

Response to reviewers' comments (EGUSPHERE-2024-590)

Dear Editor and Reviewers:

We sincerely thank you for taking the time to review our manuscript titled "*Dissolved organic matter fosters core mercury-methylating microbiome for methylmercury production in paddy soils*". Your constructive feedback has been invaluable in improving the quality and clarity of our work. In response to reviewers' comments, we have carefully revised the manuscript and highlighted all changes in yellow for your convenience. Additionally, we have provided a detailed, point-by-point response to address each of your suggestions and concerns. We deeply appreciate your thoughtful review and believe that the revisions have significantly enhanced the manuscript. We hope that the revised version meets your expectations and satisfactorily addresses all the points raised during the review process.

With great gratitude,

Dr. Bo Meng, on behalf of all co-authors

Reviewer#1 (Emi Stuart)

General comments:

The study presented by Pu et al. aimed to elucidate the underlying mechanisms of MeHg production in paddy soils, and investigated the roles of dissolved organic matter and the soil microbial community within this process. This is an interesting topic with important implications for the toxicity of rice, especially under a changing climate. I found that the paper was well-presented with clear research aims and hypotheses, and the experimental approaches were appropriate and sufficient in addressing the aims. Overall, the study is well written, particularly in the Introduction, however the language in some sections could be improved and made more precise (e.g. the Abstract and Discussion sections).

Response: Thanks for your valuable comments. We have carefully modified the manuscript following the reviewer's specific comments.

Specific comments:

Line 15-16: Please rephrase the second half of this sentence. E.g. rephrase "hotspot for soil biogeochemistry". Also, what remains poorly understood?

Response: Following the reviewer's advice, the "hotspot for soil biogeochemistry" was changed to "Dissolved organic matter (DOM) is a critical component for soil biogeochemical process". It remains poorly understood how DOM interacts with microbial communities and influences Hg methylation in paddy soils.

"Dissolved organic matter (DOM) is a critical component for soil biogeochemistry process, yet its interactions with microorganisms involved in MeHg production, remain poorly understood." (Mian text, Lines 15-17).

Line 140-144: Please justify the use of only one bacterial species to represent core Hg-methylating microorganisms. You mention that *Geobacter sulfurreducens* was a core Hg-methylating microorganism in this study but this is never explicitly stated in the Results.

Response: The use of only one bacterial species to represent core Hg-methylating microorganisms can be justified by several reasons:

- (1) **model organism benefits:** using a single, well-characterized bacterial species as a model organism allows for a more detailed and controlled study of Hg-methylation mechanisms. This approach provides clear insights into the biochemical pathways and environmental conditions that facilitate Hg-methylation.
- (2) **Simplicity and Clarity:** Focusing on one species simplifies the experimental design and data interpretation. This clarity is crucial for understanding fundamental processes without the confounding variables introduced by multiple species interactions.
- (3) **Reproducibility:** Experiments using a single species are easier to replicate, ensuring that findings are consistent and reliable. This reproducibility is essential for validating results and drawing definitive conclusions about the role of the species in Hg-methylation.
- (4) **Representative Organism:** The chosen bacterial species is likely a representative of other Hg-methylating microorganisms. By studying this species, researchers can infer similar mechanisms and behaviors in related species, providing broader ecological insights.

In this study, the microorganisms belonging to *Geobacter* were core Hg-methylating microorganisms across all paddy soils. To make it clear, the corresponding sentence was re-organized in the revised manuscript as below:

"It is worth highlighting that, in this study, microorganisms belonging to Geobacter were identified as the most significant core microorganisms for Hg methylation across all paddy soils." (Mian text, Lines 231-232).

Line 148: Is the mann-whitney U test appropriate here (typically used for comparing two groups)?

Response: Apologies for the mistake. We employed the Kruskal-Wallis test to compare microbial alpha diversity across all samples.

"The Kruskal-Wallis test was used to compare microbial alpha diversity among all samples." (Mian text, Lines 165-166).

Line 173: Please describe these significant differences and/or indicate them in the table.

Response: Following the reviewer's suggestion, the significant differences of DOC concentration and S_R of DOM were added in the revised manuscript as below:

"Furthermore, statistically significant differences in DOM concentrations (reflected by DOC concentration) and DOM composition (reflected by SR of DOM) were found in NMS, MMS and HMS (Table S2). Specifically, DOC concentration varied significantly across the three soil types, with 0.48 ± 0.13 in NMS, 0.40 ± 0.07 in MMS, and 0.30 ± 0.10 in HMS. Similarly, the SR of DOM differed markedly between NMS (1.40 ± 0.76), MMS (0.89 ± 0.09), and HMS (0.46 ± 0.09)." (Mian text, Lines 193-197).

Line 204, 280: These are only some of the dominant genera as presented in Fig. 2C (although only *Geobacter* should really be described as "dominant"). What about *Olavius* and *Granulicella*, which have a higher abundance than *Methanoregula* or *Desulfomonas*. Furthermore, the majority of the microbiome is unclassified – this should be mentioned in the Results and possibly Discussion.

Response: Sorry for the confusion. The abundance of *Olavius* and *Granulicella* is indeed higher than that of *Methanoregula* or *Desulfomonas*. In the revised manuscript, we described the three genera with the highest abundance of microorganisms in NMS, MMS, and HMS, respectively. The corresponding sentences were re-organized in the revised manuscript:

*"Although most microorganisms are not annotated, the three genera with the highest abundance in each soil type are as follows: In NMS, *Geobacter*, *Syntrophus*, and *Desulfomonas* dominate; in MMS, *Geobacter*, *Granulicella*, and *Olavius* are the most abundant; and in HMS, *Geobacter*, *Methanoregula*, and *Granulicella* prevail (Fig. 2c)."* (Mian text, Lines 227-231).

This study found that many core Hg-methylating microorganisms were not annotated. However, for those that were annotated, we discovered that the core microorganisms involved in Hg methylation in paddy soil were more diverse than previously thought. The corresponding sentences were re-organized in the revised manuscript as below:

"Although many core Hg-methylating microorganisms have not been annotated, our study emphasizes that the annotated Hg-methylating microorganisms play a much greater role in Hg methylation in paddy soils than previously thought." (Mian text, Lines 312-314).

Line 201-202: This sentence needs rewording.

Response: To express this more clearly, the corresponding sentences were re-organized in the revised manuscript as below:

"Importantly, the impact of various modules in the microbial community on MeHg production was analyzed using random forest analysis. The results revealed that the microbiome in Module 1 is a crucial bacterial group influencing soil methylmercury concentration (Fig. 2b). This group is considered the core Hg-methylating microbiome in this study." (Mian text, Lines 223-226).

Line 267-269: This should be moved to Results.

Response: According to the reviewer's suggestion, we have moved the sentence "the contributions of Hg bioavailability and redox conditions to the core Hg-methylating microbiome composition are 10% and 25%, respectively, which are much lower than that of DOM (65%)" to Results section 3.3, titled "Dissolved organic matter as indicators of core mercury-methylating microbiome composition in paddy soils."

Line 271-274: This explanation sounds a bit contradictory, as it seems to support that Hg bioavailability could have a greater contribution to the microbiome composition than what was found. I would suggest rephrasing the second sentence to “Environmental Hg may induce the persistence of some microorganisms”, and the third sentence to “Therefore, long-term Hg contamination often only elevates the abundance of specific microbial taxa capable of Hg tolerance”.

Response: We agree the reviewer's comments here. The corresponding sentences were re-organized in the revised manuscript as below:

"Hg is a toxic element to microorganisms and is usually not involved in microbial metabolism (Wang et al., 2020). Environmental Hg may induce the persistence of some microorganisms. Therefore, long-term Hg contamination often only elevates the abundance of specific microbial taxa capable of Hg tolerance (Frossard et al., 2018)" (Main text, Lines 299-302).

Figure 6 and supplementary Text S1.3, S3: Should Figure 6A have error bars? It is not clear from the supplementary methods how many samples were used to extract DOMs and whether or not these were pooled before extraction.

Response: Sorry for the confusion. The Figure 6A has no error bar because we divided the 19 paddy soil samples into NMS, MMS, and HMS based on mercury concentrations (see Table S1 for the classification results) and then obtained the homogenized NMS, MMS, and HMS samples by mixing all paddy soil samples within each group in equal proportions. We subsequently extracted the dissolved organic matter from these homogenized samples to determine the organic matter composition. To make it clear, the corresponding sentences were re-organized in the Supplementary Text S3 as below:

"The natural DOM solution was extracted from paddy soils from the second sampling campaign. First, we divided the 19 paddy soil samples into NMS, MMS, and HMS based on mercury concentrations (see Table S1 for the classification results). Then, we mixed all paddy soil samples within each group (NMS, MMS, and HMS) in equal proportions to obtain homogenized samples for each group. Next, we extracted the dissolved organic matter from the soil using a soil-to-water ratio of 1:10 (w/v). The extracted solution was then split into two portions: one for the determination of organic matter composition and the other for validation experiments." (Supplementary text, Lines 76-82).

Supplementary methods: Please provide sufficient details to allow for reproduction of your study. E.g. Details on the soil sample processing (were the analyses performed on dry or fresh soil; were soils ground/sieved, what were the weights of sample used, etc), origin of bacterial strains, incubations (vials used, form of Hg(II) added, etc), manufacturers of materials.

Response: Thank you for your valuable comment. We have included detailed information to ensure the reproducibility of our study:

1. Soil sample processing:

- **The physico-chemical analyses were performed on freeze-dried soil samples, except for the determination of soil iron and sulfur, which was performed using fresh soil.**

- Soils were ground and sieved through 200 mesh to remove debris and ensure uniform particle size.
 - For the determination of various indicators, different amounts of soil samples were used. Specifically, for mercury analysis, 0.5g of soil was required for water-soluble Hg, 0.2g for total mercury, and 0.3-0.4g for methylmercury. We have included the weights of sample for each indicator in the revised manuscript.
2. Origin of bacterial strain:
- The bacterial strain used in this study was kindly provided by Professor Peng Liang from Zhejiang Agriculture and Forestry University.
3. Incubations:
- Incubations were conducted in 100 mL glass vials with airtight caps to prevent contamination.
 - Hg(II) was added in the form of mercuric chloride (HgCl₂) at a concentration of 1.36 µg/L.
 - The vials were incubated at 33°C for 24 h.
4. Manufacturers of Materials:
- Mercuric chloride (HgCl₂) from Sinopharm Chemical Reagent Co., Ltd., China.
 - Extraction kits from MP Biomedicals, France.
 - Glass vials from BKMAM Biotechnology, China.

We have revised the manuscript to include these details in the Methods section to enhance reproducibility.

Thank you again for your insightful comments, and we hope these revisions meet your expectations.

Technical corrections:

Main text:

Line 19-20: This sentence is a bit confusing. Perhaps rephrase to "...identified that was exclusively..."

Response: Following the reviewer's advice, the sentence was rephrased to "Surprisingly, a core Hg-methylating microbiome was identified that was exclusively associated with MeHg concentration."

Line 23: 89% of?

Response: Sorry for the confusion. we have clarified this information in the revised manuscript as below:

"Structural equation model further indicated that core Hg-methylating microbiome composition significantly impacted soil MeHg concentration, contributing to 89% of the observed variation" (Main text, Lines 22-23).

Line 28: Change to "(a core Hg-methylating microorganism)"

Response: Done.

Line 94-99: Also add analysis of low-molecular-weight organic acids

Response: Thank you for your suggestion. We have now included the analysis of low-molecular-weight organic acids in Materials and methods section 2.1, titled "Soil sampling and physico-chemical analysis"

Line 113-139: Please move amplicon sequencing method details out of this section and into a new section below (preferably) or rename the title to include amplicon sequencing.

Response: Thank you for your suggestion. We will move the amplicon sequencing method details to section 2.3, titled "Amplicon sequencing and bioinformatic analysis".

Line 119: Please define *hgcAB* this as compared to the *hgcA* gene.

Response: Sorry for the confusion. *hgcAB* refers to a gene cluster that includes both the *hgcA* and *hgcB* genes, which together are crucial for the process of mercury methylation in microorganisms. The *hgcA* gene specifically encodes for a corrinoid protein that is essential for methylating inorganic mercury, while the *hgcB* gene encodes a small iron-sulfur protein that functions in electron transfer. When referring to *hgcAB*, it encompasses the full operon responsible for this biochemical pathway, whereas *hgcA* refers specifically to the methylating enzyme component. To make it clear, the corresponding sentences were re-organized in the revised manuscript as below:

*"In addition, the abundance of the Hg-methylating gene *hgcA* (which encodes a corrinoid protein essential for methylating inorganic Hg) was quantified in an Applied Biosystem 7500." (Main text, Lines 126-127).*

*"The detection and taxonomic identification of the *hgcAB* gene (full operon responsible for Hg methylation pathway) was performed with *marky-coco* (Capo et al., 2023)." (Main text, Lines 134-135).*

Line 140-144: The wording could be made clearer (e.g. "...we incubated *Geobacter sulfurreducens* PCA (*G. sulfurreducens* PCA, a core Hg-methylating microorganism in this study) with Hg²⁺ and a natural DOM solution extracted from either NMS, MMS, or HMS soils.")

Response: Thank you for your valuable comment. We agree that the original wording could be improved for clarity. Following your suggestion, we have revised the sentence as follows:

*"we incubated *Geobacter sulfurreducens* PCA (*G. sulfurreducens* PCA), identified as a core Hg-methylating microorganism in this study, with Hg²⁺, and a natural DOM solution extracted from NMS, MMS, or HMS soils." (Main text, Lines 158-160).*

Line 148: This sentence could be made clearer (e.g. “Hg-methylating microbial communities across differentially-polluted soils were compared by analysing dissimilarity matrices using Bray-Curtis distance and visualized using principal coordinates analysis (PCoA) and Adonis with the "ade4" and "vegan" packages”)

Response: Thank you for the suggestion. The revised sentence, 'Hg-methylating microbial communities across differentially-polluted soils were compared by analyzing dissimilarity matrices using Bray-Curtis distance and visualized using principal coordinates analysis (PCoA) and Adonis with the 'ade4' and 'vegan' packages,' is indeed clearer and more concise. We will update the text accordingly.

Line 158: Provide citations for psych and Gephi

Response: Thank you for the reminder. The appropriate citations are as follows:

"Revelle, W. 2023. psych: Procedures for Psychological, Psychometric, and Personality Research. Northwestern University, Evanston, Illinois, USA. <https://CRAN.R-project.org/package=psych>" (Main text, Lines 716-717).

"Bastian, M., Heymann, S. and Jacomy, M. 2009. Gephi: An Open Source Software for Exploring and Manipulating Networks. In Proceedings of the Third International ICWSM Conference (pp. 361-362). <https://gephi.org>" (Main text, Lines 579-580).

Line 177: Change >> to >

Response: Done.

Line 183-184: Repetitive and confusing sentence. Consider changing to “hgcA gene-based taxonomic profiles of paddy soils reveal changes in Hg-methylating microbial community compositions across different levels of Hg pollution”

Response: Thank you for your suggestion. The revised sentence, 'hgcA gene-based taxonomic profiles of paddy soils reveal changes in Hg-methylating microbial community compositions across different levels of Hg pollution,' is indeed clearer and more concise. We will update the text accordingly.

Line 282: Zhong et al. 2024 (Soil Geobacteraceae are the key predictors of neurotoxic methylmercury bioaccumulation in rice) could be cited here

Response: Thank you for the recommendation. Citing Zhong et al. (2024) would indeed strengthen our discussion by providing additional context on the role of *Geobacteraceae* in methylmercury bioaccumulation. We will incorporate this citation into the relevant section to enhance the overall narrative.

Line 292: Typo – “citric acid”

Response: Thank you for pointing out the typo. We will correct 'citric acid' in the manuscript. We appreciate your attention to detail.

Supplementary:

Line 23-24: Should change to “Water-soluble Hg (representing Hg bioavailability) in paddy soils was extracted according to Shi et al. with slight modifications (Shi et al., 2005).” As you only followed their protocol for extraction, not quantification. Also was the rest of their extraction protocol followed as described? If so please specify.

Response: Thank you for the suggestion. We will revise the sentence to 'Water-soluble Hg (representing Hg bioavailability) in paddy soils was extracted according to Shi et al. with slight modifications (Shi et al., 2005),' to accurately reflect that we followed their protocol for extraction but not for quantification. Additionally, the extraction methods for water-soluble SO_4^{2-} , NO_3^- , and dissolved organic carbon were similar; however, the water-to-soil ratios used for each were different. We will clarify these differences in the Supplementary texts to ensure the methodology is accurately described.

Line 85-86 Please reword for clarity E.g. “To monitor abiotic Hg methylation, the aforementioned experiment was conducted as described above, without addition of bacterial cells.”

Response: Thank you for the suggestion. We will reword the sentence for clarity as follows: 'To monitor abiotic Hg methylation, the aforementioned experiment was conducted as described above, without addition of bacterial cells.' This revision will be included in the Supplementary Text S3.

Figures:

Fig. 1A: Change axis label to “proportion of community abundance” or change values to percentages. Remove second “phyla” in second sentence.

Response: Thank you for the suggestions. We will update the axis label to 'proportion of community abundance' accordingly. Additionally, we will remove the redundant 'phyla' in the second sentence. These revisions will be reflected in the revised manuscript.

Fig. 1B: The inconsistent use of colour in Figures 1b and 1c to represent the different treatments may confuse readers. Please consider making these the same colours or removing colour altogether from Fig. 1B.

Response: Thank you for pointing out the inconsistency in the use of color in Figures 1b and 1c. We will standardize the color scheme across both figures to ensure consistency. This adjustment will help avoid any potential confusion for readers. We appreciate your attention to this detail and will make the necessary revisions.

Fig. 1 and S5: Please add more details to the figure captions to distinguish these two figures.

Response: Thank you for the suggestion. We will add more details to the captions for Fig. 1 and S5 to clearly distinguish between the two figures. This will help ensure that their content and differences are easily understood by readers. We appreciate your feedback and will update the captions accordingly.

"Figure 1: Taxonomic profiles of Hg-methylating microbial communities in paddy soils based on amplicon sequencing. (a) Microbial community composition in differently polluted paddy soils. Phyla with low abundance grouped together under "other phyla". (b) Microbial diversity (based on the Chao1 index) in differently polluted paddy soils. (c) Principal coordinates analysis (PCoA) based on Bray-curtis distance showing the overall pattern of Hg-methylating microbial communities in differently polluted paddy soils. NMS, non-Hg polluted paddy soils (n = 23); MMS, moderate Hg-polluted paddy soils (n = 13); HMS, high Hg-polluted paddy soils (n = 10)." (Main text, Lines 215-219).

"Fig. S5. (a) Hg-methylating microbial community composition in different paddy soils based on metagenomic sequencing. Phyla with low abundance phyla grouped together under "other phyla". (b) Principal coordinates analysis (PCoA) based on Bray-curtis distance showing the overall pattern of Hg-methylating microbial communities in paddy soils. NMS, non-Hg polluted paddy soils (n = 15); MMS, moderate Hg-polluted paddy soils (n = 5); HMS, high Hg-polluted paddy soils (n = 2)." (Supplementary text, Lines 115-119).

Fig. S4 and S7: Change "significant difference" to "statistically significant predictor"

Response: Thank you for the suggestion. We will change 'significant difference' to 'statistically significant predictor' in the Supplementary figures to more accurately reflect the analysis. We appreciate your attention to this detail and will make the necessary revision.

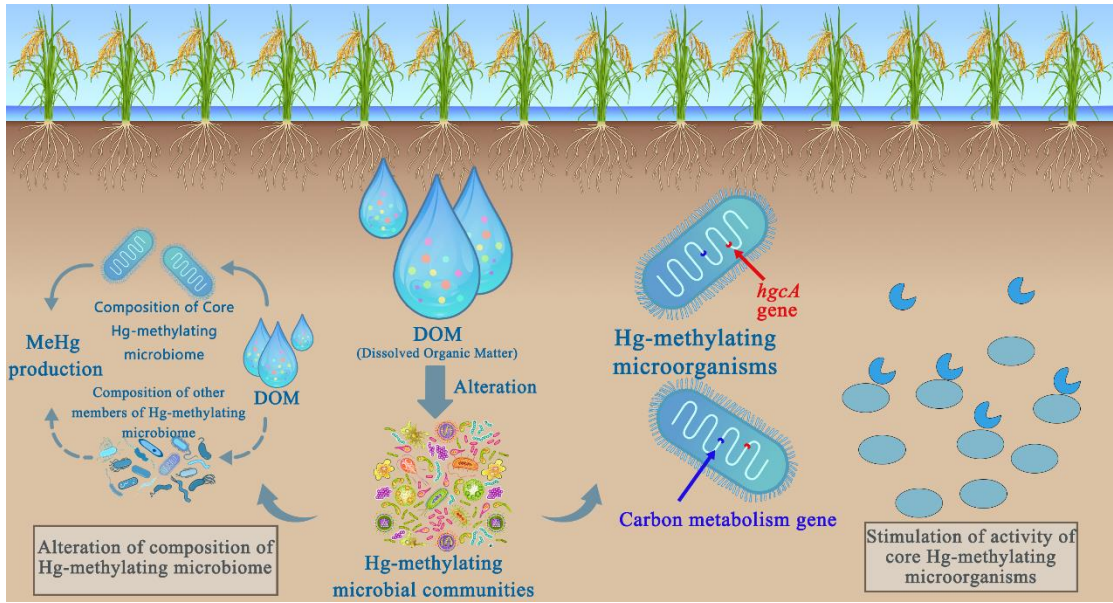
Suggestions for the graphical abstract:

I suggest that the text box on the left should read: "Alteration of composition of Hg-methylating microbiomes", as you show both the core and non-core microbiomes in the figure.

Response: Thank you for the suggestion. We will update the text box on the left to read: 'Alteration of composition of Hg-methylating microbiomes,' as this better reflects that both core and non-core microbiomes are shown in the figure. We appreciate your feedback and will make this revision.

The middle section of the figure (i.e. the downward arrow and the image of the microbial communities) seems to be repetitive of the left section of the figure

Response: Thank you for pointing out the redundancy. We change it to avoid repetition with the left section. We appreciate your feedback and will make the necessary adjustments to improve the clarity and effectiveness of the figure.



Reviewer#2 (aoxue Yin)

General comments

This manuscript presents an insightful study investigating the relationship between dissolved organic matter (DOM) and methylmercury (MeHg) production in paddy soils, particularly focusing on the core microbiome responsible for mercury methylation. The study employs advanced genomic and metagenomic techniques to identify key microbial taxa involved in mercury methylation and explores the impact of DOM on the activity of these microorganisms. The authors highlight that DOM plays a pivotal role in shaping the composition and functional activity of the Hg-methylating core microbiome, which in turn regulates MeHg production. The conclusions are supported by extensive field sampling, genome-resolved metagenomic analysis, and controlled incubation experiments.

Response: Thank you for your valuable feedback on our manuscript. We appreciate your positive comments regarding the investigation of the relationship between dissolved organic matter (DOM) and methylmercury (MeHg) production in paddy soils, especially focusing on the core microbiome responsible for mercury methylation.

In response to your comments, we have carefully revised the manuscript. We hope the changes address all concerns and improve the overall quality of the work.

Comment 1: The discussion section could further expand on how the findings integrate with broader environmental mercury cycling processes. A more detailed comparison with existing literature on Hg-methylating microbes in non-paddy soils would enhance the paper's contribution to microbial ecology.

Response: Thank you for your valuable comment. We have expanded the discussion section to better integrate our findings with broader environmental mercury cycling processes, as you suggested. Specifically, we highlight how dissolved organic matter (DOM) impacts mercury methylation not only in paddy soils but also in other soil environments, comparing our findings with existing literature on Hg-methylating microbes in non-paddy soils. To enhance clarity, the corresponding sentences were added in the revised manuscript as below:

"DOM's influence on microbial Hg methylation has been observed in other ecosystems, such as wetlands and sediments, where DOM shapes microbial community structures to promote methylmercury (MeHg) production. For instance, in wetlands, DOM-bound Hg has been found to change the community assembly of mercury for methylating microbes (Fagervold et al., 2014). This highlights the broader ecological significance of DOM's role in promoting Hg methylation and suggest that DOM-driven microbiome modulation is a critical process across diverse environments." (Main text, Lines 337-341).

Comment 2: The manuscript discusses DOM composition but lacks specific details on the types of organic compounds most relevant to stimulating Hg-methylating activity. Including additional chemical analyses of DOM could strengthen the conclusions.

Response: Thank you for your valuable comment. While we did not conduct additional chemical

analyses, we did examine the composition of dissolved organic matter (DOM) across paddy soils with varying levels of Hg contamination (NMS, MMS, and HMS). Our findings revealed that low-molecular-weight organic acids such as oxalic acid, formic acid, tartaric acid, and acetate were highly abundant in these soils, with their concentrations being highest in NMS soils, followed by MMS, and lowest in HMS. These compounds are known to serve as key carbon sources for Hg-methylating microorganisms, particularly in enhancing their activity.

Although we do not have direct data on the influence of aromatic compounds and humic substances on Hg methylation, it is possible to hypothesize that their more complex structures may make them less bioavailable or more resistant to microbial degradation, potentially leading to a weaker stimulation of Hg-methylating activity compared to low-molecular-weight organic acids. This hypothesis warrants further investigation in future studies. To clarify, we have added the corresponding sentences to the revised manuscript as below:

"While aromatic compounds and humic substances were not directly analyzed in this study, their complex structures likely reduce Hg bioavailability or slow microbial degradation, resulting in weaker effects on Hg methylation compared to low-molecular-weight organic acids. Future research could integrate direct Hg speciation measurements with detailed DOM compositional analyses to better understand how specific DOM components and Hg species interact to influence microbial Hg methylation." (Main text, Lines 332-336).

Comment 3: The authors hypothesize that DOM stimulates microbial activity through metabolic pathways but do not delve deeply into the mechanistic underpinnings. Further discussion on the microbial metabolic pathways involved, possibly supported by metabolomic data, would improve the mechanistic understanding.

Response: Thank you for your insightful comment. We fully acknowledge the importance of understanding the microbial metabolic pathways involved in DOM-stimulated Hg methylation, as it is crucial for elucidating the underlying mechanisms of this process. Although our current study primarily focused on identifying core Hg-methylating microorganisms and analyzing DOM composition, we agree that a deeper exploration of the metabolic pathways involved would significantly enhance the mechanistic understanding of how DOM influences these microorganisms.

In our study, we observed that different DOM compounds, particularly low-molecular-weight organic acids such as oxalic acid, formic acid, and acetate, were utilized by key Hg-methylating microorganisms (e.g., *Geobacter* and *Desulfovibrio* species). These microorganisms are known to employ key metabolic pathways such as the TCA cycle (tricarboxylic acid cycle) and acetate metabolism, which could be directly linked to the utilization of DOM components. For instance, *Geobacter sulfurreducens* and *Desulfovibrio desulfuricans* preferentially use acetate, which feeds into the TCA cycle, providing electrons for anaerobic respiration and potentially influencing Hg methylation rates (Hu et al., 2013; Liu et al., 2018b). Additionally, *Methanoregula* and *Methanosarcina* species involved in methanogenesis could be utilizing formate and acetate through the methanogenesis pathway, further contributing to Hg methylation (Sakai et al., 2010; Schöne et al., 2022).

While we did not include metabolomic data in this study, we recognize the need for future research in this area. Incorporating metabolomic profiling would be highly beneficial for identifying key

metabolites and providing more detailed insights into the specific metabolic pathways influenced by DOM, such as acetate fermentation, methanogenesis, and electron transfer processes involved in Hg methylation. To enhance clarity, the corresponding sentences were added in the revised manuscript as below:

"Although metabolomic data were not included in this study, future research incorporating such analyses could provide valuable insights into how specific DOM components influence microbial metabolism and Hg methylation, revealing key metabolites and pathways such as acetate fermentation, methanogenesis, and electron transfer processes." (Main text, Lines 325-328).

Minor Comments

Comment 4: The introduction is well-written but could benefit from a clearer explanation of the broader ecological importance of Hg methylation in paddy fields versus other environments.

Response: Thank you for your constructive comment. We appreciate your suggestion to provide a clearer explanation of the broader ecological significance of Hg methylation in paddy fields compared to other environments. We have expanded the introduction to address this point:

"Compared to other environments such as wetlands and aquatic sediments, paddy fields present unique ecological conditions that make them significant hotspots for Hg methylation. The frequent flooding and draining cycles, high organic matter content, and dynamic redox conditions in paddy soils create an environment that supports high levels of microbial activity, particularly Hg-methylating microorganisms (Yin et al., 2013). These conditions not only enhance MeHg production but also increase the likelihood of MeHg entering the food web through rice consumption, posing significant health risks (Zhang et al., 2010). Understanding Hg methylation in paddy fields is therefore crucial, as rice is a critical exposure route for MeHg in humans." (Main text, Lines 43-49).

Comment 5: Line16 suggest "remains"

Response: Thank you for your suggestion. We have revised the sentence as recommended.

Comment 6: Line 64 "...other factors also play roles in MeHg production..."

Response: Thank you for your comment. We have revised the sentence as suggested.

Comment 7: Line 62, Line 237, Line 299, Lne 321 suggest unify the description of "low-molecular-weight DOMs" throughout the maus.

Response: Thank you for your comment. We have unified the description of "low-molecular-weight DOMs" throughout the manuscript for consistency.

Comment 8: Line 255 Whether the results support the argument that "MeHg concentration was strongly linked to hgcA gene abundance even compared to abiotic factors", it seems to be no description of the relevant results

Response: Thank you for your comment. Our results demonstrated that the contribution of *hgcA*

gene abundance to MeHg concentration was stronger compared to the contributions of Hg bioavailability and redox conditions. Specifically, alteration of the core Hg-methylating microbiome composition, which is closely linked to *hgcA* gene abundance, significantly regulated soil MeHg concentration ($\lambda = 0.84$, $p < 0.001$) (Fig. 4). In comparison, the contributions of Hg bioavailability (10%) and redox conditions (25%) were much lower than that of DOM (65%) (Fig. 4), further supporting the link between *hgcA* gene abundance and MeHg concentration, even in the presence of varying abiotic factors. To enhance clarity, the corresponding sentences were re-organized in the revised manuscript as below:

*"Additionally, SEM result showed that the core Hg-methylating microbiome composition, which is closely linked to *hgcA* gene abundance, significantly regulated soil MeHg concentration ($\lambda = 0.84$, $p < 0.001$) (Fig. 4). In comparison, the contributions of Hg bioavailability and redox conditions to the core Hg-methylating microbiome composition are 10% and 25%, respectively, which are much lower than that of DOM (65%) (Fig. 4)." (Main text, Lines 246-250).*

Reviewer#3 (Anonymous reviewer)

Comment 1: In the abstract, DOM was crucial in determining core Hg-methylating microbiome composition (65%), what does the 65% mean, please explain it in the text.

Response: Thank you for your insightful question. The "65%" mentioned in the abstract refers to the contribution of DOM to the composition of the core Hg-methylating microbiome. This value was calculated using structural equation modeling (SEM) results based on the method described by Tao et al. (2015). Specifically, in SEM, the contribution of a variable to its target variable was calculated as the square of its path coefficient divided by the sum of the squares of all path coefficients pointing to the same target variable. Using this formula, we calculated the contribution of DOM to the core Hg-methylating microbiome, and the result showed that DOM contributed 65%.

To ensure clarity, we have revised the Materials and Methods section to provide a clearer explanation of the contribution calculation method in the revised manuscript as below:

"We further calculated the contribution of ecological parameters, including DOM, to the core Hg-methylating microbiome, and the contribution of the core Hg-methylating microbiome to MeHg production, following the approach described by Tao et al. This calculation was performed by determining the proportion of the squared path coefficient of each parameter relative to the sum of the squared path coefficients of all parameters influencing the same target variable (Tao et al., 2015)." (Mian text, Lines 181-185).

Comment 2: In the core Hg-methylating microbiome, how much does the *Geobacter* account? Why the authors use the *Geobacter* for pure incubation experiment? why not use the soil for incubation experiment? What criteria were used to select the *Geobacter*?

Response: Thank you for these excellent questions. Below, we provide detailed responses to each point:

(1) How much does the *Geobacter* account in the core Hg-methylating microbiome?

In the core Hg-methylating microbiome, *Geobacter* accounts for an average of 44.5% (36.2%, 40.5%, and 56.8% in NMS, MMS, and HMS soils, respectively) of the community, based on our analysis (Fig. 2c). This highlights its significant presence and potential role in Hg methylation within the studied system.

To explicitly include the microbial abundances of the three most abundant genera in each soil type, we have incorporated the specific values into the revised manuscript as follow:

*"Although most microorganisms are not annotated, the three genera with the highest abundance in each soil type are as follows: In NMS, *Geobacter* (36.2%), *Syntrophus* (1.7%), and *Desulfomonas* (0.4%) dominate; in MMS, *Geobacter* (40.5%), *Granulicella* (2.9%), and *Olavius* (2.9%) are the most abundant; and in HMS, *Geobacter* (56.8%), *Methanoregula* (0.6%), and *Granulicella* (2.3%) prevail (Fig. 2c)."* (Mian text, Lines 227-231).

(2) Why did the authors use *Geobacter* for pure incubation experiments? Why not use soil for incubation experiments?

The primary reason for selecting *Geobacter* for pure incubation experiments is its well-documented and dominant role in Hg methylation in various environments, as supported by prior studies (Bravo et al., 2018; Zhong et al., 2024). By using *Geobacter*, we aimed to isolate and clearly demonstrate the direct effects of DOM on Hg-methylation rates and microbial activity, without the interference of complex soil matrix effects.

While soil incubation experiments could provide additional context for ecosystem-level processes, they would introduce confounding factors, such as competing microbial communities and varying physicochemical properties of the soil. Our approach using a pure culture system allows for a controlled study to elucidate specific microbial and biochemical mechanisms.

To explicitly clarify the rationale for using *Geobacter sulfurreducens* PCA in the incubation experiments, we have added the text in the Methods section as follows:

"Geobacter was selected for these pure incubation experiments due to its dominant role in mercury methylation and its ability to isolate the effects of DOM on methylation rates without the interference of soil matrix complexity." (Mian text, Lines 160-161).

(3) What criteria were used to select *Geobacter*?

Geobacter was chosen based on several criteria: (i) Its established status as a key Hg-methylating microorganism in diverse environments, including paddy soils (Zhong et al., 2024); (ii) Its high abundance within the core Hg-methylating microbiome observed in our study (Fig. 2c); (iii) Its metabolic versatility, which makes it an ideal model organism for studying mercury methylation pathways under controlled conditions (Schaefer and Morel, 2009). These factors collectively make *Geobacter* an excellent candidate for exploring DOM-microbe interactions and their implications for methylmercury production.

We hope this explanation clarifies our rationale and methodology.

Comment 3: Why wasn't Hg speciation measured or presented to confirm the hypothesis?

Response: Thank you for your insightful suggestion. While Hg speciation measurements would undoubtedly provide additional insights into the mechanisms of Hg methylation, the primary focus of this study was to investigate the role of DOM and microbial dynamics, particularly the core Hg-methylating microbiome, in driving MeHg production in paddy soils. This decision was made to prioritize a detailed examination of how DOM influences microbial Hg methylation through changes in community composition and activity.

Additionally, indirect evidence from random forest analysis and SEM highlighted the dominant influence of DOM on the core Hg-methylating microbiome compared to abiotic factors such as redox conditions and Hg bioavailability (Fig. S4 and Fig. 4). These results support the robustness of our conclusions regarding DOM's central role in Hg methylation.

While Hg speciation measurements were beyond the scope of this study, we acknowledge their importance and propose that future research combining direct Hg speciation analyses with DOM compositional studies could further elucidate how Hg speciation interacts with DOM and microbial communities to influence Hg methylation.

To better understand, the corresponding sentences are added in the revised manuscript as below:

"Future research could integrate direct Hg speciation measurements with detailed DOM compositional analyses to better understand how specific DOM components and Hg species interact to influence microbial Hg methylation." (Mian text, Lines 334-336).

Comment 4: The discussion would improve by explaining how specific components of DOM influence the distribution of Hg-methylating gene communities?

Response: Thank you for this insightful comment. We agree that explaining how specific components of DOM influence the distribution of Hg-methylating gene communities would improve the discussion. To address this, we have revised the manuscript to elaborate on the mechanisms by which different DOM components, particularly low-molecular-weight organic acids, shape core Hg-methylating microbiomes.

Our study identified several key low-molecular-weight organic acids, such as oxalic acid, formic acid, and acetate, in paddy soils. These compounds play a pivotal role in stimulating core Hg-methylating microorganisms by providing essential carbon sources that fuel critical metabolic pathways. Specifically, *Geobacter sulfurreducens* and *Desulfovibrio desulfuricans* utilize acetate and fumarate in the TCA cycle (Hu et al., 2013; Liu et al., 2018), facilitating anaerobic respiration and electron transport processes that enhance Hg methylation rates. Similarly, methanogenic archaea like *Methanoregula* and *Methanosarcina* use formate and acetate through methanogenesis (Sakai et al., 2010; Schöne et al., 2022), further contributing to Hg methylation. These mechanisms suggest that low-molecular-weight organic acids selectively promote the growth and activity of Hg-methylating microorganisms, thereby influencing the distribution of Hg-methylating gene communities.

To better understand, the corresponding sentences are re-organized in the revised manuscript as follow:

"These findings demonstrate that DOM composition strongly influences microbial Hg methylation by stimulating key metabolic pathways. For instance, Geobacter sulfurreducens and Desulfovibrio desulfuricans use acetate and fumarate in the TCA cycle, supporting anaerobic respiration and electron transport that enhance Hg methylation (Hu et al., 2013; Liu et al., 2018b). Similarly, methanogenic archaea such as Methanoregula and Methanosarcina utilize formate and acetate through methanogenesis, further contributing to Hg methylation (Sakai et al., 2010; Schöne et al., 2022). Although metabolomic data were not included in this study, future research incorporating such analyses could provide valuable insights into how specific DOM components influence microbial metabolism and Hg methylation, revealing key metabolites and pathways such as acetate fermentation, methanogenesis, and electron transfer processes. This highlights how specific DOM components shape the core Hg-methylating microbiome and influence its role in MeHg production." (Mian text, Lines 321-329).

Comment 5: The conclusion needs to be strengthened. It currently restates results that are largely expected. Highlight the novel findings and their significance more clearly. Meanwhile, the environmental implication of this study can be discussed to demonstrate the impact of this study.

Response: Following the reviewer's suggestion, we have revised the conclusion to better emphasize

the novel findings of this study and their broader significance. Specifically, we highlighted the role of DOM in influencing the core Hg-methylating microbiome and its direct impact on MeHg production. Additionally, we briefly discussed the environmental implications of our findings, focusing on the potential effects of human activities and climate change on DOM dynamics and Hg methylation processes.

To better understand, the corresponding sentences are re-organized in the revised manuscript as follow:

"This study provides novel evidence that DOM significantly influences MeHg production by altering the composition and stimulating the activity of the core Hg-methylating microbiome. While DOM regulates the composition of other members of the Hg-methylating microbiome, its impact on MeHg production is primarily mediated through the core Hg-methylating microbiome. Using metagenomic binning and pure incubation experiments, we demonstrated that low-molecular-weight DOM directly promotes MeHg production by enhancing the metabolic activity of core Hg-methylating microorganisms. These findings underscore the central role of the core Hg-methylating microbiome in Hg cycling and highlight DOM as a critical driver of microbial Hg methylation. As human activities and climate change continue to alter DOM composition and concentration, their influence on Hg methylation dynamics warrants further investigation to better predict and mitigate Hg-related environmental and health risks." (Mian text, Lines 351-359).

Reference

- Bravo, A.G., Zopfi, J., Buck, M., Xu, J., Bertilsson, S., Schaefer, J.K., Pote, J., Cosio, C., 2018. Geobacteraceae are important members of mercury-methylating microbial communities of sediments impacted by waste water releases. *The ISME Journal* 12, 802-812.
- Hu, H., Lin, H., Zheng, W., Tomanicek, S.J., Johs, A., Feng, X., Elias, D.A., Liang, L., Gu, B., 2013. Oxidation and methylation of dissolved elemental mercury by anaerobic bacteria. *Nature Geoscience* 6, 751-754.
- Liu, Y.-R., Johs, A., Bi, L., Lu, X., Hu, H.-W., Sun, D., He, J.-Z., Gu, B., 2018. Unraveling Microbial Communities Associated with Methylmercury Production in Paddy Soils. *Environmental Science & Technology* 52, 13110-13118.
- Sakai, S., Conrad, R., Liesack, W., Imachi, H., 2010. *Methanocella arvoryzae* sp nov., a hydrogenotrophic methanogen isolated from rice field soil. *International Journal of Systematic and Evolutionary Microbiology* 60, 2918-2923.
- Schaefer, J.K., Morel, F.M.M., 2009. High methylation rates of mercury bound to cysteine by *Geobacter sulfurreducens*. *Nature Geoscience* 2, 123-126.
- Schöne, C., Poehlein, A., Jehmlich, N., Adlung, N., Daniel, R., von Bergen, M., Scheller, S., Rother, M., 2022. Deconstructing *Methanosarcina acetivorans* into an acetogenic archaeon. *Proceedings of the National Academy of Sciences* 119, e2113853119.
- Tao, S., Fang, J., Zhao, X., Zhao, S., Shen, H., Hu, H., Tang, Z., Wang, Z., Guo, Q., 2015. Rapid loss of lakes on the Mongolian Plateau. *Proceedings of the National Academy of Sciences* 112, 2281-2286.

Zhong, H., Tang, W., Li, Z., Sonne, C., Lam, S.S., Zhang, X., Kwon, S.Y., Rinklebe, J., Nunes, L.M., Yu, R.-Q., Gu, B., Hintelmann, H., Tsui, M.T.-K., Zhao, J., Zhou, X.-Q., Wu, M., Liu, B., Hao, Y., Chen, L., Zhang, B., Tan, W., Zhang, X.-X., Ren, H., Liu, Y.-R., 2024. Soil Geobacteraceae are the key predictors of neurotoxic methylmercury bioaccumulation in rice. *Nature Food* 5, 301-311.