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Warming inhibits Hg^{II} methylation but stimulates methylmercury demethylation in paddy soils

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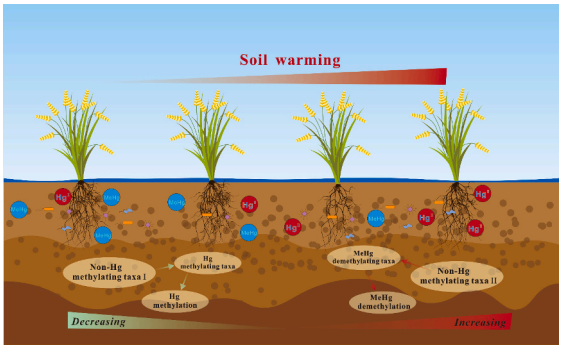
HIGHLIGHTS

- The effects of warming on Hg methylation and demethylation in paddy soils were studied.
- Warming reduces the abundance of Hg-methylating microorganisms and inhibits Hg methylation.
- Warming promotes the abundance of demethylating microorganisms and stimulate MeHg demethylation.

GRAPHICAL ABSTRACT



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ABSTRACT

Inorganic mercury (Hg^{II}) can be transformed into neurotoxic methylmercury (MeHg) by microorganisms in paddy soils, and the subsequent accumulation in rice grains poses an exposure risk for human health. Warming as an important manifestation of climate change, changes the composition and structure of microbial communities, and regulates the biogeochemical cycles of Hg in natural environments. However, the response of specific Hg^{II} methylation/demethylation to the changes in microbial communities caused by warming remain unclear. Here, nationwide sampling of rice paddy soils and a temperature-adjusted incubation experiment coupled with isotope labeling technique ($^{202}Hg^{II}$ and $Me^{198}Hg$) were conducted to investigate the effects of temperature on Hg^{II} methylation, MeHg demethylation, and microbial mechanisms in paddy soils along Hg gradients. We showed that increasing temperature significantly inhibited Hg^{II} methylation but promoted MeHg demethylation. The reduction in the relative abundance of Hg-methylating microorganisms and increase in the relative abundance of MeHg-demethylating microorganisms are the likely reasons. Consequently, the net Hg methylation production potential in rice paddy soils was largely inhibited under the increasing temperature. Collectively, our findings offer insights into the decrease in net MeHg production potential associated with increasing temperature and

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highlight the need for further evaluation of climate change for its potential effect on Hg transformation in Hg-sensitive ecosystems.

1. Introduction

The organic form of mercury (Hg), methylmercury (MeHg), causes severe harm to the human nervous and immune systems (Clarkson, 1993). Studies of Hg-contaminated areas where rice is the chief staple food have shown that rice consumption is the dominant MeHg exposure pathway for local residents (Feng et al., 2008; Liu et al., 2019a; Zhang et al., 2010). Rice is an essential staple food that accounts for approximately 10 % of the world's cultivated area (Elert, 2014; Laborte et al., 2017). In recent years, Hg-contaminated rice has been reported in many countries around the world (e.g., China, the United States, Thailand, and Indonesia) (Krisnayanti et al., 2012; Pataranawat et al., 2007; Windham-Myers et al., 2014), and these countries are important rice exporters, thus increasing the risk of MeHg exposure globally (Knoema, 2021). Studies have shown that MeHg accumulates in rice grains during the rice-growing process, and paddy soil, as a typical constructed wetland, is the primary source of MeHg in rice (Liu et al., 2021b; Meng et al., 2014, 2011). Therefore, it is urgent to understand the biogeochemical cycle of Hg in paddy soils.

Net MeHg production is controlled by the leverage between Hg^{II} methylation and MeHg demethylation (Liu et al., 2019b; Wu et al., 2022; Zhao et al., 2016a, 2016b). Hg^{II} methylation is influenced by a number of factors, such as sulfur, iron, pH, redox potential, the presence of dissolved organic matter (DOM), and Hg bioavailability (Abdelhafiz et al., 2023; Ullrich et al., 2001; Wang et al., 2021). Among these factors, the biotic methylation process mediated by microorganisms is the main way of Hg^{II} methylation in natural environments (Lin et al., 2021; Milliken et al., 2004; Parks et al., 2013). Apart from Hg^{II} methylation, MeHg demethylation is another important process that defines net MeHg formation. The MeHg demethylation process can be affected by abiotic factors (e.g., reactive oxygen species, free radicals and DOM) (Barkay and Gu, 2021); However, microbially mediated demethylation yields the major pathway for MeHg degradation in paddy soils (Strickman et al., 2022; Wu et al., 2020). Therefore, proxies affecting the activity and abundance of Hg-methylating microorganisms and demethylating microorganisms play important roles in regulating net MeHg production in paddy soils.

Climate change has unequivocally emerged as a factual reality, with the atmosphere, hydrosphere, and biosphere undergoing extensive and rapid transformations (IPCC, 2021). The repercussions of climate change can potentially disrupt the structure, composition, and functioning of global ecosystems (Grimm et al., 2013; Li et al., 2018). The increasing temperature is a crucial manifestation of climate change emphasized by the IPCC (2021), and the average surface temperature during 2011–2020 exhibited a 1.1 °C increase compared to the late 19th century average. Moreover, there is a significant variation in the average surface temperature across different latitudes in China. Specifically, southeastern China experiences a relatively high average surface temperature of 24 °C during the period from 2001 to 2020, while the northeastern and northwestern regions are characterized by low temperatures, with an annual average temperature <0 °C (Tian et al., 2022). Considering that other geological factors are the same, these temperature disparities may contribute to variations in soil methylation/demethylation processes. Alterations in microbial community composition and structure have been shown to expand to larger geographical areas in response to increasing temperature (IPCC, 2021; Tian et al., 2006; Wu et al., 2022), subsequently impacting the biogeochemical cycling of carbon, nitrogen, and sulfur (Chen et al., 2013; Galloway et al., 2008; Menzo et al., 2018). As mentioned earlier, microorganisms predominantly mediate Hg^{II} methylation and demethylation (Strickman et al., 2022; Wu et al., 2020). Previous studies have examined the effect of climate on MeHg concentrations in arctic soils, oceans and in submerged upland soils (Yang et al., 2016; Zhang et al., 2020; Zhou et al., 2022) and found that increasing temperature reduced SO_4^{2-}, thereby inhibiting the Hg methylation potential (Åkerblom et al., 2013). However, the responses of specific Hg^{II} methylation and MeHg demethylation processes to increasing temperature are still unclear. Here, we propose the following hypothesis: an increase temperature in paddy soils will influence both Hg^{II} methylation and demethylation by modulating the community structure and diversity of microorganisms.

To address this knowledge gap, we employed a multi-compound-specific isotope labeling technique ($^{202}Hg^{II}$ and $Me^{198}Hg$ tracers) to track both Hg^{II} methylation and MeHg demethylation under different incubation temperatures. Geochemical, microbial community, and metagenomic analyses were performed to decipher the underlying mechanisms of changes in Hg^{II} methylation and MeHg demethylation induced by increasing temperature. The knowledge gained from this study will improve the understanding of the response of the biogeochemical cycle of Hg in Hg-sensitive ecosystems to climate change.

2. Materials and methods

2.1. Site description and sampling

Paddy soil samples (surface soil, 1–10 cm below the soil–water interface) were collected from a nationwide sampling campaign across major rice-producing provinces in China, including Jiangxi Province (JX), Zhejiang Province (ZJ), Hunan Province (HN), Guizhou Province (GZ), Heilongjiang Province (HLJ), and Shaanxi Province (SX). A wide range of climatic variables was carefully considered in this study (Table S1). The soils from the JX, the HLJ, and the HN were recognized as background samples (no distinct Hg pollution), while the paddy soils from the ZJ, the GZ, and the SX suffered different degrees of Hg pollution (Table S1 and Text S1). The sampling campaign was conducted 50–60 days after transplantation of rice seedlings in August 2022, which was confirmed by the local agricultural department. This sampling period was chosen to reflect the optimal condition for Hg^{II} methylation (Zhao et al., 2016a, 2016b). Surface soil samples were collected from four locations within 500 m^2 of the same rice field and mixed evenly as one sample. Soil samples were collected into 500 ml sterile PP (polypropylene) bottles without any remaining headspace. All the bottles were sealed with Parafilm®, double bagged, and stored at 4 °C in an ice box until further incubation. More detailed descriptions of the sampling sites are provided in Text S1 in the Supporting Information.

2.2. Incubation experiments

Anaerobic incubation experiments were performed by mixing the soil samples in a beaker with deionized water (Milli-Q®, Millipore, USA; N_2 purged) in an oxygen-free (N_2–filled) glovebox (PLAS-LABS, USA). The resulting soil slurry (40–60 mL, 50 %–60 % moisture) was then transferred into 100 mL serum bottles (gas-tight borosilicate glass bottles) for incubation. The mean temperature of the study areas was 19.32–28.49 °C (Table S1) during the sampling season (July to September). Even considering the mean temperature range throughout the year (Table S1), the temperature gradient of 15 °C–25 °C-35 °C could well reflect the temperature changes of the whole rice growing season. To activate anaerobic microorganisms, the paddy soils were pre-incubated at 15 °C, 25 °C, and 35 °C in the dark for one week (Zhou et al., 2022). Gamma-irradiated soils (treated with 0.6 kGy h^{-1} gamma irradiation for 48 h) were treated as the sterilized control (Hao et al., 2024; Zhou et al., 2022). The experiment included factorial

combinations of three temperatures (15, 25, and 35 °C) and two treatments (sterilized control and unsterilized treatment). Each set consisted of three replicates, resulting in a total of 108 soil samples (6 sites × 2 treatments × 3 temperatures × 3 replicates). Enriched Hg isotope tracers, including $^{202}Hg(NO_3)_2$ and $Me^{198}Hg(NO_3)_2$, were spiked into soil slurries to trace Hg^{II} methylation and MeHg demethylation, respectively. The amounts of spiked $^{202}Hg^{II}$ and $Me^{198}Hg$ tracers were ~10 % and ~100 % (Table S2), respectively, of the soil ambient total Hg (THg) and MeHg concentrations (Hintelmann et al., 1995; Liu et al., 2021a; Wu et al., 2020). More details on the preparation of the $^{202}Hg^{II}$ and $Me^{198}Hg$ tracers are available in our previous studies (Liu et al., 2023a). Considering that simultaneous incubation of soil samples from different sites may lead to contamination of uncontaminated soil samples, we quickly closed the serum bottle after isotope addition and moved it outside the glove box, followed by cleaning the glove box. In addition, the incubation order was carried out from low to high according to the ambient MeHg concentration (i.e., HN: 0.29 ng/g-HLJ: 0.74 ng/g-JX: 0.77 ng/g-ZJ: 1.34 ng/g-SX: 2.53 ng/g-GZ: 2.91 ng/g).

Three bottles were destructively sampled in the glovebox after 24 h of incubation due to the systematic underestimation of rate constants over longer durations (Helmrich et al., 2021). Samples for the analysis of isotopic MeHg concentrations and DOM were collected into 50 ml PP tubes (JET®, BIOFIL, China), stored at −20 °C, freeze-dried and ground evenly through a 200-mesh sieve using an agate mortar. To investigate the impact of alterations in microbial community structure and the iron-sulfur cycle on Hg^{II} methylation and MeHg demethylation, soil slurry incubation samples for 16S rRNA gene sequencing were collected into cryogenic tubes (2 ml, Nalgene®, Thermo Scientific, USA) and stored at −80 °C for further analysis. The liquid phase of incubation soil slurry was separated by centrifugation (RCF = 2850 *g* for 10 min at 4 °C) under anaerobic conditions (in N_2-filled glovebox) for sulfide (S^{2-}) and iron (Fe^{2+} and Fe^{3+}) analysis (Cline, 1969; Viollier et al., 2000). Sulfide and iron were determined immediately (as both species are redox sensitive) using methylene blue method and ferrozine method, respectively. To investigate the potential factors contributing to abiotic demethylation, 5 g of paddy soil sample (separated from the origin slurry to replicate conditions in the incubation experiment) from each of the sampling sites was mixed with 50 ml of 10 mM sodium benzoate, and the hydroxyl radicals produced during Gamma irradiation (treated with 0.6 kGy h^{-1} for 48 h) were detected.

2.3. Analytical methods

Gas chromatography-inductively coupled plasma–mass spectrometry (GC-ICP–MS, Agilent 7700×, Agilent Technologies, Inc., USA) followed by the ethylation-purge trap method was employed to determine MeHg isotopes (Hintelmann et al., 1995, 2000). Hg^{II} methylation was monitored via the formation of $Me^{202}Hg$ from spiked $^{202}Hg^{II}$, while MeHg demethylation was assessed by the decrease in spiked $Me^{198}Hg$ over time (Hintelmann et al., 1995, 2000). The first-order methylation rate constant (K_m) and demethylation rate constant (K_d) were calculated using Eqs. (1) and (2), respectively (Hintelmann et al., 1995, 2000). More details of the Hg isotope analysis can be found in our previous works (Hao et al., 2024; Liu et al., 2023a; Wu et al., 2020)

$$K_m = \frac{\left[Me^{202}Hg\right]_{ti} - \left[Me^{202}Hg\right]_{t0}}{\left[^{202}Hg^{II}\right] \times (t_i - t_0)} \quad (1)$$

$$K_d = \frac{ln\left(\left[Me^{198}Hg\right]_{t0}\right) - ln\left(\left[Me^{198}Hg\right]_{ti}\right)}{(t_i - t_0)} \quad (2)$$

Here, $[Me^{202}Hg]_t$ and $[Me^{198}Hg]_t$ are the $Me^{202}Hg$ and $Me^{198}Hg$ concentrations, respectively, which were evaluated initially (t_0) and after 24 h (t_i); $[^{202}Hg^{II}]$ is the $^{202}Hg^{II}$ concentration in the initial spike added to the soils. More calculation details are provided in Text S2.

The pH values of the soil samples were measured using a pH electrode (SX751 pH/ORP/Cond/DO meter, SANXIN, China). The sulfide (S^{2-}) concentration in the aqueous phase of the soil slurry was determined using the Cline method (Cline, 1969). The detection limits for sulfide analysis are 0.13 μM (Cline, 1969). Ferrous (Fe^{2+}) and ferric (Fe^{3+}) cations were measured using the ferrozine method (Viollier et al., 2000). Hydroxyl radical (·OH) was quantified using a sodium benzoate probe technique described previously (Mopper and Zhou, 1990). The detection limits for hydroxyl radical analysis are 0.1 μM (Liao et al., 2019). Concentrations of SO_4^{2-} and NO_3^- were measured via ion chromatography (ICS90, DIONEX, USA). The concentration of soil dissolved organic matter (DOM, reflected by water-soluble DOC) was determined via extraction with Milli–Q water (soil: water = 1:10 *w/v*), filtration (0.45 μm polypropylene membrane filters) and determination using a total organic carbon analyzer (Vario TOC cube, Elementar, Germany). The detailed DOM characterization methods are given in Text S3. The measurement details of the soil background values for total Hg and MeHg are provided in Text S4.

2.4. Characterization of microbial communities and metagenomic analysis

Total microbial DNA in slurry samples was extracted from 0.5 g of soil using FastDNA® Spin Kits (MP Biomedicals, Irvine, CA) according to the manufacturer's protocol. The concentration and quality of the DNA were determined using the fluorescent method (Qubit™ 4 Fluorometer, Thermo Scientific, USA) (Pu et al., 2022). To characterize the soil microbial communities, the V3-V4 region of the microbial 16S rRNA gene was amplified using the universal bacterial primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACNVGGGTWTCTAAT-3′). More details on the 16S rRNA gene sequencing procedure are available in Text S5. Microbial mercury methylation genes (*hgcA*), demethylation genes (*merB*), and reduction genes (*merA*) were determined by real-time quantitative PCR (qPCR) on the ABI7500 (Applied Biosystems, USA); details are provided in Table S3. Shotgun metagenomic sequencing was performed on 9 samples after incubation with SX (i.e., 3 parallel samples of each treated SX). The result showed that the Hg^{II} methylation/demethylation rates of SX were most significantly responsive to warming. Sequencing was performed on an Illumina NovaSeq 6000 platform (paired-end 150 bp) from Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China), with a read depth of ~30 Gbp per sample. More details on the metagenomic analysis are provided in Text S6.

2.5. QA/QC and statistics

Quality control for MeHg isotope analysis of the soil samples was determined using blanks, triplicate samples, and certified reference material (CRM, CC580). The method detection limit (3σ) for MeHg isotope analysis was 0.013 ng/g. The relative standard deviation (RSD %) of triplicates was <15 %. The data are presented as the mean ± standard error (SE). The MeHg recovery from CRM ranged from 80 % to 113 %, with an average of 98 ± 11 % (n = 13).

The Kruskal–Wallis one-way ANOVA and Mann-Whitney U were employed to determine statistically significant differences ($p < 0.05$,2-tailed) between treatments. We used the linear discriminant analysis effect size ($p < 0.05$, LDA score > 3) method to identify bacterial biomarkers under increased temperature with the Galaxy computational tool v.1.0. (http://huttenhower.sph.harvard.edu/galaxy). Principal coordinate analysis (PCoA) of unweighted Euclidean distances, the Adonis test, and microbial alpha diversity were calculated and plotted using the R platform (Team, 2014) with the "vegan" package (Oksanen et al., 2017). To identify the significant drivers of K_m and K_d, a random forest model was constructed using the "randomForest" package (Liaw and Wiener, 2002).

3. Results

3.1. Variation in physicochemical parameters at different temperatures

The physicochemical parameters of the paddy soils at the different temperatures at all the sites are shown in Table S4. The concentrations of DOM, Fe^{2+}, S^{2-}, NO_3^- and SO_4^{2-} in the soil slurries varied across the different sites (K—W one-way ANOVA, $p < 0.001$; Fig. S1) but were similar among the different temperature treatments at the given sites (K—W one-way ANOVA, $p > 0.05$; Table S4). UV–vis absorption and fluorescence spectra were used to characterize the compositional structure of the DOM. $SUVA_{254}$ and S_R in this study represented the relative aromatic content (Weishaar et al., 2003) and relative molecular weight/size of DOM (Helms et al., 2008; Zhang et al., 2023), respectively. Significant changes in physicochemical parameters were not observed at most sites in this study (Table S4). DOC of the JX, the HLJ and the ZJ significantly increased with the increasing temperature, which could be attributed to the dissolution of SOM into DOM (Jiao et al., 2020). However, the compositional structure of DOC are more critical factors affecting the Hg^{II} methylation than the total DOC (Abdelhafiz et al., 2023). Therefore, the significant changes in some sites of DOC with increasing temperature in this study may not affect the Hg^{II} methylation/demethylation. Significant decreases of S^{2-} in the ZJ and the SX and the significant increases of SO_4^{2-} in the JX, the HLJ, and the GZ may imply that the sulfate reduction process is inhibited with increasing temperature (Table S4). At 35 °C treatment, the NO_3^- in the JX, the HLJ, the SX, and the GZ was significantly reduced (Table S4). It reflects that stronger denitrification at higher temperatures may inhibit the sulfate reduction process (Chen et al., 2023). Sulfate-reducing bacteria have been found to be important Hg^{II} methylating microorganisms (Compeau and Bartha, 1985; Ranchou-Peyruse et al., 2009). The inhibition of sulfate reduction process could inhibit Hg^{II} methylation. The changes in other physicochemical parameters (e.g., $SUVA_{254}$, S_R, Fe^{2+}, and pH) did not exhibit a consistent result at all sites, suggesting that their contribution to Hg^{II} methylation/demethylation during temperature changes may not be primary.

3.2. Variation in microbial communities at different temperatures

An increase in temperature induced significant decreases (K—W one-way ANOVA, $p < 0.05$; Fig. 1a and Table S5) in the bacterial alpha diversity (based on the Shannon, Chao1 and Simpson index) at all the sampling sites. Euclidean distance-based principal coordinates analysis (PCoA) showed that the overall bacterial community composition was significantly different between the temperature gradients (Fig. 1b), which was further verified by a nonparametric dissimilarity test (Adonis, $p < 0.01$). The number of *hgcA* gene copies decreased significantly with increasing temperature (K—W one-way ANOVA, $p < 0.05$; Fig. S2). Copies of the *mer* operon (*merA* and *merB*) were below the detection limit in all treatments at all sites [real-time threshold cycle (C_T) > 31].

Linear discriminant analysis (LDA) effect size (LEfSe) revealed that the 15, 25, and 35 °C treatments enriched ten taxa (*Koribacteraceae*, *Bryobacteraceae*, *Soilibacteraceae*, *Bacteroidaceae*, *Chitinophagaceae*, *BSV26*, *Anaerolineaceae*, *Gemmatimonadaceae*, *Nitrosomonadaceae* and *Pedosphaeraceae*), four taxa (*SR*, *Geobacteraceae*, *Erysipelotrichaceae* and *Nitrospiraceae*), and four taxa (*Bacillaceae*, *Clostridiaceae*, *Chromobacteriaceae*, and *Pseudomonadaceae*), respectively ($\alpha < 0.01$, LDA score > 3; Fig. 2a). Random forest analysis revealed that *Bacteroidaceae*, *Paenibacillaceae*, *Xanthobacteraceae*, *Sulfurospirillaceae*, *Clostridiaceae*, *Anaerolineaceae* and *Geobacteraceae* had significant effects on Hg^{II} methylation in paddy soils at different temperatures (Fig. 2b). In addition, the families *Sulfurospirillaceae*, *Xanthobacteraceae*, *Clostridiaceae*, *Bacteroidaceae*, *Anaerolineaceae*, *Paenibacillaceae*, *Geobacteraceae*, *Pedosphaeraceae* and *Bryobacteraceae* were identified as important predictors of MeHg demethylation in paddy soils at different temperatures (Fig. 2c). The relative abundances of the key bacterial taxa showed that the proportions of *Clostridiaceae and Chromobacteriaceae* significantly increased ($p < 0.01$) with increasing temperature (from 15 °C to 35 °C), while the proportions of other bacterial families (e.g., *Anaerolineaceae*, *Geobacteraceae*, etc.) significantly decreased ($p < 0.05$; Fig. 3 and Table S6). It is worth mentioning that the abundance of key microorganisms and bacterial alpha diversity between 15 °C and 25 °C were not as significant as those between 15 °C–35 °C or 25–35 °C, possibly because the microorganisms respond differently to different temperatures. Bárcenas-Moreno et al. (2009) found that the optimum temperature for microbial growth was about 30 °C. Soil incubation temperatures above 30 °C significantly altered the temperature response of microbial diversity and community structure (fungi and bacteria), while lower incubation temperatures had little effect. Metagenomic analysis revealed that the abundance of *Geobacter* spp. harboring the *hgcAB* gene decreased by 13.65 % from 15 °C to 25 °C and further decreased by 11.40 % from 25 °C to 35 °C (Fig. S3). Additionally, the abundance of *Anaerolineae* spp. decreased by 34.29 % and 36.04 % during the warming processes

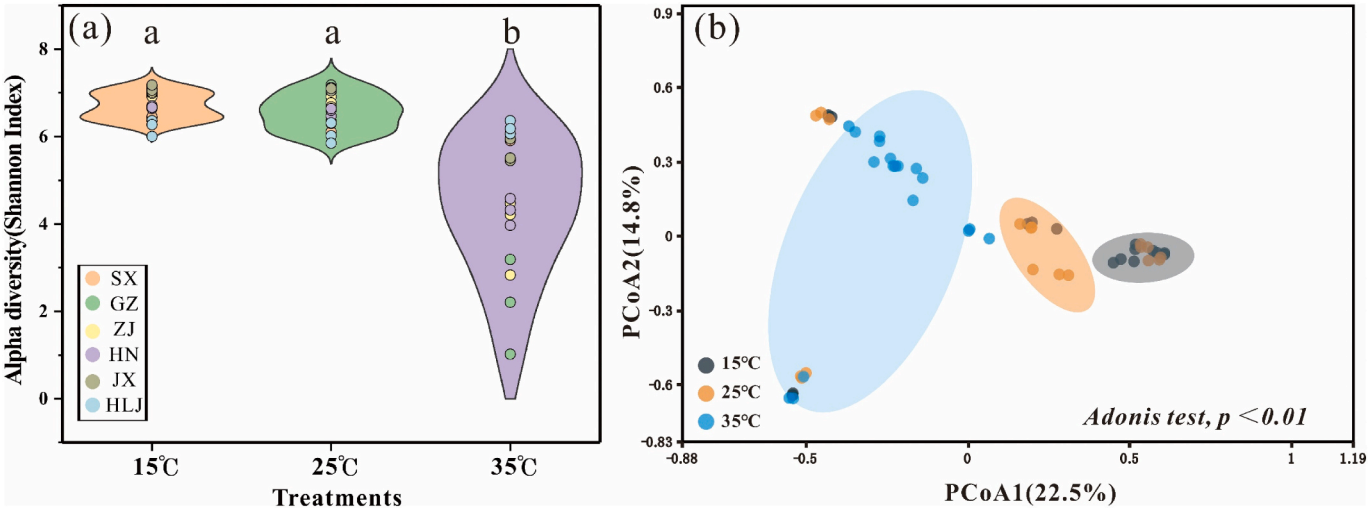

Fig. 1. Effect of temperature on bacterial diversity and community composition. **(a)** The alpha-diversity based on Shannon index. Different lowercase letters indicate that the differences between the treatments at different temperature are significant (K—W one-way ANOVA, $p < 0.05$). Orange: SX, Light Green: GZ, Yellow: ZJ, Purple: HN, Dark Green: JX, Blue: HLJ. **(b)** The beta-diversity indicated by principal coordinate analysis (PCoA) based on Euclidean distances. Different colored elliptic shades represent different treatments.

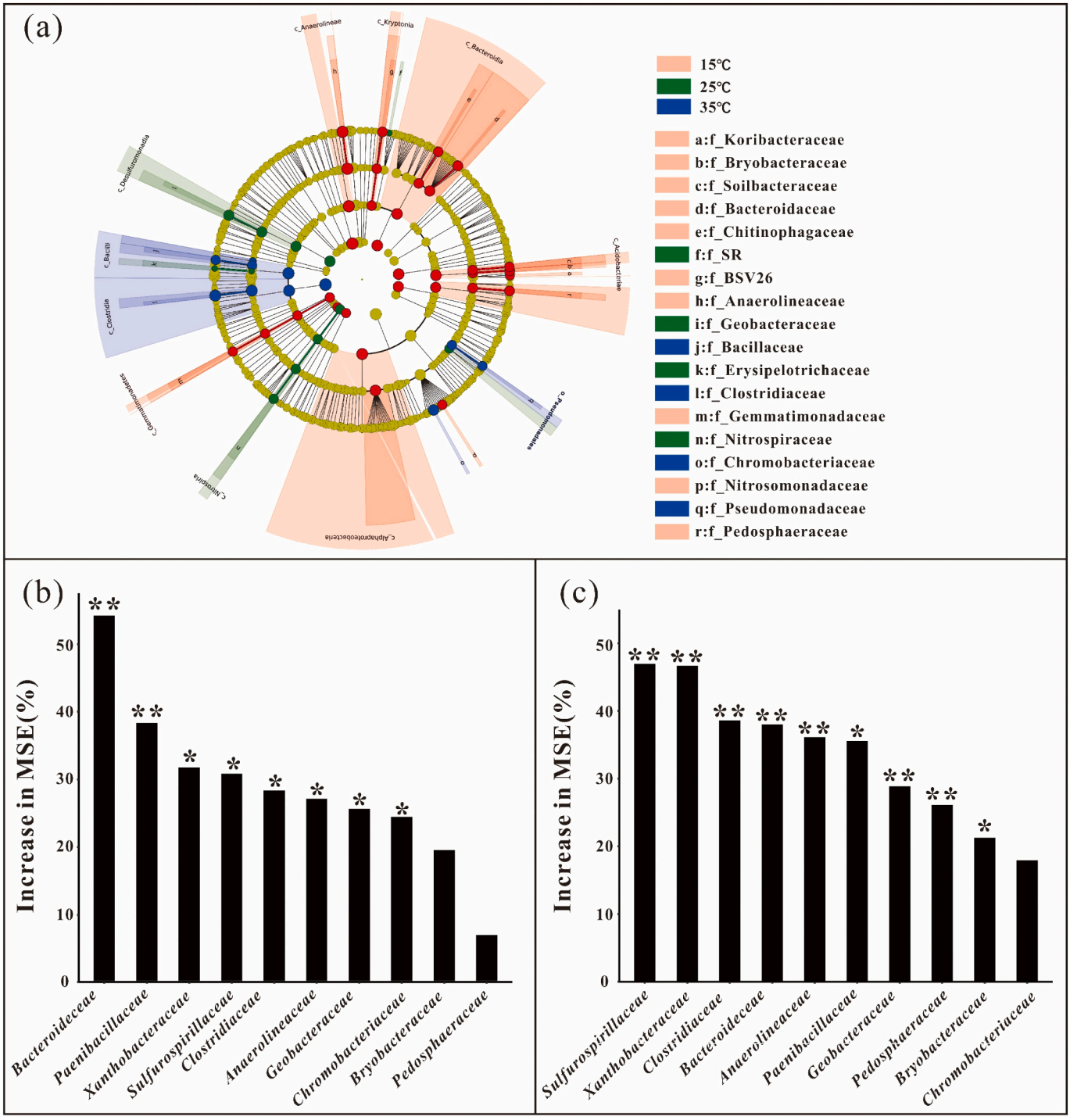

Fig. 2. Key bacterial taxa in response to temperature changes. **(a)** Linear Discriminant Analysis (LDA) showed that the bacterial taxa were enriched differently among the different temperature treatments. **(b)** The random forest model indicated the importance of the predictors for $^{202}Hg^{II}$ methylation. **(c)** The random forest model indicated the importance of the predictors for $Me^{198}Hg$ demethylation.

from 15 °C to 25 °C and 25 °C to 35 °C, respectively (Fig. S3). Conversely, there was a significant increase ($p < 0.05$) in the abundance of the demethylating bacterium *Clostridium* spp. with increasing temperature (Fig. S3).

3.3. Methylation of $^{202}Hg^{II}$ during the incubation

Our study showed that both the methylation rate constant (K_m-$^{202}Hg^{II}$, reflecting the methylation of spiked $^{202}Hg^{II}$) and the $Me^{202}Hg$ concentration in the sterilized control were significantly lower than those in the unsterilized samples at the same temperature treatment (Mann-Whitney U, $p < 0.05$; Figs. S4 and S5). Moreover, no significant difference was observed in the K_m-$^{202}Hg^{II}$ or $Me^{202}Hg$ concentrations among the sterilized controls subjected to the different temperature treatments, suggesting that microbial processes predominantly drive Hg^{II} methylation in paddy soils, while abiotic Hg^{II} methylation could even be negligible.

Interestingly, the variation trends in K_m-$^{202}Hg^{II}$ across the different temperature treatments were the same across all the sampling sites, revealing a significant decrease in K_m-$^{202}Hg^{II}$ with increasing

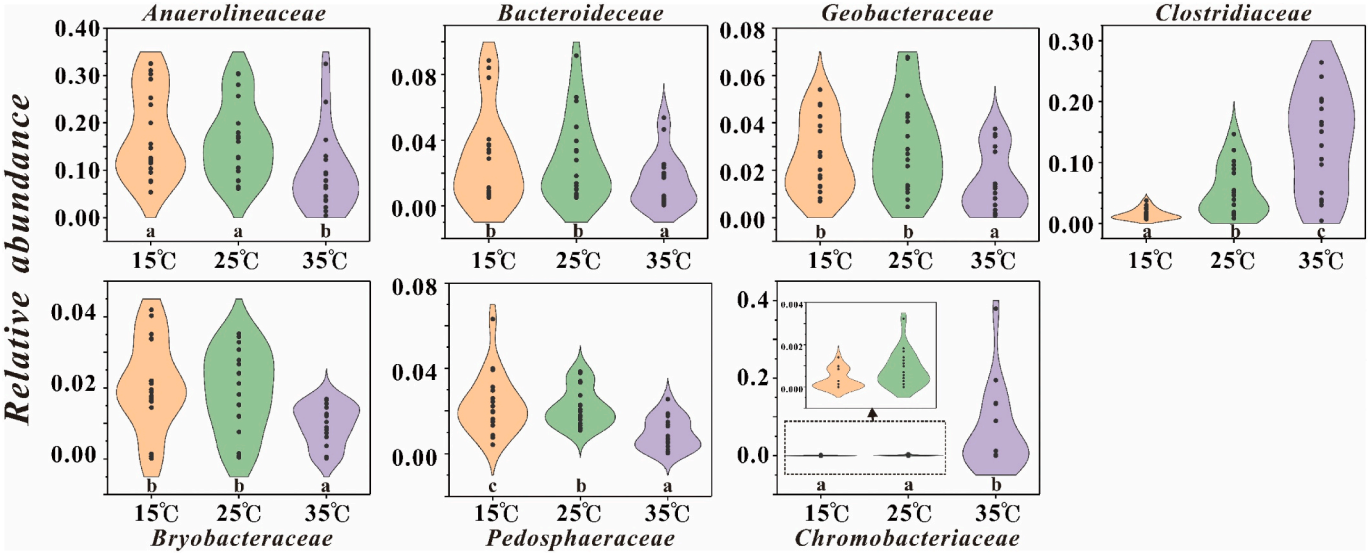

Fig. 3. Violin plot for the relative abundance of key bacterial taxa. Different lowercase letters indicate that the differences between the treatments at different temperature are significant (K—W one-way ANOVA, $p < 0.05$).

temperature (K—W one-way ANOVA, $p < 0.05$; Fig. S4). K_m-$^{202}Hg^{II}$ exhibited a decrease ranging from 0.06 to 0.53 times when the temperature increased from 15 °C to 25 °C and similarly decreased from 0.30 to 0.76 times when the temperature further increased to 35 °C. These findings suggested that increasing temperature may influence physicochemical parameters or microbial activity and thus inhibit Hg^{II} methylation. Given the consistent variation patterns of K_m-$^{202}Hg^{II}$ across the sampling sites, we integrated the K_m-$^{202}Hg^{II}$ values of all the sites at different temperatures for further discussion (Fig. 4a). The integrated K_m-$^{202}Hg^{II}$ values for all the sampling sites decreased significantly when the temperature increased from 15 °C to 35 °C (K—W one-way ANOVA, $p < 0.05$; Fig. 4a).

3.4. *Demethylation of $Me^{198}Hg$ during the incubation*

The $Me^{198}Hg$ tracer significantly degraded with increasing temperature at all sites, as indicated by the significant increase in the demethylation rate constant (K_d-$Me^{198}Hg$, reflecting the demethylation of spiked $Me^{198}Hg$) with increasing temperature during incubation (K—W one-way ANOVA, $p < 0.001$; Fig. 4b). K_d-$Me^{198}Hg$ clearly increased from 0.22 to 2.21 times when the temperature increased from 15 °C to 25 °C and similarly increased from 0.67 to 5.60 times when the temperature further increased to 35 °C. A consistent pattern was also observed for K_d-$Me^{198}Hg$ across all sites (K—W one-way ANOVA, $p < 0.05$; Fig. 4b). This observation suggested that temperature-induced changes in physical and chemical parameters or microbial communities may have facilitated MeHg demethylation in paddy soils during incubation. At the same temperature, the K_d-$Me^{198}Hg$ and degraded $Me^{198}Hg$ concentrations in the sterilized control were significantly lower than those in the unsterilized treatment at the given sites (Mann-Whitney U, $p < 0.05$; Fig. S6 and Fig. S7), with the soil from the HLJ serving as an exception. In addition to those in the HLJ treatment, no significant differences in the K_d-$Me^{198}Hg$ or $Me^{198}Hg$ concentrations in the sterilized control were observed among the different temperature treatments across the sampling sites (K—W one-way ANOVA, $p > 0.05$; Fig. S6 and S7). Taken together, the evidence shown above suggested that MeHg demethylation in paddy soil was mainly a biotic process during incubation. Notably, although the biotic demethylation at the HLJ increased with increasing temperature, the abiotic demethylation at this site was significantly greater (Fig. S6) than the biotic demethylation at the same temperature, which was different from what was observed at the other five sites. The possible reasons for the abiotic demethylation of MeHg in the HLJ will be discussed in Section 4.2.

The net Hg methylation potential (K_m-$^{202}Hg^{II}$/K_d-$Me^{198}Hg$), which

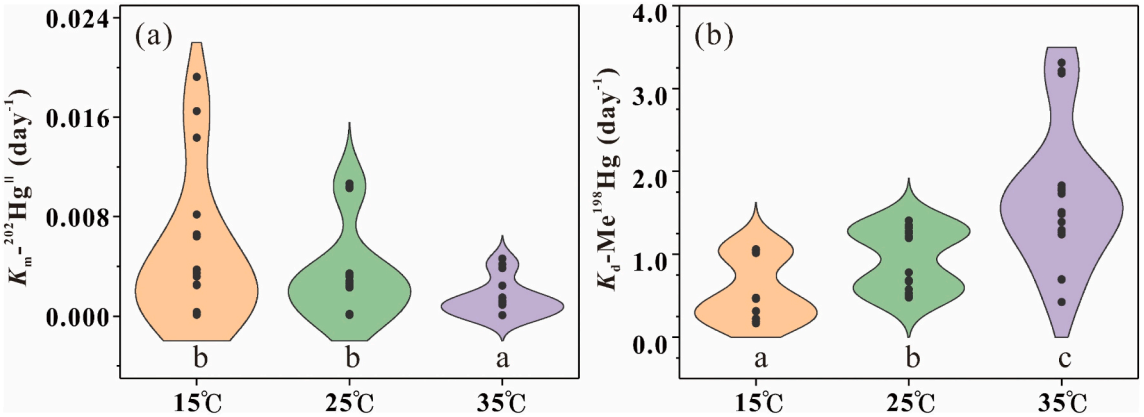

Fig. 4. Hg^{II} methylation/demethylation rate constants for all sites at different temperature. **(a)** Violin plot for the methylation rates ($K_m^{202}Hg^{II}$) for different treatments at all sites. **(b)** Violin plot for the MeHg degradation rates (K_d-$Me^{198}Hg$) for different treatments at all sites. Different lowercase letters indicate that the differences between the treatments at different temperature are significant (K—W one-way ANOVA, $p < 0.05$).

represents the balance between MeHg production and degradation (Korthals and Winfrey, 1987; Zhao et al., 2016b), decreases significantly with increasing temperature (K—W one-way ANOVA, $p < 0.05$; Fig. S8). With an increase in temperature from 15 °C to 25 °C, the net methylation potential decreased from 0.27 times to 0.85 times. Furthermore, as the temperature further increased to 35 °C, the net methylation potential decreased from 0.27 times to 0.96 times. Subsequent random forest analysis of the net Hg methylation potential revealed that microbial parameters (alpha diversity, beta diversity, and functional gene copy number) exerted greater influences than abiotic parameters (Fig. S9). These results suggest that alterations in microbial community structure and diversity induced by increasing temperature are primarily responsible for inhibiting Hg^{II} methylation and promoting MeHg demethylation.

4. Discussion

4.1. Increasing temperature inhibits Hg^{II} methylation

K_m-$^{202}Hg^{II}$ (Fig. S4) and $Me^{202}Hg$ production (Fig. S5) in the sterilized control were significantly lower than those in the unsterilized treatment, suggesting that biotic methylation plays a pivotal role in paddy soils and that abiotic methylation is negligible. The signals of enriched Hg isotope tracers revealed that K_m-$^{202}Hg^{II}$ (Fig. 4a) significantly decreased with increasing temperature at all sites. This phenomenon may be attributed to the alterations in Hg^{II} bioavailability due to changes in physicochemical parameters or changes in microbial communities (Ullrich et al., 2001; Wang et al., 2021; Zhao et al., 2016b) induced by increasing temperature. The bioavailability of Hg^{II} and the microbial activity involved in the Hg^{II} methylation process are determined by various physicochemical parameters, including the concentration of sulfur, iron and organic matter, species composition, and pH (Bravo et al., 2015; Bravo and Cosio, 2020; Skyllberg et al., 2003; Ullrich et al., 2001). Sulfur can directly affect the chemical speciation and solubility of Hg^{II} and subsequently affect its bioavailability, and higher sulfur concentrations ultimately limit the bioavailability of Hg^{II} methylation by binding to Hg^{II} to form HgS(s) (Drott et al., 2013). Gilmour and Henry (1991) demonstrated that higher pH values favor MeHg demethylation, while lower pH values stimulate Hg^{II} methylation. However, significant changes in the pH were not detected during the incubation period in this study (Table S4). Iron-sulfur cycling and the chemical speciation of dissolved organic matter are equally crucial for Hg transformation (Abdelhafiz et al., 2023; Ullrich et al., 2001; Ulrich and Sedlak, 2010), while DOM with a lower molecular weight and less humic substances was found to more readily fuel Hg^{II} methylation (Bravo et al., 2017). Additionally, the recrystallization of Fe(oxyhydr) oxides to Fe—S solids and the formation of Hg-sulfide precipitates through sulfide combination may significantly diminish the bioavailability of Hg^{II} (Skyllberg et al., 2021; Ullrich et al., 2001). The Hg^{II} methylation/demethylation responded to increasing temperature in a consistent manner (Fig. S4 and S6), even though there were significant differences in the physicochemical parameters among different sampling sites (Fig. S1). Furthermore, no significant differences were found in most of the physicochemical parameters at the same sites with the temperature increasing. A few abiotic parameters with significant changes within the same sites did not show a consistent result (Table S4). Therefore, even taking into account the possible effects of physicochemical parameters, the microbial-mediated processes remained the primary causal factor (Liu et al., 2021a; Wu et al., 2020) because (i) no significant changes were observed in most abiotic parameters during the incubation process (Table S4), (ii) a decrease in microbial diversity and alteration of community structure were associated with increasing temperature (Fig. 1a and b), and (iii) random forest analysis of the net Hg methylation potential (K_m-$^{202}Hg^{II}$/K_d-$Me^{198}Hg$) further confirmed that microbial parameters (alpha diversity, beta diversity, and functional gene copy number) exerted greater influence than abiotic parameters (Fig. S9).

In this study, seven key bacterial taxa closely linked to increasing temperature were identified (Fig. 2): *Anaerolineaceae*, *Bacteroideceae*, *Geobacteraceae*, *Clostridiaceae*, *Chromobacteriaceae*, *Bryobacteraceae* and *Pedosphaeraceae* (Fig. 3). The relative abundances of the Hg-methylating taxa *Anaerolineaceae, Bacteroideceae* and *Geobacteraceae* were significantly associated with *hgcA* gene abundance (Fig. S10), suggesting that these bacterial taxa were potential Hg-methylating microorganisms in response to increasing temperature. The *Anaerolineaceae family* has been reported to contain the *hgcA* gene, which is capable of Hg^{II} methylation (Gionfriddo et al., 2020; Liu et al., 2023c) and has versatile metabolic abilities for carbohydrate fermentation, and for which acetate is produced (Xia et al., 2016). In addition, the family *Bacteroideceae*, as a potential Hg-methylating taxon (Gionfriddo et al., 2020; Liu et al., 2023c), not only participates in Hg^{II} methylation but also provides a carbon source for denitrification by degrading complex organic matter from soil (Gao et al., 2022). Furthermore, the *Geobacteraceae* family is also known to be a Hg-methylating taxon (Liu et al., 2018; Zhou et al., 2022). Our metagenomic results from SX samples confirmed that *Geobacter* spp., *Anaerolineae* spp. and *Bacteroidetes* spp. were Hg-methylating microorganisms, and the abundance of *Geobacter* spp. and *Anaerolineae* spp. decreased significantly with increasing temperature, which may explain the inhibition of Hg^{II} methylation (Fig. S3).

In addition, positive correlations were observed between non-Hg methylating taxa I (*Bryobacteraceae and Pedosphaeraceae*) and Hg methylating taxa (Fig. S11). Significant correlations between the abundance of *Bryobacteraceae* and *Pedosphaeraceae* and *hgcA* gene were not detected in this study, indicating that these bacteria are not directly involved in Hg^{II} methylation. However, the family *Bryobacteraceae* not only has potential for phosphorus transport, mineralization and solubilization (Camargo et al., 2022) but also has been found to be capable of nitrogen reduction (Dedysh et al., 2017). Moreover, the family *Pedosphaeraceae* plays an important role in sediment nutrient circulation, and its order *Pedosphaerales* has potential for metal resistance (Ni et al., 2015; Zhang et al., 2019). As previously described, these bacteria may affect the composition of the Hg-methylating microbial community by providing nutrients for microorganisms, thus regulating Hg^{II} methylation (Carrell et al., 2021; Liu et al., 2019b). Increasing temperature may decrease the abundance of non-Hg methylating taxa, thereby inhibiting the growth of Hg-methylating taxa and decreasing Hg^{II} methylation. With increasing temperature, microbial diversity decreases (Cardinale et al., 2012; Nottingham et al., 2022; Wu et al., 2022), leading to the gradual occupation of ecological niches by more competitive microorganisms, thereby inhibiting the growth of less competitive microorganisms (Wiedenbeck and Cohan, 2011). Therefore, the inhibition of Hg^{II} methylation with increasing temperature can be explained by the decrease in the relative abundance of non-Hg-methylating taxon I and Hg-methylating taxa (Fig. 3 and S3).

4.2. Increasing temperature stimulates MeHg demethylation

Our study showed significantly lower K_d-$Me^{198}Hg$ (Fig. S6) and $Me^{198}Hg$ (Fig. S7) production in the sterilized control than in the unsterilized treatment across the sampling sites, with the soil from the HLJ serving as an exception, suggesting that biotic demethylation plays a pivotal role in paddy soils. Notably, abiotic demethylation at the HLJ site predominated (Fig. S6) over the other five sites. Considering that MeHg can be degraded directly or indirectly by abiotic means through photolysis or photochemical reactions (Black et al., 2012; Lehnherr and St. Louis, 2009), for example, that MeHg can be degraded by hydroxyl radicals (·OH) produced by photolysis (Zhang et al., 2017), we then quantified the ·OH produced during γ-irradiation sterilization. The amount of ·OH produced at the HLJ site during γ-irradiation was significantly greater than that at other sites (Fig. S12), and the production of ·OH was attributed to the high Fe^{2+} concentration (Hammerschmidt and Fitzgerald, 2010; Liu et al., 2023b). The photo-Fenton

reaction involves photochemical reduction of thermodynamically stable Fe^{3+} to Fe^{2+}, which can react with naturally ubiquitous H_2O_2 to yield ·OH, Fe^{3+} and OH- (Hammerschmidt and Fitzgerald, 2010). Therefore, the abiotic demethylation at the HLJ site could be explained by the ·OH degradation of MeHg caused by the high Fe^{2+} concentration at the HLJ site (Fig. S1). In addition to ·OH, some reactive oxygen species (ROS) such as singlet oxygen and superoxide anions have been reported to induce degradation of MeHg (Zhang et al., 2010; Luo et al., 2020; Tang et al., 2024). In this study, only hydroxyl radicals were quantified, which may underestimate the degradation of MeHg by various ROS produced after irradiation. However, even though the data supporting this hypothesis is limited, the contribution of ROS to demethylation cannot be ignored, and further work is needed to support this observation.

K_d-$Me^{198}Hg$ significantly increased with increasing temperature at all sites (Fig. 4b), indicating that increasing temperature facilitates MeHg demethylation. As indicated in Section 4.1, the primary factor contributing to the inhibition of Hg^{II} methylation and promotion of MeHg demethylation in this study can be explained by the alteration of the microbial community composition during the incubation periods. A strong relationship was observed between the relative abundance of *Clostridiaceae* and K_d-$Me^{198}Hg$ (Fig. S10), suggesting that *Clostridiaceae* plays an important role in MeHg demethylation in paddy soils. The family *Clostridiaceae* has been confirmed to be a potential MeHg demethylating taxon (Marvin-DiPasquale et al., 2000; Narita et al., 2000; Wang et al., 2021; Zhou et al., 2020). The metagenomic findings substantiated that *Clostridium* spp. are MeHg demethylating microorganisms, and their abundance increased with increasing temperature (Fig. S3). Our study revealed a positive correlation between non-Hg-methylating taxa II (*Chromobacteriaceae*) and MeHg-demethylating taxa (*Clostridiaceae*) (Fig. S11). Furthermore, previous studies have shown that the family of *Chromobacteriaceae* does not demethylate MeHg, indicating that these bacteria may not be directly involved in MeHg demethylation. However, the family of *Chromobacteriaceae* has also been reported to undergo nitrate reduction (Schreiber et al., 2023), chitin hydrolysis, and glucose fermentation (Chen et al., 2021) processes that are potentially coupled to the Hg cycle. Warming represents different effects on different microbial lineages and/or functional lines, that is, the warming effect varies greatly among different microbial functional groups (Zhou et al., 2012). Microorganisms with unique survival strategies will have a competitive advantage, which can occupy ecological niches and inhibit the growth of other microorganisms (Shu and Huang, 2021; Wiedenbeck and Cohan, 2011). Reimer et al. (2021) found that *Firmicutes* is one of the few bacterial taxa that can tolerate high temperatures due to their sporulation ability. Therefore, high temperature as an advantage for their survival may lead to an increase in their relative abundance in the microbial community (Wu et al., 2015, 2022). In this study, under the condition of increasing temperature and the presence of heavy metal stress (Hg), the MeHg demethylated taxa (i.e., *Clostridium*, *Firmicutes*, carrying Hg resistance genes) have higher relative abundance because they can tolerate high temperatures and resist Hg stress through efflux mechanisms (Chen et al., 2020), while Hg methylated taxa may lose their competitive advantage in the face of Hg stress and high adaptation costs. Consequently, our study suggested that increasing temperature stimulates MeHg demethylation through (i) promoting an increase in the abundance of MeHg demethylation taxa such as *Clostridiaceae* and (ii) increasing the abundance of non-Hg methylating taxa II (*Chromobacteriaceae*) to promote the growth of MeHg demethylation taxa.

5. Conclusion and implications

The current work provides the first in-depth understanding of how temperature affects Hg^{II} methylation and MeHg demethylation, highlighting the underlying process and mechanisms involved. The leverage between these two processes controlled the net Hg methylation potential, resulting in a decrease in the net Hg methylation potential with increasing temperature. Although MeHg production is affected by a variety of factors, our results highlight that temperature-induced changes in microbial community composition are also important even when considering differences in physicochemical parameters caused by different geological conditions. We summarize the alterations in the microbial community that affect Hg^{II} methylation/demethylation during warming: increasing temperature inhibits Hg^{II} methylation by (1) decreasing the activity and abundance of Hg-methylating microorganisms and (2) decreasing the abundance of non-Hg-methylating microorganisms that are "mutually beneficial" to Hg-methylating microorganisms. Moreover, increasing temperature stimulates the demethylation of MeHg by (1) increasing the abundance of demethylating microorganisms and (2) increasing the abundance of non-Hg methylating microorganisms that are "mutually beneficial" to demethylating microorganisms. An increase in temperature is responsible for this effect by reducing microbial diversity, allowing more competitive microbes to take up ecological niches. Our study highlights the importance of temperature-induced alterations in microbial community composition as an important factor affecting MeHg production in rice field ecosystem. Since rice field ecosystem is a typical constructed wetland, our findings are expected to have reference significance for the response of Hg^{II} methylation/demethylation processes to climate change in sensitive ecosystems (e.g., wetland ecosystem). Furthermore, despite the limitations of the study, the microbial mechanisms behind the changes in microbial community composition and MeHg production caused by climate change should be emphasized.

CRediT authorship contribution statement

Qianshuo Zhang: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Qiang Pu:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Zhengdong Hao:** Writing – review & editing, Data curation. **Jiang Liu:** Writing – review & editing, Funding acquisition, Formal analysis. **Kun Zhang:** Writing – review & editing, Formal analysis. **Bo Meng:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Xinbin Feng:** Writing – review & editing, Resources, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.172832.

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