**Response to reviewers' comments**

Dear Emi Stuart:

Thanks for taking the time to review our manuscript "***Dissolved organic matter fosters core mercury-methylating microbiome for methylmercury production in paddy soils***". We sincerely appreciate your constructive feedback, which has greatly improved the quality and clarity of our work. We have carefully modified the manuscript based on your constructive comments. The changes are highlighted in yellow for your easy reference. Additionally, we provided a detailed point-by-point response to each of your comments below. We believe that these revisions have significantly enhanced the manuscript, and we are grateful for your valuable input. We hope that the revised version meets your expectations and addresses all the concerns raised during the review process.

With great gratitude,

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

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) represents 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 15-16).

# 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 218-220).

# 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 155-156).

# 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 SR 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 ± 0.10in 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 181-185).

# Line 204, 280: These are onl 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 Desulfuromonas. 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 *Methanorelula* 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 215-218).

**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 303-305).

# 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 211-214).

# 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)"* (Mian text, Lines 288-291).

# 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.**

1. **Origin of bacterial strain:**

* **The bacterial strain used in this study was kindly provided by Professor Peng Liang from Zhejiang Agriculture and Forestry University.**

1. **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.**

1. **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 h*gcAB*, 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 118-119).

*"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 126-127).

# 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 suggestion. Revising the sentence to "…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" does indeed make the wording clearer. We will update the text accordingly.**

# Line 148: This sentence could be made clearer (e.g. “Hg-methylating microbial communities across differentally-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 SO₄²⁻, NO₃⁻, 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 203-207).

*"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.**

