle and delivered a PAR level approximating that of the growth chambers. After incubation, the assay was halted by freezing the collected soil in liquid nitrogen and the time recorded. Soil samples were removed from the liquid nitrogen bath and stored frozen at -20° C until they were lyophilized, homogenized, and stored in the dark until analysis.

K_{meth} assays were performed by comparing the amount of Me^{200}Hg formed over the course of the incubation to the remaining I^{200}Hg (a proxy for ^{200}Hg(II). K_{demeth} was assessed by measuring the amount Me^{201}Hg remaining at the end of the experiment and comparing it to the T^{201}Hg concentration at the end of the experiment. The T^{201}Hg concentration was a proxy for the total amount of Me^{201}Hg added at the beginning of the incubation, since the ^{200}Hg(II) solution was of very high purity and thus all of the ^{201}Hg species added were in the methylated form at the beginning of the assay. K_{meth} and K_{demeth} could then be calculated using the equations from Drott et al. 2008:

$$K_{meth} = [Me^{200}Hg]_{t24}/([T^{200}Hg]*t)$$

$$K_{demeth} = -Ln([Me^{201}Hg]_{t24}/[T^{201}Hg])/t$$

Ambient MeHg, as well as Me^{200}Hg and Me^{201}Hg concentrations that are directly applicable to the added enriched isotopes (i.e., in excess of natural abundance) were simultaneously analyzed using isotope dilution-gas chromatography-ICPMS (Hintelmann et al., 2000). Ambient THg concentrations, as well as the concentrations of $T^{200}Hg$ and $T^{201}Hg$ that are directly applicable to the added enriched isotopes were analyzed using isotope dilution-cold vapour-ICPMS as described in Strickman and Mitchell 2017. Additional information on sample preparation is available in Supplementary Text 3. The K_{meth} and K_{demeth} values obtained in this way do not necessarily capture rates of transformation of ambient MeHg and IHg species, because the tracer has a higher bioavailability than ambient species (Hintelmann et al., 2002). Therefore, K_{meth} and K_{demeth} are properly referred to as potential rates constants. Quality assurance and quality control (QA/QC) measurements were collected during Hg and MeHg determinations to ensure that the accuracy and precision of the instruments fell within acceptable ranges. QA/QC measurements included blanks, duplicates, recovery of standard reference materials, and calculation of minimum detection limits (MDLs) for each isotope-specific Hg species of interest. These data are available in Supplementary Table 1.

ANCILLARY SOIL ANALYSES

Using archived freeze-dried soil, we determined the soil organic matter percentage and the concentrations of total sulfur (S) and water-extractable sulfate. Soil organic matter was measured using combustion at 550º C for four hours. Sulfur and sulfate analyses were conducted at the University of Washington School of Environment and Forestry Analytical center. For total sulfur analyses, soils were digested using EPA-3050 and analyzed using EPA 200.7 on a Thermo-Scientific 6300 ICP spectrometer. For sulfate analyses, samples were water-extracted before being run on a Dionex DX-120 ion chromatograph using EPA method 300.0.

Estimation of root carbon exudation rate

Preliminary data analysis indicated that root exudation rate might help explain experimental results. We conducted another experiment in the summer of 2018 to estimate the rate of root exudation from three M-206 rice plants grown under identical growth conditions. The rate of root exudation of total carbon was estimated at the tillering phase using the calcium sulfate incubation method of Aulakh et al. (2001), described in Supplementary Text 4.

STATISTICAL ANALYSES

All variables were assessed for normality using q-q plots, frequency distribution histograms, and comparison of mean and median values, and transformed if necessary, using log, natural log, or square root transformations to approximate a normal distribution. Treatment level comparisons of means were made using the Welch's T-Test at $\alpha \leq 0.05$, which compares the means between two populations but does not rely on an assumption of equal variances (Delacre et al., 2017). To search for statistically significant differences in the variance of the data, we used Levene's test, again at $\alpha \leq 0.05$. The Levene's test assesses whether two samples come from populations with the same variance (Glass, 1966), and can be used as a stand-alone analysis to explore differences in two datasets in terms of their variance. (Nordstokke et al., 2019). Correlative relationships between individual variables were explored using a Pearson correlation matrix based on normalized values at $\alpha \leq 0.05$. Statistical analysis was conducted with R version 3.6.2 with RStudio 1.5.5019 (R Core Team, 2016). For all tests, n = 6.

RESULTS

AMBIENT INORGANIC MERCURY AND METHYLMERCURY

Ambient IHg concentrations ranged between c. 44.9—63.1 ng/g. Inorganic Hg concentrations did not differ between the vegetated and non-vegetated rhizoboxes (T-test p = 0.66, Levene's p = 0.81; Figure 2).

Ambient MeHg concentrations were low but detectable in all samples, ranging between 0.01—0.12 ng/g in vegetated and 0.07—0.25 ng/g in non-vegetated rhizoboxes. While mean soil MeHg concentrations did not differ between treatments based a T-test (T-test p = 0.37), we did observe a significant difference in the variance of the MeHg concentrations from the two treatments (Levene's p-value = 0.0003). A visual inspection of the data indicated that this outcome was due to a narrowed range of MeHg concentrations in the vegetated treatment, as well as an elimination of greater MeHg concentrations in this treatment in comparison to the distribution of MeHg concentrations in the non-vegetated treatment (Figure 2).

MERCURY METHYLATION AND DEMETHYLATION POTENTIAL RATES CONSTANTS AND METHYLMERCURY CONCENTRATIONS IN VEGETATED AND NON-VEGETATED SOILS

Methylation and demethylation potential rates constants and %-MeHg

The potential rate constant for mercury methylation (K_{meth}) was low but detectable in all replicates (mean ± standard deviation 0.011 ± 0.008 day^{-1} in vegetated, vs. 0.014 ± 0.066 day^{-1} in non-vegetated rhizoboxes). Similar to soil ambient MeHg concentrations, there was no difference in mean K_{meth} values between vegetated and non-vegetated treatments (T-test p = 0.37) but the variance in K_{meth} values was significantly different between the two treatments (Levene's p = 0.009). As with MeHg concentrations, visual inspection of the potential rate data (Figure 2) indicated that this result was due to an elimination of higher K_{meth} values in the vegetated treatment relative to the non-vegetated treatment. Mercury demethylation was also observed in all samples, with K_{demeth} values that ranged between 0.93 and 2.08 day^{-1}. These values are in the same range as the only other data available on mercury demethylation rates rice paddy soils (0.1—1.5 day^{-1}), which were obtained from plots within Hg-contaminated cultivated paddy fields in China (Zhao et al., 2016). Neither the mean nor variance in K_{demeth} differed between treatments (T-test p-value = 0.99, Levene's p-value = 0.29; Figure 2).

In vegetated boxes, average %-MeHg values were approximately two thirds (0.12 ± 0.03 %) of those in non-vegetated boxes (0.26 ± 0.13 %). There was a statistically significant difference in the variance of %-MeHg values between the two treatments (Levene's p-value = 0.004), with a visual pattern matching that seen in MeHg concentration and K_{meth} data: smaller variance and elimination of greater values in the vegetated treatment relative to the non-vegetated treatment.

Pearson correlations of all Hg variables revealed no correlation between K_{meth} and any other Hg variable in the vegetated boxes. In the non-vegetated boxes, by contrast, K_{meth} was strongly and positively correlated with both soil MeHg concentration (r = 0.85, p = 0.033; Figure 4) and %-MeHg (r = 0.88, p = 0.022). K_{demeth} had a significant negative relationship with both %-MeHg and soil MeHg concentrations in vegetated boxes only (%-MeHg: r = -0.82, p = 0.044, MeHg concentration: r = -0.84, p = 0.038). There was no relationship between K_{demeth} and any Hg variable in the non-vegetated boxes.

Ancillary soil analyses and root exudation rates

Sulfur content in the soil ranged between 253 and 355 µg/g (Figure 2). Sulfur concentrations in vegetated soils were slightly lower than in non-vegetated soil (275 ± 26 µg/g vs. 305 ± 47 µg/g), but this pattern was not significant in terms of either mean (T-test, $p = 0.22$) or variance (Levene's $p = 0.22$). Soil sulfate ranged between 2.10 ± 0.47 µg/g in vegetated and 2.10 ± 0.31 µg/g in non-vegetated respectively. Sulfate did not differ between treatments (T-test, $p = 0.89$, Levene's $p = 0.36$). Soil organic matter percentages were 7.45 ± 0.17 % in the vegetated boxes and 7.23 ± 0.19 % in non-vegetated boxes. Organic matter % (OM-%) was elevated in the vegetated treatment (Figure 2), but this pattern was slightly below the threshold of significance (T-test $p = 0.065$). Soil organic matter % did not differ in terms of variance (Levene's $p = 0.79$). Root exudation rates at tillering were 2.54 ± 2.02 mg C/plant/day ($n = 3$).

Soil total sulfur concentrations displayed a significant positive relationship with K_{meth} rates only in vegetated boxes ($r = 0.90$, $p < 0.01$; Figure 4). To explore the effect of S on the bioavailability of IHg for methylation, we compared the IHg/total S ratio to MeHg, %-MeHg, K_{meth}, and K_{demeth} (Hg variables) There was a significant negative relationship between the Hg/S ratio and %-MeHg only in vegetated boxes ($r = - 0.81$, $p = 0.049$). No relationships were found between any Hg variable and S or Hg/S ratio in the non-vegetated boxes. Sulfate did not display a correlation between any Hg variable in either treatment. Organic matter % had no correlations with any Hg variable in the vegetated treatment, but in the non-vegetated boxes, OM-% correlated negatively with both %-MeHg ($r = -0.84$, $p = 0.037$) and K_{meth} ($r = -0.84$, $p = 0.036$).

Discussion

Soil mercury Speciation and Transformation Rates

Observed soil ambient IHg levels were 2—3 times higher than the average aquatic (Fleck et al., 2016) or terrestrial (Obrist et al., 2016) IHg concentrations in Western North America, reflecting the history of 19th century gold mining contamination of the region where the paddy soil was obtained (Donovan et al., 2016). While the IHg levels in this study were lower than anticipated (100—300 ng/g) based on study sites located <10 km away (Marvin-DiPasquale et al., 2014), they do concur well with rice paddy sites in the upstream area of the Yolo Bypass located north of where our study soil was collected (57 ± 4 ng/g) (Tanner et al., 2018). This heterogeneity of IHg soil burdens within closely located field sites reflects the multiple sources of eroded soil to the Yolo Bypass, which differ in their IHg concentrations (Donovan et al., 2016). As expected, IHg concentrations were indistinguishable between the vegetated and non-vegetated rhizoboxes, indicating that the experimental soil was well homogenized and that gross differences in total IHg substrate did not underlie observed treatment-level patterns in MeHg dynamics within the context of our experiment. Although methylation and demethylation were both occurring in this system, which was confirmed by the isotope tracer measurements, these processes acted upon less than 1% of the total mercury in the system (Figure 2) and did not create a significant difference in the pool of IHg between treatments.

The soil MeHg concentrations in this experiment (0.068—0.25 ng/g) were approximately half of those from rice paddies within a 10 km radius of our soil-collection area (0.60—2.24 ng/g (Marvin-DiPasquale et al., 2014; Windham-Myers et al., 2009, 2014b). However, these locations also had IHg concentrations that were 4—6 times higher than those measured in our experimental soil. Taken together, our soil MeHg and IHg concentrations were comparable to the nearby, less-contaminated site investigated by Tanner et al. (2018) (MeHg 1.0 ± 0.2 ng/g; IHg 57 ± 2.0 ng/g). Furthermore, the %-MeHg values of most previous studies (0.6 — 0.2%) in the region are comparable to those found here (0.11—0.42%) (Windham-Myers et al., 2009, 2014b) . We therefore conclude that limited IHg supply, rather than unrealistically low K_{meth} values, helps explain the low MeHg concentrations in our experimental boxes.

Mercury methylation potential rate constants in this study (0.006—0.030 day^{-1}) were within the lower range of those observed in both working and experimental rice paddies in California (0.009—0.170 day^{-1}) (Marvin-DiPasquale et al., 2014; Windham-Myers et al., 2009, 2014b) and were substantially higher than those found in Chinese rice paddies (0.0002—0.0014 day^{-1}; L. Zhao et al., 2016). K_{demeth} was detected in all replicates (0.93—2.1 day^{-1}); these values were on the higher end of K_{demeth} measured in Hg-contaminated, vegetated rice paddy soils in China (0.1—1.5 day^{-1}) (Zhao et al., 2016); the only other published study, to our knowledge, in which K_{demeth} was measured in paddy soil. While microbial mercury demethylation is an important process controlling MeHg concentrations in the environment, it unfortunately receives less attention than mercury methylation, due to analytical challenges including the preparation of the MeHg isotope tracer solution and the wide range of potential rates, which makes it difficult to estimate the required amount of demethylation tracer. Our contribution of both K_{meth} and K_{demeth} values collected simultaneously is the first such dataset obtained from California rice paddies, and allowed us to compare the relative methylation and demethylation rates constants between California and Chinese rice paddies. It is interesting to note that the ratio of mean K_{demeth} to K_{meth} rates in vegetated soil in our study (127) was much lower than the ratio obtained in China by Zhou et al. 2016 (1341). While preliminary, this observation suggests a greater proportional importance of demethylation in the Chinese sites.

METHYLMERCURY PRODUCTION IN SOILS WITH AND WITHOUT PLANTS

The presence of plants altered the functioning of the MeHg cycle in terms of the importance of methylation or demethylation to the final soil MeHg concentration.

Methylation was a driver of soil MeHg concentrations in both treatments, but its importance varied between soil with and without plants. The importance of methylation in non-vegetated soils was clearly evidenced by a positive correlation between soil MeHg concentration and both K_{meth} and %-MeHg. In vegetated soils, while there was not a significant correlation between MeHg and K_{meth} or %-MeHg, we did observe a marked concurrence between these variables in terms of a significantly compressed variance and reduced upper ranges of values (Figure 2). These results show statistically that the presence of plants lowered the variability of MeHg production, and suggest that MeHg production was a control on final MeHg concentrations in vegetated soils, although the relationship was not as clear as in non-vegetated soils. In contrast, demethylation was a driver of soil MeHg concentration only in vegetated soils. In the vegetated treatment, we observed a significant negative correlation between K_{demeth} and both MeHg concentration and %-MeHg. These relationships were absent in non-vegetated soils.

These results indicate that in soils with plants, demethylation was the clearest control on MeHg concentration, and that methylation was a secondary control. In soil without plants, the rate of Hg methylation was the most dominant determinant of soil MeHg burdens. These differential controls on MeHg concentrations between vegetated and non-vegetated treatments appeared to be a result of plants reducing the variance and highest rates of methylation, while exerting less of an effect on demethylation.

Methylation

Influence of plants on IHg bioavailability for methylation

The availability of sulfur may help explain the differences in MeHg concentrations and production in vegetated compared to non-vegetated soil. Total sulfur concentration correlated positively with K_{meth} in vegetated soils only, but sulfate showed no relationship with K_{meth} or MeHg in either treatment. This result argues against sulfate-limitation of sulfate-reducing Hg methylators. Rather, we observed tentative evidence that sulfur supply affected K_{meth} by mediating the bioavailability of IHg for methylation. While high concentrations of sulfide can reduce IHg bioavailability by precipitation (Hsu-Kim et al., 2013; Skyllberg et al., 2006) sulfide-Hg nanoparticles formed in the presence of low-molecular-weight dissolved organic matter are actually more bioavailable for methylation than other Hg species (Pham et al., 2014; Zhang et al., 2012). We observed a significant negative correlation between %-MeHg and the Hg:S ratio in vegetated soils only. Percent MeHg is a long-term, integrated measure of the methylation capacity of a system based on Hg and MeHg species with realistic bioavailabilities for transformation, in contrast with K_{meth} which represent a short-term snapshot of the process based on a high-bioavailability Hg(II) tracer (Hintelmann and Evans, 1997). Therefore, the fact that %-MeHg declined as the Hg:S ratio increased (i.e. as the supply of S declined relative to Hg) suggests that the bioavailability of Hg for methylation depended on a relatively ample supply of sulfur. This correlation was not apparent in the non-vegetated soils, suggesting a plant-mediated role that should be further explored.

Preliminary evidence of limitations on IHg bioavailability were also observed in the non-vegetated treatment. Soil organic matter content correlated significantly with $K_{meth,}$ but the effect was negative, and observed in the non-vegetated treatment only, suggesting that binding of IHg to stable organic carbon compounds may have helped modulate the methylation process (He et al., 2019). The relationship of IHg bioavailability to organic carbon supply is relatively complex. While low-molecular weight, easily decomposed (labile) organic carbon molecules can increase methylation by supporting the metabolism of Hg methylators, high-molecular-weight organic matter particles with many thiol binding sites complex with Hg and reduce its bioavailability for methylation (He et al., 2019; Ravichandran, 2004). The organic matter pool in the non-vegetated boxes lacked the input of labile organic carbon from plant root exudation or decomposing biomass, reflected in the borderline-significant reduced OM-% of non-vegetated soils (T-test $p = 0.065$). Our results suggest that, in the absence of plants, soil organic carbon content can exert the opposite effect, and reduce K_{meth}. A similar result has been found in marine sediments (Hammerschmidt et al., 2008), and, interestingly, Ma et al. 2019 detected differences in the predictive power of OM-% on soil MeHg between bulk and rhizosphere paddy soil. We speculate that the lack of relationship between OM-% in the vegetated treatment may be related to the supply of labile organic carbon from root exudates and decomposition, thus offsetting the binding effect of the background soil OM. Taken together, these findings suggest that soil OM-% may have different effects on K_{meth} and MeHg in vegetated vs. non-vegetated soils.

Lack of a plant-related stimulation of mercury methylation

Our result that soil MeHg concentration and K_{meth} values had less variance and reduced upper values in vegetated soils relative to non-vegetated soils contrasts with several previous studies, which have observed MeHg soil concentrations up to 600% higher in vegetated compared to non-vegetated soils or in rhizosphere compared to bulk soil (Ma et al., 2019; Sun et al., 2011; Windham-Myers et al., 2009, 2014b; Zhao et al., 2018). We offer two potential explanations for these different results. Firstly, a relatively low supply of organic carbon from the M-206 rice plants may explain the absence of a plant-related stimulation of MeHg production in this project. Our measured exudation rate for this cultivar was 2.54 mg C/plant/day, which is at the lower end of rice root exudation rates collected using comparable methods (c. 4.4—14 mg C/plant/day) (Aulakh et al., 2001). Observational and experimental studies have attributed the MeHg stimulation effect in rice paddy soil to the leakage of organic carbon from rice roots, which enhances the activity of the Hg methylating flora (Liu et al., 2013; Wang et al., 2015; Windham-Myers et al., 2009; Zhao et al., 2018). However, the carbon stimulation effect may be dependent on the degree of carbon exudation. In a pot study by J.-Y. Zhao et al (2018) comparing MeHg concentration changes over time in the soil around two varieties of rice, only the rice variety with a high estimated exudation rate stimulated MeHg production relative to a non-vegetated control, while the low-exudate cultivar had final soil MeHg levels that were on average c. 40% lower than the control (Zhao et al., 2018). J.-Y. Zhao et al. attributed this result to the fact that the root exudation rate for the low-exudation variety that was approximately 1/3 that of the high-exudation variety. We posit that the absence of a MeHg-stimulation effect in the vegetated boxes of our experiment was at least partly explained by the low root exudation rates of the M-206 plants. Our results combined with those of J.-Y. Zhao et al. (2018) indicate that the magnitude of organic carbon exuded from plants may be more significant in modulating mercury methylation than previously realized.

Secondly, the densely rooted nature of the vegetated boxes could have reduced the ability of the experimental plants to draw Hg species from bulk soil into rhizosphere soil. As plants transpire, solutes are drawn towards the roots in the transpiration-driven stream of soil water. This transpiration-driven flow brings distally produced MeHg, and possibly also bioavailable IHg, from the bulk soil into the rhizosphere soil, representing a subsidy to the soil near plant roots (Windham-Myers et al., 2009, 2014b). This Hg subsidy could obscure the endogenous rates of MeHg methylation and demethylation in the rhizosphere. While we cannot confirm that the entirety of each vegetated rhizobox within our study was truly rhizosphere soil, soil profiles were very densely colonized by roots, and densely rooted containers have previously been used by other researchers to simulate rhizosphere soil (Regier et al., 2012; Shuman and Wang, 2008; Sun et al., 2011; Wang et al., 2014). The dense rooting could have eliminated any possible Hg subsidy from the bulk soil, which may have allowed demethylation to degrade a greater proportion of the MeHg in soil around plant roots. Supporting this possibility, studies that eliminated transpiration-driven flow between bulk and rhizosphere soil through the use of fully separated and densely rooted containers observed no significant differences (Regier et al., 2012), or even decreases of up to 40% for some treatments (J.-Y. Zhao et al., 2018) in soil MeHg concentration with plants compared to soil without plants. In comparison, the majority of the studies which observed a stimulation of MeHg by the presence of plants (Ma et al., 2019; Sun et al., 2011; Windham-Myers et al., 2009, 2014b; Yin, 2020) took place in experimental or field conditions that maintained a physical connection between bulk and rhizosphere soil.

Demethylation

In sharp contrast to methylation, demethylation showed little response to the presence of plants; to the best of our knowledge, this is the first exploration of Hg demethylation in soil with and without plants in a freshwater environment. In wetland systems, biotic demethylation is a relatively generalist process that is effected by multiple functional groups spread across a wider taxonomic distribution than mercury methylation (Boyd and Barkay, 2012; Li and Cai, 2012; Marvin-DiPasquale et al., 2000). Mercury demethylation in anoxic, low-MeHg environments, including rice paddies, is largely carried out through oxidative demethylation (Marvin-DiPasquale et al., 2000; Zhou et al., 2020), a process that degrades MeHg to Hg^{2+}, CO_2, and CH_4 (Marvin-DiPasquale and Oremland, 1998; Oremland et al., 1991). The ecology of oxidative demethylators is poorly understood, but the process is known to be carried out in other environments by anaerobic sulfate reducers, methanogens (Oremland et al., 1991), and methanotrophs (Lu et al., 2017). In addition, a wide taxonomic distribution and functionally diverse consortia of mercury demethylators has recently been identified in rice paddy soil, including representatives of Catenulisporaceae, Frankiaceae, Mycobacteriaceae, and Thermomonosporacea (Zhou et al., 2020). Our results suggest that this functional and taxonomic diversity allowed the mercury demethylating microbial consortia to operate equally well in both vegetated and non-vegetated soils.

Despite the non-significant difference in K_{demeth} between the two treatments, there was a significant and negative relationship between K_{demeth} and MeHg soil concentrations as well as K_{demeth} and %-MeHg in vegetated soils. These negative correlations suggest that MeHg degradation was a controlling factor for rhizosphere soil MeHg concentrations; this finding is in concurrence with the only previous study of demethylation in rice paddy soil, which found that K_{demeth} helped predict soil and grain MeHg burdens (Zhao et al., 2016). Similar correlations were not present in soils without plants.

Our results indicate that demethylation is a potentially valuable mechanism for reducing MeHg accumulation in the rhizosphere. The robustness of K_{demeth} to the biogeochemical patterns that affected K_{meth} suggests that management interventions that reduce K_{meth}, such as limiting organic carbon supply or reducing sulfur over-fertilization (Liu et al., 2016; Tang et al., 2019b), may have little to no impact on K_{demeth}. Further studies are needed to determine whether K_{demeth} in rice paddy soils is rapid or slow in comparison to other environments, and how much variability exists in the rate of this process under different agricultural approaches, in different geographical regions, and in response to different climates. A better understanding of these questions would determine the feasibility of agricultural strategies to increase demethylation in paddy fields.

CONCLUSION

Our results emphasize that the presence of plants does not have a uniformly stimulatory effect on Hg methylation. Rather, it is likely that the plant MeHg-stimulation effect is dependent upon the degree of organic carbon exudation from plant roots, plant impacts on sulfur speciation and cycling, the degree of transpiration-driven flux of Hg species from bulk soil into rhizosphere soil, and potentially other biogeochemical characteristics of the rhizosphere. Interestingly, demethylation occurred at similar rates in both planted and unplanted soils, but controlled MeHg content in planted soils only. The effects of rhizosphere biogeochemical gradients on mercury methylation and demethylation should be further explored, with an emphasis on factors—such as root exudation—that vary significantly between rice cultivars in order to identify rice cultivars that support lower rates of methylation or higher rates of demethylation in the soil surrounding their roots.

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FIGURE CAPTIONS

Figure 1: Schematic diagram of construction (A) and isotope placement and recovery procedures (B). A: rhizobox with rice paddy soil, rice plants, overlying water layer, and vinyl and rigid polycarbonate facings. B: methylation and demethylation assay workflows.

Figure 2: Left panel, from top to bottom; comparison of ambient IHg concentration, ambient MeHg concentration, %-MeHg, and mercury methylation potential rates constants (K_{meth}). In title, a denotes a non-significant result, while b denotes significance at α of 0.05. Pairs denote Levene's test result followed by T-test result. Right panel, from top to bottom; mercury demethylation potential rates constants (K_{demeth}), total sulfur, sulfate, and organic matter contents of soil. All charts present a comparison of vegetated (n = 6) to non-vegetated (n = 6) rhizoboxes. The shaded area of each figure represents the 75th to 25th interquartile range (IQR); the central line represents the median; whiskers represent the highest data value that falls below 1.5x the IQR. Stars represent data falling beyond 1.5x the IQR. The same information is available in a screen-reader accessible tabular format in Supplementary Table 2.

Figure 3: Pearson correlations between Hg variables for non vegetated (left) and vegetated (right) soil treatments. Pearson correlation r and p values are displayed beneath each plot. Points represent transformed data.

Figure 4: Pearson correlations between Hg variables and ancillary variables for non vegetated (left) and vegetated (right) soil treatments. Pearson correlation r and p values are displayed beneath each plot. Points represent transformed data.

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