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Relative importance of aceticlastic methanogens and hydrogenotrophic methanogens on mercury methylation and methylmercury demethylation in paddy soils

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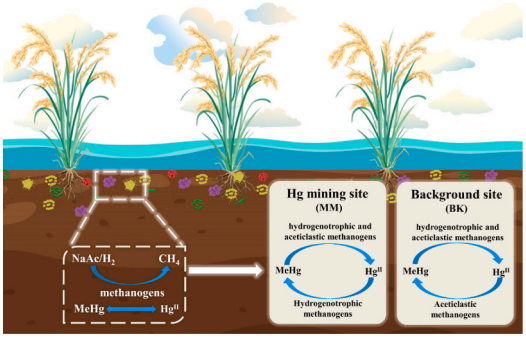
HIGHLIGHTS

- Roles of different types of methanogens on Hg methylation and demethylation in paddy soils were studied.
- Aceticlastic methanogens are the potential Hg methylation communities at control site.
- Hydrogenotrophic methanogens are involved in MeHg production as Hg level increased.
- Both aceticlastic and hydrogenotrophic methanogens facilitate MeHg degradation in paddy soils.

GRAPHICAL ABSTRACT



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ABSTRACT

The accumulation of methylmercury (MeHg) in paddy soil results from a subtle balance between inorganic mercury (e.g., Hg^{II}) methylation and MeHg demethylation. Methanogens not only act as Hg methylators but may also facilitate MeHg demethylation. However, the diverse methanogen flora (e.g., aceticlastic and hydrogenotrophic types) that exists under ambient conditions has not previously been considered. Accordingly, the roles of different types of methanogens in Hg^{II} methylation and MeHg degradation in paddy soils were studied using the Hg isotope tracing technique combined with the application of methanogen inhibitors/stimulants. It was found that the response of Hg^{II} methylation to methanogen inhibitors or stimulants was site-dependent. Specifically, aceticlastic methanogens were suggested as the potential Hg^{II} methylators at the low Hg level background site, whereas hydrogenotrophic methanogens were potentially involved in MeHg production as Hg levels increased. In contrast, both aceticlastic and hydrogenotrophic methanogens facilitated MeHg degradation across the sampling sites. Additionally, competition between hydrogenotrophic and aceticlastic methanogens was observed in Hg-polluted paddy soils, implying that net MeHg production could be alleviated by promoting

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aceticlastic methanogens or inhibiting hydrogenotrophic methanogens. The findings gained from this study improve the understanding of the role of methanogens in net MeHg formation and link carbon turnover to Hg biogeochemistry in rice paddy ecosystems.

1. Introduction

Methylmercury (MeHg), the organic form of mercury (Hg), is neurotoxic to humans and can be bioaccumulated and biomagnified along food chains (Meng et al., 2014; Beckers and Rinklebe, 2017; Liu et al., 2022b). Catastrophic poisoning episodes of birds, fish, and mammals have been reported in the late 1900s due to MeHg (Rumbold et al., 2001). The previous theory that the consumption of seafood is the main route of human MeHg exposure has been shown to not apply to certain populations in South and East Asia (Clarkson, 1993). This is related to MeHg accumulation in rice grain (Meng et al., 2010; Meng et al., 2011), which is the staple food for many of the continent's populous countries. In inland China, rice consumption is the predominant pathway for human MeHg exposure, particularly in Hg-polluted areas (Feng et al., 2008; Li et al., 2017). Furthermore, MeHg-contaminated rice has been reported by numerous researchers around the world (Lenka et al., 1992; Appleton et al., 2006; Krisnayanti et al., 2012; Rothenberg et al., 2014; Rothenberg et al., 2015; Aslam et al., 2020). Consequently, there has been an increased focus on the production of MeHg in paddies, which are the most widespread of constructed wetland ecosystems.

In situ methylation of inorganic mercury (Hg^{II}) in paddy soils results in the accumulation of MeHg in rice grain (Strickman and Mitchell, 2017; Liu et al., 2021b). The bioavailability of Hg^{II}, as well as factors such as the pH, redox potential, presence of dissolved organic matter (DOM), sulfur, iron, and microbial activity, all regulate MeHg formation (Ullrich et al., 2001; O'Connor et al., 2019; Wang et al., 2021; Abdelhafiz et al., 2023). Of these factors, microorganisms play a pivotal role in the production of MeHg in natural environment (Milliken et al., 2004), including sulfate-reducing bacteria (SRB), iron-reducing bacteria (IRB), methanogens (Hamelin et al., 2011; Yu et al., 2012), and some putative non-Hg methylators (Liu et al., 2019). The demethylation of MeHg is the opposite of Hg^{II} methylation (Barkay and Gu, 2022). Microbially-mediated demethylation was recognized as the major pathway for MeHg degradation in flooded paddy soils (Wu et al., 2020; Strickman et al., 2022). Currently, reductive demethylation (RD) and oxidative demethylation (OD) are two known pathways that regulate biotic demethylation. The latter (i.e., OD) mainly occurs in anoxic environments (e.g., wetlands and paddy soils), and the main bacteria involved in this process are SRB and methanogens (Oremland et al., 1991; Marvin-Dipasquale and Oremland, 1998). In particular, methanogens not only act as Hg^{II} methylators (Yu et al., 2013; Blum et al., 2017), but may also be involved in biotically-mediated MeHg demethylation in natural environments (Pak and Bartha, 1998).

Our previous study suggested that methanogen-mediated methanogenesis promoted the demethylation of MeHg in Hg-polluted paddy soils (Wu et al., 2020). Additionally, it has also been reported that methanotrophs can degrade MeHg through the binding of MeHg with methanobactin (Lu et al., 2017; Kang-Yun et al., 2022). These findings suggest that methanogenesis may be closely coupled with the net formation of MeHg in paddy soils but there have been few studies on this. It is known that methanogens consist of hydrogenotrophic methanogens and aceticlastic methanogens. Hydrogenotrophic methanogens can reduce CO_2 to CH_4 with H_2 as the major electron donor; whereas aceticlastic methanogens activate acetate to acetyl coenzyme A (acetyl-CoA) (Liu and Whitman, 2008). However, the relative importance of these two methanogens in Hg^{II} methylation and MeHg demethylation in paddy soils remains unknown.

Herein, it was hypothesized that different types of methanogens play divergent roles in Hg^{II} methylation and MeHg demethylation in rice paddy soils due to their different metabolic pathways. To verify this hypothesis, a joint incubation experiment with specific metabolic inhibitors (i.e., CH_3F) or stimulants (i.e., NaAc, H_2/CO_2) for different types of methanogens was carefully designed. Isotopically-enriched Hg tracers including $^{202}Hg^{II}$ ($^{202}Hg(NO_3)_2$) and $Me^{198}Hg$ ($Me^{198}HgNO_3$) were employed to trace the specific Hg^{II} methylation and MeHg demethylation in paddy soils during incubation, simultaneously. The objectives of this study were to identify the role of different types of methanogens in Hg^{II} methylation and MeHg demethylation, as well as the underlying mechanisms. The results of this study will provide a better understanding of the influence of methanogenesis on net MeHg production in rice paddy ecosystems.

2. Materials and methods

2.1. Site description and sampling

Two paddy fields in Guizhou Province, China, were selected for this study. One (Hg mining site, MM, 109°12′10.08″E, 27°30′38.88″N) is in a large-scale abandoned Hg mining region in the Wanshan Hg mining area, which is mainly contaminated by legacy Hg mining wastes. The other one (background control site, BK, 106°31′0.48″E, 26°24′58.32″N) is in the rural area close to Guiyang City. There is no distinct Hg pollution source around this site. The climate and geological conditions of the two sites are similar (Liu et al., 2022a; Pu et al., 2022). Detailed information on the sampling sites is available in our previous studies (Wu et al., 2020; Liu et al., 2023).

The sampling campaign was conducted in August 2021 (50–60 days after the transplanting of rice seedlings). Surface soil (1–10 cm below the soil-water interface) was collected in 1 L gas-tight brown glass bottles without remaining headspace. Overlying water samples (1–3 cm above the soil-water interface) were carefully collected using a pre-cleaned syringe and then transferred into gas-tight glass bottles (no headspace). All the bottles were double-packed into Ziplock bags to avoid any cross-contamination. Samples were transported to the laboratory in a cooler within 1 day and stored at 4 °C in the dark until the commencement of the incubation experiment. It's noted that the Hg-polluted paddy soils were stored for 30 days before the incubation while the Hg uncontaminated soils were incubated in 5 days.

2.2. Incubation experiments

Anaerobic incubation experiments were performed in an O_2-free (filled by N_2) glovebox (PLAS-LABS, USA). Soil samples were mixed and stirred to homogenize in a 2 L beaker by adding the corresponding overlying water. The moisture content of the prepared slurries (BK and MM) was around 55 %. The prepared soil slurries (30 mL) were divided into 100 mL gas-tight borosilicate glass bottles for further incubation. Lueders and Friedrich (2000) found that the available terminal electron acceptors (SRB and IRB) which can compete with methanogens were exhausted, the microbial metabolic processes of SRB and IRB were largely stopped (Fe^{3+} and SO_4^{2-} reduction) in anoxically incubated rice field soil slurries after 7 days. To minimize the influences of other reduction reactions (e.g., sulfate and iron reduction) on methanogenesis, all of the samples in bottles were preincubated for one week (25 °C, in the dark) in the glovebox. Parallel experiments were then conducted with five treatments in triplicate with soil samples from both BK and MM (Table 1). Untreated soils functioned as the control. Gamma-irradiated soils (16 kGy gamma irradiation) were treated as the abiotic control (McNamara et al., 2003). Specific inhibitors or stimulants were

Table 1
Amounts of specific inhibitors/stimulants added for each incubation experiment.[a]

Treatments	Quantity/Dose	Effects	Reference
Control	no addition	control	
Abiotic control	16 kGy Gamma-ray irradiation	sample sterilization (abiotic)	(McNamara et al., 2003)
NaAc	5 mM	promotes aceticlastic methanogens	(Liu and Conrad, 2010)
H_2/CO_2	under H_2/CO_2 mixture (4:1, *v/v*) at 1.5 kPa	promotes hydrogenotrophic methanogens	(Long et al., 2017)
CH_3F	2 %	inhibits aceticlastic methanogens	(Conrad and Klose, 1999)

[a] 1 mM molybdate (Na_2MoO_4) solution was added for all the treatments.

employed in this study to distinguish the role of different types of methanogens in Hg^{II} methylation and MeHg demethylation (Table 1). Sodium acetate (NaAc) and H_2/CO_2 were used to stimulate aceticlastic and hydrogenotrophic methanogens, respectively (Liu and Conrad, 2010; Long et al., 2017). Methyl fluoride (CH_3F) was selected to inhibit aceticlastic methanogens (Frenzel and Bosse, 1996). Molybdate (Na_2MoO_4) was added to the soil slurries for each treatment to inhibit SRB during incubation (Stams et al., 2005; Wu et al., 2020). Enriched Hg isotope tracers including $^{202}Hg(NO_3)_2$ and $Me^{198}HgNO_3$ were spiked into soil slurries to trace the Hg^{II} methylation and MeHg demethylation, respectively. The amounts of spiked $^{202}Hg^{II}$ and $Me^{198}Hg$ tracers were ~ 10 % and ~ 100 % of soil ambient total Hg (THg) and MeHg, respectively (Wu et al., 2020; Liu et al., 2022a). More details on the preparation of the $^{202}Hg^{II}$ and $Me^{198}Hg$ tracers are provided in Text S1 (Supplementary Information, SI).

The incubation was conducted in the dark at room temperature (25 °C) for 12 h due to the systematic underestimation of rate constants over longer durations (Helmrich et al., 2022). Two specific subsampling periods were set at the 0th and the 12th h during the incubation. However, due to the periods of incubation, bottle preparation, and subsampling, the actual time series for MeHg was 4, 16 h. Aliquots from three random bottles were destructively sampled in the glovebox after measuring the CH_4 concentrations in the headspace of the incubation bottles. Samples for the analysis of isotopic MeHg concentrations and DOM were collected into 50 mL polypropylene (PP) tubes (JET, BIOFIL, China), stored at −20 °C, and then freeze-dried and ground evenly through a 200-mesh sieve using an agate mortar. The soil samples for 16S rRNA sequencing were collected into cryogenic tubes (2 mL, Thermo Scientific, Nalgene®, USA), and stored at −80 °C. Samples for sulfide (S^{-II}) and iron (Fe^{II} and Fe^{III}) analysis were collected into 50 mL PP tubes (JET, BIOFIL, China) and were extracted by centrifugation (RCF = 2850 *g* for 10 min at 4 °C) and filtration (0.45 μm PES filter, JIN TENG®, China).

2.3. Analytical methods

Gas chromatography-inductively coupled plasma mass spectrometry (GC-ICP-MS, Agilent 7700×, Agilent Technologies Inc., USA) followed the ethylation-purge trap method was employed to determine MeHg isotopes (Hintelmann et al., 1995; Hintelmann et al., 2000; Meng et al., 2018; Liu et al., 2023). 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; Hintelmann et al., 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; Hintelmann et al., 2000; Liu et al., 2023):

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

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

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 4 or 16 h (t_i); $[^{202}Hg^{II}]$ is the $^{202}Hg^{II}$ concentration in the initial spike added to the soils. More calculation details are shown in Text S2.

The CH_4 in the headspace of each incubation bottle at the 16th h was collected using a Pressure-Lok precision analytical syringe (VICI, USA) and then directly injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID) (Yuan et al., 2014). Sulfide (S^{-II}) in the aqueous phase of soil slurry was determined using the Cline method (Cline, 1969). Ferrous (Fe^{II}) and ferric (Fe^{III}) cations were measured using the ferrozine method (Viollier et al., 2000). The detailed DOM characterization methods are given in Text S3.

DNA was extracted from soil slurry using a FastDNA® Spin Kit for Soil (MP Bio medicals, USA) with a Fastprep bead beater (MO BIO Laboratories, Inc., USA). The DNA concentration was measured by a Nanodrop 2000 spectrophotometer (Thermo, USA). The extracted DNA samples were stored at −80 °C for further analysis. Primers Delta-F, 5'-GCCAACTACAAGMTGASCTWC-3′ and Delta-R, 5'-CCSGCNGCRCAC-CAGACRTT-3′, targeting the *hgcA* gene (Christensen et al., 2016), were used for quantitative real-time PCR (qPCR) on ABI7500 (Applied Biosystems, USA). The reaction parameters were 94 °C for 3 min; 40 cycles of 95 °C for 15 s, 65 °C for 30 s; and 72 °C for 4 min. To characterize the soil microbial communities, the V3-V4 region of the microbial 16S rRNA gene was amplified using universal bacterial (515F: 5'-GTGY-CAGCMGCCGCGGTAA-3′, R806: 5'-GGACTACNVGGGTWTCTAAT-3′,) and archaeal (524F: 5'-TGYCAGCCGCCGCGGTAA-3′, Arch958R: 5'-YCCGGCGTTGAVTCCAATT-3′) primers. More details on the 16S rRNA sequencing and Real-time qPCR are available in Text S4.

2.4. QA/QC and statistics

Quality control for MeHg isotope analysis in 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^{-1}. The deviation of the triplicate samples was generally <15 % of the mean concentration. The data are presented as the mean ± standard error (SE). An average MeHg concentration of 75.8 ± 6.6 μg·kg^{-1} (n = 16) was obtained from CC580, which was comparable to the certified value of 75.5 ± 3.7 μg·kg^{-1}. The standard gas CH_4 (0.1 % CH_4 and 0.1 % CO_2 in N_2, mol/mol) was provided by the SWCHEM Southwest Institute of Chemical Co., Ltd. Quality analyses were all performed using SPSS 22.0 (IBM®, IL, USA) and Origin 2019 (OriginLab®, MA, USA). *t*-test was employed to deduce statistically significant differences (p < 0.05, 2-tailed) between different treatments.

3. Results and discussion

3.1. Methane production

Methane (CH_4) production reflected the activity of methanogens during the incubation, providing direct evidence of methanogenesis. At the BK, the CH_4 concentrations in the H_2/CO_2 treatment (promoting hydrogenotrophic methanogens, 0.32 ± 0.02 μM) were significantly higher than those in the control (0.27 ± 0.01 μM, p < 0.05, Fig. 1a). As expected, the lowest CH_4 concentration was observed in the CH_3F treatment (0.18 ± 0.01 μM, p < 0.05, Fig. 1a), in which CH_3F was used to inhibit aceticlastic methanogens. In contrast, when NaAc was used to promote aceticlastic methanogenesis, the CH_4 concentration in the NaAc treatment (0.22 ± 0.03 μM) was similar to that in the control (p > 0.05, Fig. 1a).

At the MM, the CH_4 concentrations in the H_2/CO_2 treatment (0.64 ±

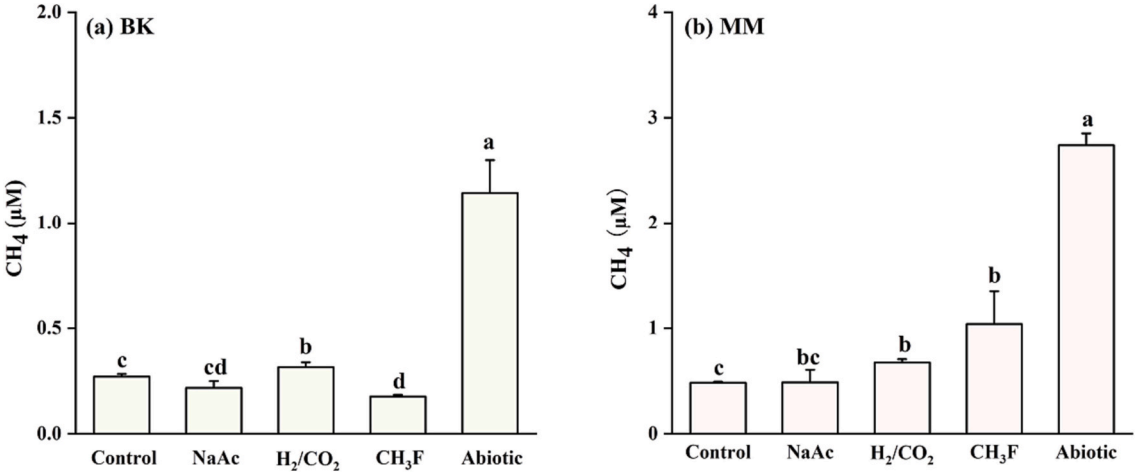

Fig. 1. Methane (CH_4) production at the BK (a) and the MM (b) during the incubation across the different treatments. Error bars represent the standard error (±SE) for replicates ($n = 3$). Different lower-case letters indicate the significant difference between treatments at the 16th h (*t*-test, $p < 0.05$).

0.03 μM) were 1.3 times higher than those in the control (0.48 ± 0.01 μM) during the incubation period, suggesting methanogenesis was promoted by the addition of H_2/CO_2 ($p < 0.05$, Fig. 1b). The CH_4 concentrations in the NaAc and CH_3F treatments for the MM were different to expectations: although NaAc was expected to promote aceticlastic methanogenesis, comparable CH_4 concentrations were observed in the control and NaAc treatments ($p > 0.05$). The CH_3F treatment displayed a high cumulative CH_4 output (1.04 ± 0.31 μM) compared to the control ($p < 0.05$, Fig. 1b).

Unexpectedly, highly elevated CH_4 concentrations were observed in the abiotic treatment for both the BK and the MM during the incubation ($p < 0.05$, Fig. 1). This could be attributed to the decomposition of soil organic matter (SOM) under gamma irradiation. Gamma irradiation was confirmed to change the compositional structure of SOM, and promote the degradation of organic matter (Salonius et al., 1967; Lensi et al., 1991; Berns et al., 2008). The UV–vis absorption and fluorescence spectra of soil DOM revealed that the intensity of peak B (and peak T) and the S_R value in the abiotic treatment were significantly higher than those in the control ($p < 0.05$, Fig. S5). This implies that more low-molecular-weight organic compounds were released after irradiation (Helms et al., 2008; Fleck et al., 2014; Qin et al., 2020). Thus, it was inferred that the production of CH_4 in the gamma-irradiated samples was via an abiotic process and mainly from SOM decomposition (Wang et al., 2013).

3.2. Relative abundances and population dynamics of methanogens

Methanogens accounted for 3.9–4.5 % and 13.7–16.9 % of the archaeal communities at the BK and the MM, respectively (Fig. S3c and d). *Methanobacterium* (1.9–3.6 %, hydrogenotrophic methanogens) and *Methanosarcina* (1.8–2.3 %, aceticlastic methanogens) were the most abundant methanogen genera at the BK. Six methanogens genera were identified at the MM, including two aceticlastic methanogens (*Methanoseta*, *Methanosarcina*) and four hydrogenotrophic methanogens (*Methanobacterium*, *RC-I*, *Methanocella*, *Methanoregula*) (Fig. S3d). At the MM, *Methanoseta* represented the highest relative abundance (5.3–6.9 %), followed by *Methanobacterium* (3.9–5.8 %) and *Methanosarcina* (1.5–2.1 %); the relative abundances of each other methanogen was below 2 % (*RC-I*, 1.1–1.5 %; *Methanocella*, 0.9–1.2 %; *Methanoregula*, 0.7–1.0 %) (Fig. S3d).

At the BK, we note that the relative abundances of *Methanobacterium* and *Methanosarcina* were relatively low in the soil, and there was no significant difference between 4th h and 16th h within the treatments with aceticlastic methanogens in the CH_3F treatment as an exception. However, the relative abundance of hydrogenotrophic methanogens in the H_2/CO_2 treatment at the 16th h was generally higher than that at the 4th h ($p = 0.2$, Fig. 2a). The relative abundance of aceticlastic methanogens in the NaAc treatment at the 16th h was higher than that at the 4th h (Fig. 2c). The relative abundance of aceticlastic methanogens at the 16th h was significantly lower than that at the 4th h in the CH_3F treatment ($p < 0.05$, Fig. 2c). The abundances of aceticlastic methanogens and hydrogenotrophic methanogens showed similar results (Fig. S4a and c). All these results indicate that the inhibitors (i.e., CH_3F) and stimulants (i.e., NaAc, H_2/CO_2) addition were functioning as expected at the BK during incubation.

At the MM, there was no significant difference in the relative abundances of aceticlastic methanogens among the control, NaAc, H_2/CO_2 and CH_3F treatments either at the 4th h or 16th h ($p > 0.05$, Fig. 2d). The relative abundance of hydrogenotrophic methanogens in the NaAc treatment at the 16th h was significantly lower than that at the 4th h ($p < 0.05$, Fig. 2b). Additionally, a higher relative abundance of hydrogenotrophic methanogens was observed in the H_2/CO_2 and CH_3F treatments compared with the control either at the 4th h or 16th h ($p < 0.05$, Fig. 2b). The abundances of hydrogenotrophic methanogens were increased with the H_2/CO_2 or CH_3F addition (Fig. S4c). Besides, the abundance of aceticlastic methanogens showed an increase in the NaAc treatment while those decreased in the H_2/CO_2 and CH_3F treatments (Fig. S4d). It could find that the abundance of aceticlastic methanogens and hydrogenotrophic methanogens have an opposite change within the treatments during the incubation at the MM.

3.3. The role of different methanogens in methane production

The abundance of hydrogenotrophic methanogens and the formation of CH_4 were enhanced by the addition of H_2/CO_2 for both the BK and the MM (Figs. 1 and 2). This suggests that H_2/CO_2 was stimulating hydrogenotrophic methanogenesis in the incubation slurries. A lower CH_4 concentration and relative abundance of aceticlastic methanogens were obtained in the CH_3F treatment than those in the control at the BK. This indicates that aceticlastic methanogens were theoretically inhibited after the addition of CH_3F (Frenzel and Bosse, 1996; Hao et al., 2013). Additionally, there was no significant difference in the relative abundances of hydrogenotrophic methanogens between the control and CH_3F treatments at the BK ($p > 0.05$, Fig. 2a). Meanwhile, the abundance of aceticlastic methanogens in the H_2/CO_2 treatment was comparable with that in the control during the incubation at the BK (Fig. 2c). All the results described above indicate that aceticlastic methanogens (e.g., *Methanosarcina)* and hydrogenotrophic methanogens (e.g., *Methanobacterium)* may occupy different niches in the paddy soils at the BK. Therefore, the competition between aceticlastic methanogens and

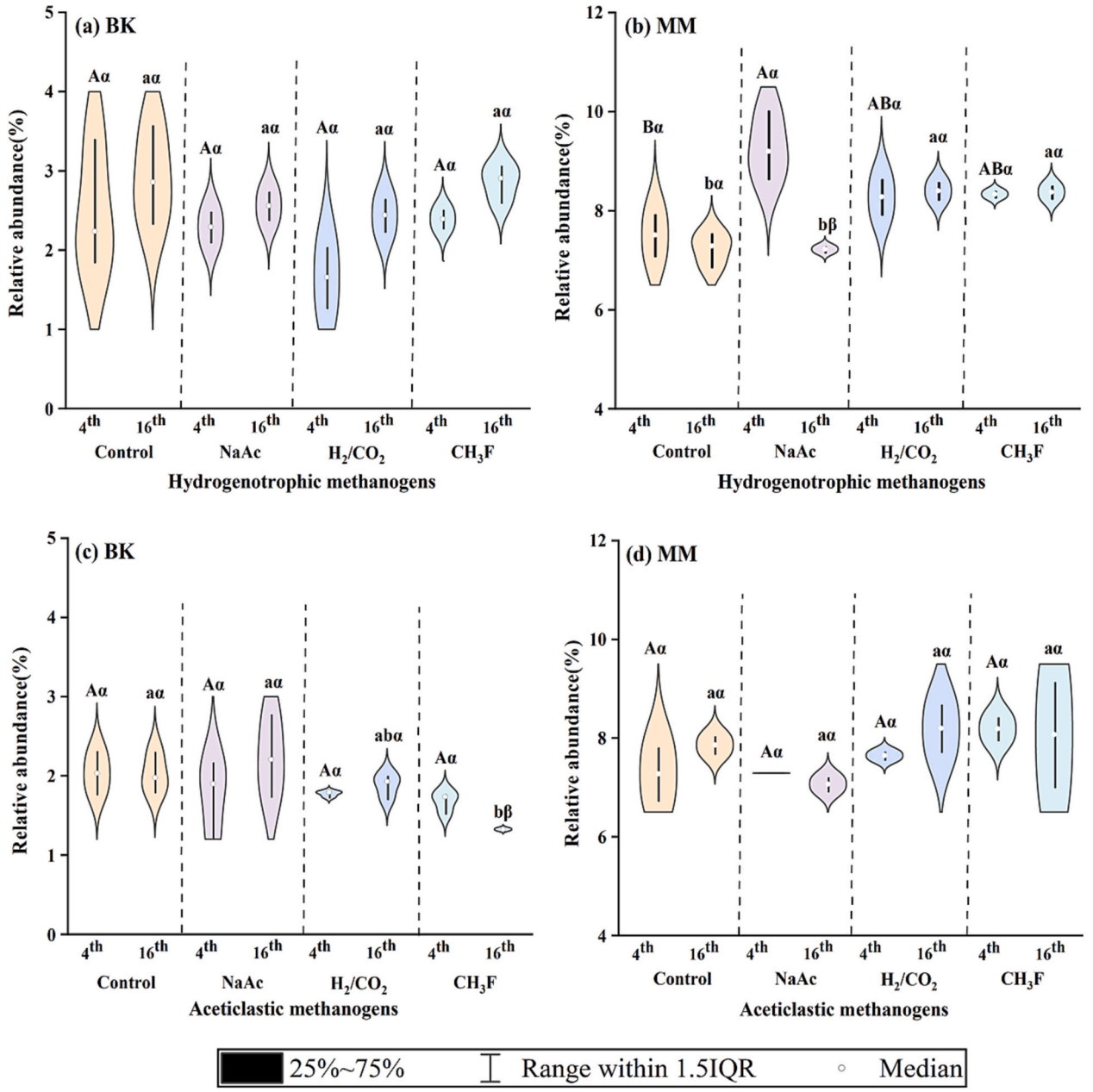

Fig. 2. Relative abundances of different methanogen genera at the BK (a, c) and the MM (b, d) during the incubation across the different treatments after 4th and 16th hours. Different capital letters and lower-case letters indicate the significant difference between treatments at different times (t-test, $p < 0.05$). Different Greek letters suggest that the differences within each treatment at different times are significant (t-test, $p < 0.05$).

hydrogenotrophic methanogens was absent at the BK.

At the MM, higher CH_4 concentrations were found in the CH_3F treatment during incubation compared to the control (Fig. 1b). Although a non-significant difference in the relative abundance of aceticlastic methanogens between the control and CH_3F treatments was observed at the MM, the relative abundance of hydrogenotrophic methanogens in the CH_3F treatment was significantly higher than that in the control during the incubation (4th h and 16th h, Fig. 2b and d). The inhibition of aceticlastic methanogens including *Methanosaeta*, is likely to promote the activity of hydrogenotrophic methanogens (Hao et al., 2012). This suggests that hydrogenotrophic methanogens and aceticlastic methanogens were potentially competitive at the MM during the incubation.

NaAc was employed as the substrate for aceticlastic methanogens in this study (Nolla-Ardevol et al., 2012). However, no discernable differences in CH_4 concentrations between the control and NaAc treatments were observed during the incubation for both the BK and the MM (Fig. 1b). It should be noted that the relative abundance of aceticlastic methanogens in the NaAc treatment increased with NaAc addition, while the relative abundance of hydrogenotrophic methanogens were not significant change during the incubation at the BK (Fig. 2c). In fact, it is noted that some *Methanosarcina* species can both growth on H_2 and CO_2 and grow on NaAc, either the aceticlastic or the hydrogenotrophic methanogenesis pathway is functional in *Methanosarcina* (Jetten et al., 1992; Thauer et al., 2008; De Vrieze et al., 2012). It was plausible that NaAc attenuated the hydrogenotrophic methanogenesis pathway of *Methanosarcina* at the BK. Hydrogenotrophic methanogens were inhibited in the NaAc treatment at the MM (Fig. 2b). The aceticlastic methanogenesis yield more energy than the hydrogenotrophic methanogenesis (Lyu et al., 2018). Therefore, although aceticlastic methanogenesis was promoted in the NaAc treatment, similar CH_4 concentrations were observed between the control and NaAc treatments, possibly due to the inhibition of hydrogenotrophic methanogenesis.

3.4. The role of hydrogenotrophic and aceticlastic methanogens in Hg^{II} methylation

The use of metabolic inhibitors/stimulants and stable isotope tracers enabled the relative importance of different methanogens in Hg^{II} methylation and MeHg demethylation to be determined at the given sites. Although sulfate reduction and iron reduction processes are important in Hg^{II} methylation and MeHg demethylation. Our results showed that the preincubation and the addition of molybdate in all treatments for BK and MM resulted in the inhibition of sulfate reduction and the extremely high ratio of Fe^{II}/TFe and rare relative abundance of *Geobacter* genus in paddy soils implying that IRB could be negligible during incubation (Text S6). The much higher Hg^{II} methylation rates at the MM were observed than those at the BK, which is different from the result in our previous study (Liu et al., 2022a). Ambient MeHg concentrations in the control treatment at the MM during the incubation were also much higher than the background MeHg concentration (Tables S1 and S4). The possible reason is that the Hg-polluted soils were stored for about 30 days, and preincubation was conducted for one week before adding Hg^{II} isotope tracers for further incubation. The metabolic processes of methanogens could be potentially accelerated after the preincubation (Lueders and Friedrich, 2000). Both the relative abundances of methanogens and CH_4 concentrations at the MM were higher than those at the BK indicating that methanogens were more active at the MM. Therefore, the conditions at the MM could be more favorable to Hg^{II} methylation than that at the BK in this study.

Similar distribution patterns of $^{202}Hg^{II}$ methylation and $Me^{198}Hg$ demethylation were found among the five treatments when plotting the K_m and K_d at the 4th h vs the corresponding values at the 16th h. However, more rapid methylation of $^{202}Hg^{II}$ and demethylation of $Me^{198}Hg$ were observed after a shorter incubation (i.e., 4 h) when compared with an extended-time incubation (i.e., 16 h) at both sites ($p < 0.05$, Fig. 3 and Fig. S6). Moreover, the differences in K_m and K_d among the treatments for BK or MM at the 4th h were more evident than those at the 16th h. This was consistent with our previous finding that the Hg methylation and demethylation processes were more active during the initial incubation period (Liu et al., 2022a). Besides, possible reactions like adsorption and precipitation of dissolved Hg^{II} tracer may influence MeHg concentration and result in undervaluation of K_m with longer incubation periods. Thus, the shorter incubation time can decrease the uncertainty to K_m values (Olsen et al., 2018; Helmrich et al., 2022). Our previous study also found that the K_m and K_d had a sharp decrease from 4 to 16 h and a slight decrease thereafter, and the K_m and K_d values among different treatments showed more significant statistical difference at the 4th h while the difference may not be significant after 16 h (Chen et al., 2023; Liu et al., 2023). Therefore, the following discussion on the role of hydrogenotrophic and aceticlastic methanogens in Hg^{II} methylation and MeHg demethylation will logically focus on the K_m and K_d at the 4th h.

At the BK, the highest K_m was found in the NaAc treatment ($13.03 \pm 0.84\ 10^{-3}$ day^{-1}, $p < 0.05$, Fig. 3a). Comparable K_m values for the control ($7.48 \pm 0.18\ 10^{-3}$ day^{-1}), H_2/CO_2 ($7.14 \pm 1.67\ 10^{-3}$ day^{-1}) and CH_3F ($7.86 \pm 1.32\ 10^{-3}$ day^{-1}) treatments were obtained at the BK (Fig. 3a). At the MM, there was no discernable difference in K_m between the NaAc treatment ($53.76 \pm 0.86\ 10^{-3}$ day^{-1}) and the control ($54.1 \pm 1.80\ 10^{-3}$ day^{-1}) ($p > 0.05$, Fig. 3b). The methylation rate constants (K_m) in the H_2/CO_2 and CH_3F treatments were significantly higher than that in the control during the incubation at the MM ($p < 0.05$, Fig. 3b).

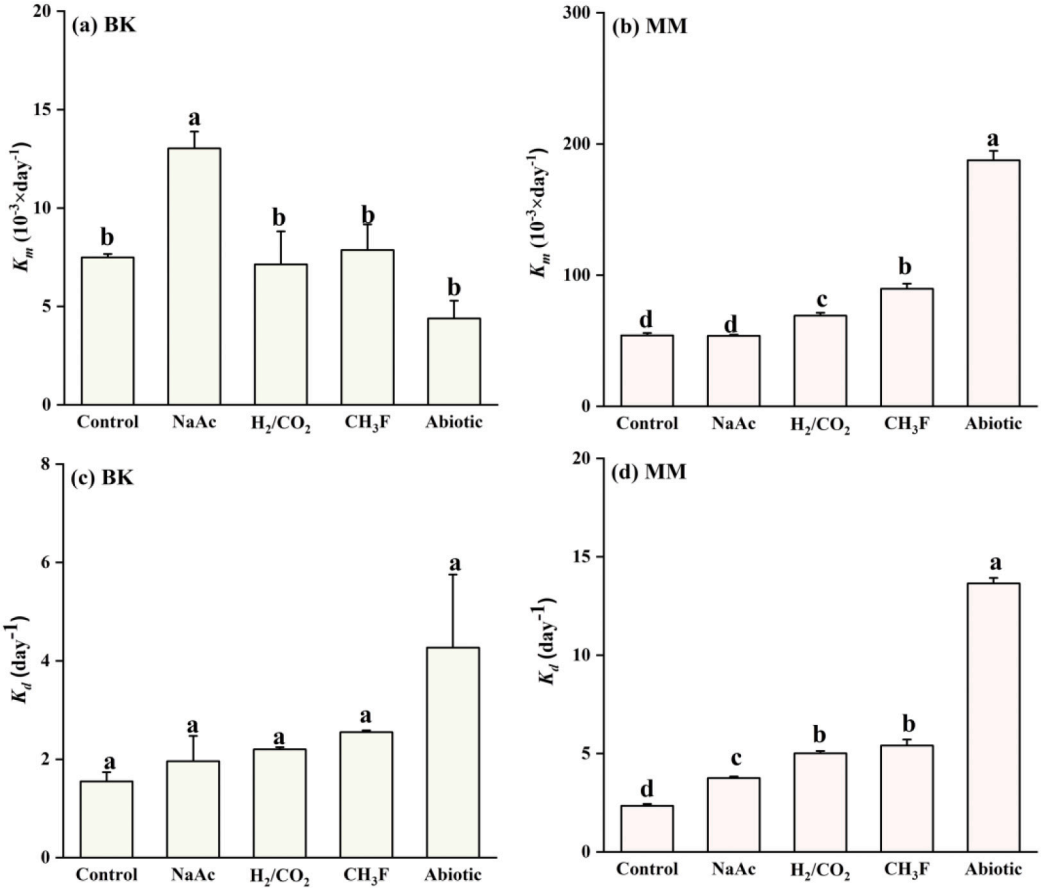

Fig. 3. The methylation rate constant (K_m) and demethylation rate constant (K_d) (c and d) at the BK (a and c) and the MM (b and d) during the incubation across the different treatments at the 4th h. Error bars indicate the standard error (±SE) for replicates ($n = 3$). Different capital letters and lower-case letters indicate the significant difference between treatments at different times (t-test, $p < 0.05$).

The highest K_m was observed in the abiotic treatment at the MM (Fig. 3b).

Hydrogenotrophic methanogens and aceticlastic methanogens played divergent roles in Hg^{II} methylation at the BK. Firstly, the activity of hydrogenotrophic methanogens and the corresponding CH_4 production was promoted in the H_2/CO_2 treatment compared to the control (Fig. 1a). However, the expected increase in K_m was absent in the H_2/CO_2 treatment (Fig. 3a), suggesting that the dominant hydrogenotrophic methanogen (i.e., *Methanobacterium*) did not facilitate Hg^{II} methylation. The possible reason is that the *Methanobacteria* class (e.g., *Methanobacterium*) does not carry *hgcA* genes, and therefore, unable to produce MeHg (Yu et al., 2013; Gilmour et al., 2018).

Our result showed that aceticlastic methanogens represented relatively low abundances though SRB was inhibited during the incubation at the BK (Fig. 2a, c). Previous study also found that sulfate-reduction rather than methanogenesis was the main driver of MeHg formation at the background site (Wu et al., 2020). However, NaAc can act as source substrates and influence the activity of aceticlastic methanogens (Hao et al., 2013; Roth et al., 2021). Besides, the relative abundance and copy numbers of aceticlastic methanogens were generally increased in the NaAc treatment during the incubation when compared to those in the control (Fig. 2c and Fig. S4c), which indicated that aceticlastic methanogens was promoted in the NaAc treatment at the BK. When compared with control, significantly higher methylation rate constant (K_m) was observed in the NaAc treatment (Fig. 3a) suggesting that aceticlastic methanogens were potentially involved in Hg^{II} methylation (Gilmour et al., 2013; Bae et al., 2019; Wang et al., 2020). However, comparable K_m values were observed between the control and CH_3F treatments at the BK (Fig. 3a). It is well known that NaAc is a broad-spectrum carbon source that is utilized by numerous microbes including aceticlastic methanogens (Roh et al., 2002; Brasen and Schonheit, 2004; Wan et al., 2015). Although aceticlastic methanogens were inhibited in the CH_3F treatment (Figs. 1a and 2c), there were still some microorganisms (i.e., *Proteobacteria*, *Chloroflexi*) which not only use acetate as a carbon source but also perform Hg^{II} methylation. Therefore, the comparable K_m values between the control and CH_3F treatments could be attributed to the promotion of microorganism activity (i.e., *Proteobacteria*, *Chloroflexi*) in the CH_3F treatment at the BK. It was evidenced by the higher relative abundance of *Proteobacteria* and *Chloroflexi* in the CH_3F treatment compared to the control at the BK (Fig. S3).

The Hg^{II} methylation (K_m) response to methanogen inhibitor or stimulant addition treatments was highly site-specific, which complicates MeHg cycling in rice paddy. At the MM, hydrogenotrophic methanogens were promoted by the addition of H_2/CO_2 (Fig. 2b) or the addition of CH_3F (Fig. 2b); both increased the production of CH_4 ($p < 0.05$, Fig. 1b). Additionally, the addition of NaAc yielded comparable CH_4 production and K_m values compared to the control treatment at the MM. This suggests that aceticlastic methanogens mediated Hg^{II} methylation was less pronounced at the MM. *Methanosaeta* (5.3–6.9 %) was observed as the dominant aceticlastic methanogen at the MM (Fig. S3). Previous studies have reported that *Methanosaeta* was not a dominant microorganism in Hg^{II} methylation (Du et al., 2021). *Methanosaeta* does not contain unique corrinoid proteins, which differs from *Methanosarcina* (i.e., aceticlastic methanogens at the BK) (Jetten et al., 1991; Ferry, 1992). Additionally, *Methanosaeta* probably does not contain *hgcAB* orthologues (Podar et al., 2015). Therefore, Hg^{II} methylation was unlikely to proceed via *Methanosaeta* at the MM.

In contrast, hydrogenotrophic methanogens at the MM played an important role in Hg^{II} methylation. This was evidenced by the higher relative abundance of hydrogenotrophic methanogens and the elevated K_m values in the H_2/CO_2 treatment compared with those in the control (Figs. 2 and 3). Furthermore, *Methanocella* and *Methanoregula*–as typical hydrogenotrophic methanogens–belong to *Methanomicrobia* and were previously recognized as potential Hg^{II} methylating microorganisms (Liu et al., 2021a; Roth et al., 2021). During the process of methanogenesis, hydrogenotrophic methanogens produce CH_3-H_4MPT which was the CH_3-THF analogue (Thauer, 1998; Hendrickson et al., 2007; Ma et al., 2019). It suspected that CH_3-H_4MPT (H_4SPT) may be catalyzed by a methyltransferase (MeTr), and the methyl group was utilized in Hg^{II} methylation like CH_3-THF (Parks et al., 2013; Tang et al., 2020). It should be noted that the data supporting this hypothesis are limited. Thus, further work is still desirable to ascertain this process. Notably, both the K_m values and the relative abundance of hydrogenotrophic methanogens in the CH_3F treatment were higher than those in the control treatment (Figs. 2b and 3b). This further indicates that competition between aceticlastic methanogens and hydrogenotrophic methanogens occurred in methanogenesis and Hg^{II} methylation at the MM. Meanwhile, promoting hydrogenotrophic methanogens or suppressing aceticlastic methanogens at the MM would have a similar effect on Hg^{II} methylation (Fig. 3b). This is further evidenced by the relative distribution patten and increase of the ambient MeHg concentrations in the H_2/CO_2 and CH_3F treatments (Table S4).

It is observed that $^{202}Hg^{II}$ in gamma-irradiated slurries produced more $Me^{202}Hg$ (higher K_m) than other treatments at the MM ($p < 0.05$, Fig. 3b). The abiotic treatment also yielded comparable K_m values to the control treatment at the BK (Fig. 3a). During the incubation, the activity of microorganisms was ideally inhibited in the abiotic treatment at the BK and the MM (Text S7). Therefore, the observed $Me^{202}Hg$ formation in gamma-irradiated slurries was attributed to the abiotic pathway (Skyring and Thompson, 1966; Salonius et al., 1967; Lensi et al., 1991). Copies of the *hgcA* gene in the abiotic treatment were significantly lower than those in the control ($p < 0.05$, Fig. S7). The decomposition of SOM in the abiotic control potentially provided active methyl donors and therefore promoted abiotic Hg^{II} methylation (Siciliano et al., 2005; Miller et al., 2009). However, the exact processes of abiotic methanogenesis and the related Hg^{II} methylation as well as the underlying mechanism in gamma-irradiated slurries still require further study.

3.5. The role of hydrogenotrophic and aceticlastic methanogens in MeHg demethylation

Similar to the variation patterns of K_m, higher demethylation rate constant (K_d) values were generally observed at the 4th h than at the 16th h for both BK and MM ($p < 0.05$, Fig. 3 and Fig. S6). In alignment with our previous study, the paddy soils at MM displayed higher K_d values than that at BK (Liu et al., 2022a). At the BK, the K_d values in the NaAc treatment (1.96 ± 0.51 day^{-1}) were generally higher than those in the control treatment (1.55 ± 0.19 day^{-1}) (Fig. 3c). The K_d values in the H_2/CO_2 treatment (2.20 ± 0.05 day^{-1}, $p = 0.135$) and CH_3F treatment (2.55 ± 0.04 day^{-1}, $p = 0.063$) were 1.4–1.7 times higher than those in the control treatment (1.55 ± 0.19 day^{-1}) (Fig. 3c). At the MM, the NaAc treatment (3.76 ± 0.08 day^{-1}), H_2/CO_2 treatment (5.01 ± 0.12 day^{-1}), and CH_3F treatments (5.41 ± 0.30 day^{-1}) yielded significantly higher K_d values than the control treatment (2.34 ± 0.10 day^{-1}) ($p < 0.05$, Fig. 3d). Moreover, the K_d values in the NaAc treatment were significantly lower than those in the H_2/CO_2 and CH_3F treatments ($p < 0.05$, Fig. 3d). The highest K_d values were observed in the abiotic control treatment at the BK and the MM ($p < 0.05$, Fig. 3c and d). Notably, K_d values showed similar distribution patterns among the treatments both for the BK and the MM (Fig. 3c and d). However, the significant differences of K_d values among the treatments were limited at the MM but less pronounced at the BK, which could be attributed to the lower relative abundance of methanogens at the BK compared to that at the MM (Fig. 2). Besides, previous study also found that methanogenesis played more important role in MeHg degradation as Hg concentrations increased (Wu et al., 2020).

In this study, MeHg demethylation was promoted in the NaAc and H_2/CO_2 treatments during incubation for both BK and MM (Fig. 3c and d). This suggests that both aceticlastic and hydrogenotrophic methanogens were potentially involved in MeHg demethylation in paddy soils. Furthermore, we observed higher K_d values in the H_2/CO_2 treatment at the BK and the MM when compared to those in the NaAc treatment

(Fig. 3c and d), implying that hydrogenotrophic methanogens rather than aceticlastic methanogens were the primary MeHg demethylators in paddy soils. There was likely a coupling relationship between the MeHg demethylation process and methanogenesis at the MM, as indicated by the significant correlation between the CH_4 concentrations and K_d values in the control, NaAc, H_2/CO_2 and CH_3F treatments at the MM (Fig. S8). Our previous study suggested that methanogenesis was associated with MeHg degradation in paddy fields (Wu et al., 2020). However, the mechanism of methanogen-mediated MeHg demethylation in paddy soils remains unclear and needs further exploration.

Gamma irradiation yielded a higher K_d than the control for both sites during the incubation. The qPCR results suggested that the activity of microbes including methanogens was inhibited by gamma irradiation in the abiotic control treatment as mentioned above (Section 3.4). A previous study also showed that MeHg concentrations decreased consistently in sterilized soil during the initial incubation period (Zhou et al., 2020). This is possibly due to the formation of reactive oxygen species (e.g., hydroxyl radicals) upon gamma irradiation (Tuominen et al., 1994), which have been reported as MeHg scavengers that can oxidatively cleave the C—Hg bond, leading to the rapid degradation of MeHg (Suda et al., 1991; Gardfeldt et al., 2001). However, the data that support this hypothesis are limited. Thus, a comparison and evaluation of gamma irradiation and other sterilization approaches (e.g., autoclaving) used for soil incubation experiments is urgently needed.

4. Conclusions

In this study, different types of methanogens were found to play divergent roles in both Hg^{II} methylation and MeHg demethylation in paddy soils. Furthermore, Hg^{II} methylation responses to methanogen inhibitors or stimulants were site-dependent. The results of Illumina sequencing and CH_4 concentrations showed that H_2/CO_2 and NaAc addition enhanced the microbial activity of hydrogenotrophic and aceticlastic methanogens for the given sites, respectively. In the background paddy fields, the higher K_m and K_d values in the NaAc treatment suggested that aceticlastic methanogens were involved in Hg^{II} methylation and MeHg demethylation. H_2/CO_2 addition did not stimulate Hg^{II} methylation, indicating that hydrogenotrophic methanogens were not the potential Hg^{II} methylators in the background paddy soils. In the Hg-contaminated paddy soils, higher K_m and K_d values were observed in the H_2/CO_2 treatment than in the control, suggesting that hydrogenotrophic methanogens were involved in Hg^{II} methylation and MeHg demethylation. The NaAc treatment yielded higher K_d values when Hg^{II} methylation was not facilitated at the Hg-contaminated site. This indicated that aceticlastic methanogens could mediate MeHg degradation at the Hg-contaminated site. Finally, competition between hydrogenotrophic and aceticlastic methanogens was observed at the MM, implying that net MeHg production could be alleviated by promoting aceticlastic methanogens or inhibiting hydrogenotrophic methanogens in Hg-polluted paddy soils. In addition, our findings showed that the addition of methanogen inhibitors in the background paddy soils and addition of aceticlastic stimulants like NaAc in the Hg-contaminated paddy soils can inhibit the formation of CH_4 to some extent. Therefore, it has important implications for global climate change while paddy soils were thought as one main anthropogenic source of methane emission (Saunois et al., 2020).

CRediT authorship contribution statement

Zhengdong Hao: Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. **Lei Zhao:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing. **Jiang Liu:** Data curation, Formal analysis, Funding acquisition, Writing – review & editing. **Qiang Pu:** Data curation, Formal analysis, Writing – review & editing. **Ji Chen:** Formal analysis, Writing – review & editing. **Bo Meng:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Xinbin Feng:** Resources, Writing – review & editing.

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.2023.167601.

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