nature portfolio

Peer Review File

Microbial potential to mitigate neurotoxic methylmercury accumulation in farmlands and rice

Corresponding Author: Professor Yu-Rong Liu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors are presenting findings about methylmercury degradation by rice paddy microorganisms based on incubation with labeled methylmercury coupled to DNA-SIP, metagenomic and tests on living bacterial strains.
The topic is relevant from different perspectives, including food safety and environmental microbiology, adding important information on the identity of methylmercury degraders and their potential impact on food safety. Referenced literature is adequate and conclusions fit with the aims and the results of the experiments.
The manuscript is generally well written, with minor revisions needed in the English and some typos (i.e., Line 49: Change “Hg methylating” with “Hg-methylating”; Lines 56-58: Revise English of the sentence “Subsequently … degraders.”; Line 152: Add final s to “Arenimona” and “Dechloromona”).
In the results section, since "Materials and methods" are at the end, authors should provide a few details for clarity (i.e., the platform used to perform long-read sequencing; the fact that 16S genes were quantified with qPCR).
There is one experimental point that is absolutely not clear for me in Lines 162-166: how was DNA pooling managed? According to what is written, heavy DNA was pooled with 16S rRNA copies, but I think this does not make sense. Authors should clarify well this point.

Reviewer #2

(Remarks to the Author)
The manuscript by Zhou et al provide interesting new data and findings about microbial mercury detoxification in farmland soil and also present some back of the envelope estimates of how ambient microbial mercury demethylation processes can lower the health burden related to human mercury exposure. The methods used to estimate rates and identify active microbial populations are state of the art and the data appear very convincing to this reviewer and my main critique relates to the uncertain modeling of the health hazards from MeHg exposure (where some modifications and further discussion are warranted).

(1) Line 104: Does these concentrations account for in situ MeHg concentration? Background concentrations may be substantial.
(2) Line 109-117: The degradation efficiency is arbitrarily defined as % degraded over a 28 day period. It would be much more useful to provide degradation rates.
(3) Line 199-211, 454-485: The estimates of health risks are critically depending on a number of rather uncertain assumptions and the estimates should be presented as such (acknowledging uncertainties). I also wonder how the “degradation efficiency” (fraction degraded MeHg in 28 days) come into play here. Why limit the assessment to this period? Do you assume that degradation halts after 28 days? The data does not support that. All in all I feel that a proper sensitivity analysis of this modeling effort is warranted to illustrate/assess the uncertainties in the numbers presented.
(4) General and line 270-272: Carrying a gene does not necessarily mean it is being used or expressed un der the given environmental conditions. I don’t thin k you can make the claim that the TCA cycle or assimilatory sulfate reduction is linked to MHg demethylation here. For that you need transcriptomic or proteomic data (or activity measuements). Uisng this indirect data to claim that SRB and methanogens play an important role in oxic MeHg demethylation is speculative.
(5) Line 317-319: How did you assess the purity of the synthesized compounds?
(6) Please provide a motivation for duration of the experiment (28 days). Would a shorter or longer incubation time change the major results?

(7) Line 369: How was the fractionation done? Is there a risk of cross-contamination between fractions?
(8) Line 376-378: The indicated reference is not the original one for the indicated primers. Please use the original reference and mention something about the taxonomic coverage since this is an important parameter.
(9) Line 387-388: These are very old primers known to miss several groups (even entire phyla). A cautionary discussion about the potential to overlook some active MeHg demethylators is warranted.
(10) Line 499-501: There are plenty of easy access and persistent data archives available for depositing sequence data. This contribute to reproducible science and I would rplease ask the authors to deposit their data in such repositories before publication can ber considered. There is really now excuse for not doing so.
(11) Ther reference list is very long and for sure it can be trimmed to remove redundancies without compromising the rigor of the article.
(12) Figure 5 is hard to interpret and can be removed while the numbers can be presented in running text.

Minor:
Title (line 1): “unraveling” is a vague term and is not really needed in the title.
Line 32-33: Strange sentence. I would write “are known to degrade CH3Hg+ in pure culture”.
Line 34: The metagenome assembled genomes are fictional population genomes and as such they are not involved in any biological process (the organisms they were assembled from are on the other hand).
Line 36+89: This is not an assessment, but an estimate or prediction (depending on how you see it).
Line 136: “enrichment” should be “13C enrichment”
Line 146: “dominated both in the” is redundant and can be deleted.
Line 156: “putative microbial taxa” is awkward and pointless. Do you mean Candidate taxa?
Line 160: Clarify that you mean “metagenome sequencing and reconstruction of metagenome assembled genomes”.
Line 163: What are these requirements? Is it a fixed limit or depending on service provider?
Line 225-226: This is an incorrect statement. Please specify that this relates to degradation of organic substrates for energy acquisition.
Line 233: Remove “the”, since many more taxa may be shared beyond the most abundant.
Line 239: “especially” rather than “specifically”.
Line 283: “to decreases of” does not fit here. Revise.
Line 287: Reading the sentence one can interpret this as if you actually were able to enhance the in situ activities of these putative demethylators, but I think this is only a suggestion, right?
Line 341: “accurate” is not the right word.
Line 403: What is “optimized” sequence? You mean “trimmed or quality controlled?
Line 415: Remove “proceeded”

Reviewer #3

(Remarks to the Author)
The authors attempted to elucidate the microbial communities and their functions involved in degrading highly toxic methylmercury (CH3Hg) through SIP and metagenomic analyses, using DNA extracted from soils treated with CH3Hg. Additionally, they calculated the potential for CH3Hg degradation in various soils across China.

The key flow of this manuscript is summarized as follows:
(1) CH3Hg was added to High-Hg contaminated paddy soil, High-Hg contaminated upland soil, Low-Hg contaminated paddy soil, and Low-Hg contaminated upland soil. The CH3Hg degradation rates were determined (Fig. 1, Supplementary Fig. 1).
(2) A SIP analysis was conducted using DNA extracted from soils treated with CH3Hg, focusing on High-Hg contaminated paddy soil and Low-Hg contaminated upland soil. Quantitative PCR targeting 16S rRNA was performed, and the community structures of the peak fractions were analyzed via amplicon sequencing. Arenimonas, Dechloromonas, and MM2 predominantly assimilated 13C in High-Hg contaminated paddy soil, while Ramlibacter and Sumerlaea were identified in Low-Hg contaminated soil, suggesting their involvement in CH3Hg degradation (Fig. 2, 3, Supplementary Fig. 2, 3).
(3) Among these, isolates of Dechloromonas (the second most abundant) and Methylovorus (possibly related to the fifth most abundant Methylotenera) were obtained from DSMZ or another source, and their ability to degrade CH3Hg was experimentally demonstrated (Supplementary Fig. 4).
(4) Shotgun metagenomics was performed on DNA extracted from soils (13CH3Hg-treated?), and MAGs were constructed. More than half of the constructed MAGs were derived from Actinobacteria, while bacteria identified as dominant in (2) were absent. The authors examined the CH3Hg degradation pathways and other metabolic pathways encoded in the genomes of these MAGs (Fig. 4, Supplementary Fig. 5).
(5) Using metagenomic analysis, the relative abundance of various metabolic pathways was compared between soils treated with 12CH3Hg and 13CH3Hg (Supplementary Fig. 6-9).

Major Comments:
(2) Analysis of High-Hg contaminated upland soil and Low-Hg contaminated paddy soil
・There is no mention about High-Hg contaminated upland soil and Low-Hg contaminated paddy soil in this manuscript. Why were these soil types not selected for subsequent analysis? This information is missing throughout the manuscript.
・The Discussion states that similar bacteria contributed to CH3Hg degradation in soils located 500 km apart. Does this mean that upland and paddy soils were being compared? Given that the potential for CH3Hg degradation across different Hg contamination levels within China was calculated, wouldn’t it be more appropriate to compare to each other among

paddy soils? If upland soils were analyzed, the Introduction should provide a clear rationale, and the comparisons should extend to upland soils as well.

(3) Selection of Dechloromonas and Methylovorus
・Why were Dechloromonas and Methylovorus selected? Dechloromonas is the second most abundant, and Methylovorus, possibly related to Methylotenera, ranks fifth. Is it unnecessary to examine other bacteria?

(4) Issues with the constructed MAGs
This is considered the most critical issue.
・More than half of the constructed MAGs were derived from Actinobacteria, and none of the bacteria identified in the SIP analysis as contributors to CH3Hg degradation were present.
・Actinobacteria are known to have high GC content, as does the second most abundant Gemmatimonas. Since the MAGs were constructed from metagenomic data of the heavy fractions, it is possible that the bacteria identified are not contributors to 13CH3Hg degradation but were enriched due to their high GC content. The authors should evaluate whether genes from Actinobacteria or Gemmatimonas are significantly more abundant in soils treated with 13CH3Hg than in soils treated with 12CH3Hg. If discrepancies arise between the metagenomic and SIP analyses, the reasons for these differences should be addressed.
・If the study aims to discuss the microbial communities and their functions involved in CH3Hg degradation, greater emphasis should be placed on bacteria identified through SIP analysis as 13CH3Hg degraders. The metabolic pathways associated with functional genes from these bacteria should be evaluated via metagenomics.
Specifically, the microbial origins of functional genes obtained through metagenomics should first be identified. Functional genes derived from microbes identified as 13CH3Hg degraders in the SIP analysis should be prioritized for discussion.
Materials and Methods of Shotgun Metagenomic Sequencing
Was shotgun metagenomics conducted only for High-Hg contaminated paddy soil?
The authors should specify which fraction from which day was used.

(5) Discussion of metabolic pathways from metagenomic analysis
The abundance of various pathways obtained from metagenomic analysis of soils treated with 12CH3Hg and 13CH3Hg is discussed, but no significant differences were observed between 12C and 13C.
Based on these results, it is difficult to discuss microbial functions associated with 13CH3Hg degradation.

General Impression
Overall, the analysis and discussion of the samples possibly with high degradation potential (as demonstrated in (1)) seems to be insufficient.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have dealt diligently and effectively with my previous comments (and those from the other reviewers). The manuscript is now much more clear and less speculative. I have only a few remaining questions and suggested edits as outlined below.
(1) The way the manuscript is written it's still hard to decipher the actual methyl mercury concentrations in the microcosms. It is stated that the systems were spiked with ca 45 microgram/kg (line 364) but it's also stated that the final concentration was 50 microgram/kg (line 360), and looking at the original features of the soils, the in situ methylmercury concentration seems to vary quite a bit from less than one to a few micrograms per kg (Supplementary table 1). I also don't see how this agrees with results presented in figure 1 and supplementary figure 1 where the measured concentration of methylmercury varies quite a lot and typically is way below 50 microgram/kg. How was in situ methyl mercury was handled in estimating the degradation efficiency? Please clarify this. How did you analytically distinguish between 13C-methylmercury and 12C-methylmercury? (fig 1A,B)? Many questions left unanswered here.

Line 185-187, 300-304: The absence of merAB genes in the "heavy" fraction is interpreted as if another methylmercury pathway is in operation. But can't it just be that the methyl group that is produced in the demethylation is not assimilated into DNA to any major extent? In such a case the demethylating microbes would still be in the isotopically "light" fractional. Assimilation into biomass is a critical assumption in this work and it is not a law of nature, so alternative explanations should at least be mentioned.

Line 155/156: should read "the 12CH3Hg+ treatment"

Line 176: What is "combination analyses"? Should this read "Combined analyses"?

Line 279: "The no significant difference" is awkward and should be revised. "similar"?

Line 334: "enormously" should be deleted. Unclear what it means.

Line 467: "sequence" should read "sequences" and "sequences were"

Reviewer #3

(Remarks to the Author)
While the authors have made meaningful progress in refining their analysis and the overall quality of the paper, several issues still need to be addressed, as outlined below.

L184–204:
Most of the genes discussed in the main text are either absent from Supplementary Fig. 6 or not indicated as significantly enriched. This discrepancy suggests that either the figure needs to be replaced with a more representative version, or the corresponding descriptions in the main text should be substantially revised to align with the data shown.

L187–189:
References should be added here.

L297–304:
Why were fdoG (Gemmatimonadota) and gcvP (Actinobacteria) highlighted as significantly enriched among the numerous genes related to carbon metabolism? While the shift in focus toward carbon metabolism is understandable due to the absence of significant differences in merA and merB, the rationale behind this focus should be more explicitly justified (as described above: L187-189's reference). Additionally, the biological or ecological significance of these specific genes must be elaborated upon in greater detail.
Currently, the discussion risks appearing selective or post hoc—as if only the genes that support a convenient narrative were chosen from Supplementary Fig. 6.

To strengthen the discussion, it is essential to clarify the reasoning behind the observed enrichment of bacteria lacking merA and merB under the 13C-Hg treatment and to ensure that all descriptions accurately reflect the underlying data.

Response letter

Responses to reviewer's comments:

Reviewer #1 (Remarks to the Author):

Authors are presenting findings about methylmercury degradation by rice paddy microorganisms based on incubation with labeled methylmercury coupled to DNA-SIP, metagenomic and tests on living bacterial strains.

The topic is relevant from different perspectives, including food safety and environmental microbiology, adding important information on the identity of methylmercury degraders and their potential impact on food safety. Referenced literature is adequate and conclusions fit with the aims and the results of the experiments.

The manuscript is generally well written, with minor revisions needed in the English and some typos (i.e., Line 49: Change "Hg methylating" with "Hg-methylating"; Lines 56-58: Revise English of the sentence "Subsequently … degraders."; Line 152: Add final s to "*Arenimona*" and "*Dechloromona*").

1. Thank you very much for the positive and constructive comments on our manuscript. We have carefully checked the manuscript and corrected English typos. Please see our revisions in lines 52, 62-64 and 165.

In the results section, since "Materials and methods" are at the end, authors should provide a few details for clarity (i.e., the platform used to perform long-read sequencing; the fact that 16S genes were quantified with qPCR).

2. We have supplemented the necessary details of methods in the result section. For instance, we have now provided information on the long-read sequencing of 16S rRNA genes using the PacBio R RS II platform and the quantification of 16S rRNA gene abundances by real-time quantitative PCR. Please see our revisions in lines 136-138, 145-148 and 176-184.

There is one experimental point that is absolutely not clear for me in Lines 162-166: how was DNA pooling managed? According to what is written, heavy DNA was pooled with 16S rRNA copies, but I think this does not make sense. Authors should clarify well this point.

3. Thanks for your valuable comment. We conducted the DNA-SIP experiments with three replicates for both the $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ treatments. Due to insufficient ^{13}C-labeled DNA from a single extraction for shotgun metagenomic sequencing, DNA was extracted three times from the same soil for each replicate. The DNA from each extraction was subjected to DNA-SIP gradient fractionation. After DNA-SIP fractionation, composite samples were formed by combining the fractions with the highest abundance of 16S rRNA gene from the $^{13}CH_3Hg^+$ treatment for

sequencing. We have clarified these important points in the revised manuscript (lines 179-184 and 476-480).

Reviewer #2 (Remarks to the Author):

The manuscript by Zhou et al provide interesting new data and findings about microbial mercury detoxification in farmland soil and also present some back of the envelope estimates of how ambient microbial mercury demethylation processes can lower the health burden related to human mercury exposure. The methods used to estimate rates and identify active microbial populations are state of the art and the data appear very convincing to this reviewer and my main critique relates to the uncertain modeling of the health hazards from MeHg exposure (where some modifications and further discussion are warranted).

4. Thank you very much for your positive comments and valuable feedback on our work. We also greatly appreciate your insightful critique regarding the uncertainties in the modeling of health hazards from CH_3Hg^+ exposure, which substantially improves our manuscript. In response, we have now expanded the discussion on the assumptions and uncertainties in the health risk estimates, providing a clearer explanation of the key parameters (e.g., microbial degradation efficiency and soil CH_3Hg^+ concentration) and their contributions to variability in the results (lines 324-328 and 529-540). Additionally, we conducted uncertainty and sensitivity analyses to assess the robustness of our conclusions and identified the parameters with the most significant influence on health risk estimates (lines 560-574). The results underscore the critical role of microbial degradation efficiency and soil CH_3Hg^+ concentration in mitigating health risks. We have also clarified the rationale for our modeling framework while discussing potential avenues for future improvements, including the integration of larger datasets and more comprehensive uncertainty analysis methods (lines 328-334). Please find our point-by-point responses to your concerns below.

(1) Line 104: Does these concentrations account for in situ MeHg concentration? Background concentrations may be substantial.

5. We appreciate your concern. The background CH_3Hg^+ concentrations may be substantial in general CH_3Hg^+ transformation studies; however, soil microorganisms assimilated only small amounts of ^{13}C from $^{13}CH_3Hg^+$. Considering the relatively high cost of DNA-SIP, we increased the spiked CH_3Hg^+ concentration (~45 μg kg^{-1}) to ensure sufficient ^{13}C enrichment with $^{13}CH_3Hg^+$ for effectively tracing ^{13}C-consuming microorganisms and identifying those involved in $^{13}CH_3Hg^+$ degradation. Meanwhile, this spiked concentration is comparable to CH_3Hg^+ concentrations observed in polluted farmland soils from Hg mining areas (ranging from 0.14 to 67 μg kg^{-1})[1]. We have clarified this important point in lines 361-368 of the revised manuscript.

(2) Line 109-117: The degradation efficiency is arbitrarily defined as % degraded over a 28 day period. It would be much more useful to provide degradation rates.

6. Thanks for this valuable suggestion. We have now calculated the degradation rates of $^{13}CH_3Hg^+$ over the incubation period (Supplementary Fig. 2). The rates were generally greater than 0, indicating continuous degradation of $^{13}CH_3Hg^+$. The degradation rates showed an initial increase followed by a decline in later stages, likely due to the depletion of the substrate $^{13}CH_3Hg^+$. Moreover, the highest degradation rates were observed on day 14 in the High_P soil (2.95 μg kg^{-1} d^{-1}) and Low_U soil (1.43 μg kg^{-1} d^{-1}), whereas peak rates occurred on day 3 in the High_U soil (1.57 μg kg^{-1} d^{-1}) and Low_P soil (5.30 μg kg^{-1} d^{-1}). To better evaluate the CH_3Hg^+ degradation potential across different soils, we combined the analysis of degradation rates with degradation efficiency[2, 3]. This integrated approach provides a more comprehensive assessment, as relying solely on degradation rates makes it challenging to identify the soil with the highest CH_3Hg^+ degradation potential. Our analysis revealed both high CH_3Hg^+ degradation efficiency and rate in the High_P and Low_U soils, providing valuable insights into the microbial processes involved. Please see our revisions in lines 124-130 and Supplementary Fig. 2.

(3) Line 199-211, 454-485: The estimates of health risks are critically depending on a number of rather uncertain assumptions and the estimates should be presented as such (acknowledging uncertainties). I also wonder how the "degradation efficiency" (fraction degraded MeHg in 28 days) come into play here. Why limit the assessment to this period? Do you assume that degradation halts after 28 days? The data does not support that. All in all I feel that a proper sensitivity analysis of this modeling effort is warranted to illustrate/assess the uncertainties in the numbers presented.

7. We appreciate the reviewer's insightful comments. Below, we provide detailed responses to these concerns.

(1). Role of microbial degradation efficiency and rationale for the incubation period

Microbial degradation efficiency (the percent of CH_3Hg^+ degraded over 28 days by the microbial community) was incorporated into the model to calculate changes in rice CH_3Hg^+ concentrations, which subsequently affected the estimated daily intake (EDI) and health risk (HQ and ΔIQ). This efficiency was quantified by comparing the difference in CH_3Hg^+ concentration between sterilized and non-sterilized soil samples over the incubation period. It is important to note that the observed degradation efficiencies during the 28-day period represent net changes, accounting for both production and degradation processes. We chose microbial degradation efficiency over degradation rate as it provides a standardized index. While degradation rates vary over time, microbial degradation efficiency offers a relatively stable estimate of microbial CH_3Hg^+ degradation potential in environments.

The 28-day incubation period was chosen based on the balance between observed CH_3Hg^+ degradation and microbial activity. During this period, 41%-88%

of CH_3Hg^+ degradation occurred, with degradation rates slowing significantly towards the end. Shorter incubation periods, such as 14 days, were insufficient to achieve measurable ^{13}C enrichment in CH_3Hg^+ degraders in DNA-SIP experiments. Conversely, longer incubation periods could result in cross-feeding effects among microbial groups, potentially confounding the identification of key CH_3Hg^+ degraders. Moreover, extended incubation time may lead to re-methylation of inorganic Hg, obscuring our focus on CH_3Hg^+ degradation. Importantly, our model does not assume degradation halts after 28 days. We recognize that both methylation and demethylation processes occur simultaneously in soils, making it difficult to determine the exact time when CH_3Hg^+ degradation halts. Previous studies have shown that CH_3Hg^+ production stabilizes at day 28 of incubation[4]. Another study found that approximately 30% of CH_3Hg^+ added into the soils was decomposed during a 28-day incubation period but that the total amount of Hg did not change[5], indicating an equilibrium between methylation and demethylation processes. This suggests that CH_3Hg^+ degradation likely reaches a stable state by 28 days. Therefore, we selected the 28-day period as it provides a balance, ensuring sufficient ^{13}C enrichment for downstream sequencing analysis while minimizing the risk of confounding factors. We have clarified these points in lines 381-384 and 529-540.

(2). Uncertainties and sensitivities in health risk estimates

The health risk estimates inherently depend on multiple assumptions, which may introduce uncertainties into the model. To address this, we conducted additional one-at-a-time sensitivity analysis and uncertainty analysis based on interval methods[6, 7] (Supplementary Tables 5-7). We identified the most important parameters for hazard quotient (HQ) and IQ decrements (ΔIQ) from microbial degradation efficiency, soil CH_3Hg^+ concentration, bioaccumulation factor (BAF), and rice ingestion rate. The results showed that microbial degradation efficiency and soil CH_3Hg^+ concentration were the most important, with microbial degradation efficiency having the highest sensitivity coefficients for HQ and ΔIQ (Supplementary Tables 5-6). For instance, when microbial degradation efficiency increased from 8.16% to 64.26% under identical conditions, the reduction in HQ increased from 0.0095 to 0.0749, representing an 8-fold increase. In contrast, varying other parameters (e.g., soil CH_3Hg^+ concentration or BAF) resulted in much smaller changes in HQ and ΔIQ. These results highlight that microbial degradation efficiency is the most critical factor in mitigating the health risks.

We further conducted an uncertainty analysis based on interval methods by varying key parameters within their observed ranges (Supplementary Table 7), following the previous study[7]. We quantified potential variability in HQ and ΔIQ, which ranged from 0.0027-0.3301 and 0.0006-0.0681, respectively. The results consistently demonstrated that microbial degradation efficiency is the key factor in reducing health risks, with its influence far surpassing other parameters like soil CH_3Hg^+ concentration or BAF. These analyses revealed the critical role of microbial degradation efficiency in mitigating the health risks, while also

emphasizing the importance of considering other factors contributing to overall uncertainty. We acknowledge the limitations of these analyses, which assume parameter independence and do not account for joint variability or probabilistic distributions. These methods were chosen due to the limited size of our dataset, which precluded the use of more advanced probabilistic approaches like Monte Carlo simulations. Therefore, further studies expanding the dataset are warranted to reinforce and consolidate the findings presented in this study, though the field sampling and experimental analyses are enormously challenging. We have now discussed these important points in the revised manuscript (Supplementary Tables 5-7, lines 324-334 and 560-574).

In summary, we demonstrate that microbial degradation of CH_3Hg^+ plays a significant role in mitigating the health risks by assessing uncertainties in key parameters, providing a clear rationale for the 28-day incubation period, and performing a detailed sensitivity analysis. These clarifications and additional analyses have been incorporated into the revised manuscript (Supplementary Tables 5-7, lines 324-334, 381-384, 529-540 and 560-574).

(4) General and line 270-272: Carrying a gene does not necessarily mean it is being used or expressed under the given environmental conditions. I don't think you can make the claim that the TCA cycle or assimilatory sulfate reduction is linked to MeHg demethylation here. For that you need transcriptomic or proteomic data (or activity measurements). Using this indirect data to claim that SRB and methanogens play an important role in oxic MeHg demethylation is speculative.

8. We agree that the presence of genes does not necessarily imply it is actively expressed or functional under specific environmental conditions. As such, we have now removed the previously speculative statements regarding the linkages between the TCA cycle, assimilatory sulfate reduction, and CH_3Hg^+ degradation, as well as the assumption of the important role of SRB and methanogens in oxic CH_3Hg^+ demethylation. Instead, we now emphasize the potential multifunctional roles of microbial taxa involved in $^{13}CH_3Hg^+$ degradation, including their potential in carbon, sulfur, and nitrogen cycling. Using $^{13}CH_3Hg^+$-DNA-SIP combined with metagenomics, we identified the microbial taxa and metabolic pathways involved in CH_3Hg^+ degradation. Our analysis recovered five fairly complete MAGs linked to $^{13}CH_3Hg^+$ degradation. Importantly, these MAGs lacked the *merA* and *merB* genes (Fig. 4B), which are crucial for the demethylation of CH_3Hg^+. This suggests that *mer*-mediated reductive demethylation is unlikely to be the dominant $^{13}CH_3Hg^+$ degradation pathway in these taxa, and this is further supported by the absence of significant differences in the relative abundance of *mer* gene abundance between the $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ treatments at the community level (Fig. 4A; Supplementary Fig 6). In contrast, we observed significant enrichments of genes associated with carbon and nitrogen metabolism in both MAGs and metagenomic analyses, particularly those involved in the H_4F pathway, which plays a critical role in one-carbon metabolism[8]. This result indicated that oxidative degradation via one-

carbon metabolism could be the primary pathway for CH_3Hg^+ degradation in the paddy soil. Please see our revisions in lines 271-288.

(5) Line 317-319: How did you assess the purity of the synthesized compounds?

9. We assessed the purity of the synthesized $^{13}CH_3Hg^+$ compounds using the MERX automated methylmercury analysis system, which provides precise determination at picogram (pg) levels. This system integrates distillation, ethylation, gas chromatography, and atomic fluorescence detection, offering high sensitivity and specificity for methylmercury detection. The purity was evaluated by comparing the measured $^{13}CH_3Hg^+$ concentration with the theoretical value based on the synthesis procedure. The close match between the measured and theoretical concentrations suggests a high level of purity. In addition, we performed liquid chromatography to check for organic impurities. The initial purity of the synthesized compound was about 70%, but further purification through extraction and back-extraction to remove inorganic components. The purity of the synthesized compound then increased over 90%. Finally, we measured the actual concentration of spiked CH_3Hg^+ in soils for subsequent analysis of CH_3Hg^+ degradation efficiency, thereby minimizing potential impacts from purity. We have supplemented these details in lines 350-358 and 394-397.

(6) Please provide a motivation for duration of the experiment (28 days). Would a shorter or longer incubation time change the major results?

10. That is an important point. As responded in #7, the experiment duration of 28 days was carefully selected based on literature and our pre-experiments[4, 5]. This duration can balance both the degradation of $^{13}CH_3Hg^+$ and the sufficient ^{13}C enrichment in soil microorganisms. The mean concentrations of $^{13}CH_3Hg^+$ in the soils ranged from 5.16 μg kg^{-1} to 28.05 μg kg^{-1} after 28 days of incubation, with biotic degradation efficiencies reaching 41%-88%. This result demonstrates active microbial $^{13}CH_3Hg$ degradation occurred during this period. Moreover, previous studies have suggested that CH_3Hg^+ degradation likely reaches a stable state by 28 days[4, 5]. For the $^{13}CH_3Hg^+$-DNA-SIP experiments, a shorter incubation period would not allow sufficient ^{13}C incorporation into the $^{13}CH_3Hg^+$ degraders. Conversely, a longer incubation time could result in cross-feeding between microbial groups. Moreover, extended incubation time may lead to re-methylation of inorganic Hg, obscuring our focus on CH_3Hg^+ degradation. Previous studies suggest that CH_3Hg^+ degradation likely reaches a stable state by 28 days[4, 5]. Therefore, the 28-day duration was chosen as an optimal balance, ensuring sufficient ^{13}C enrichment for downstream sequencing analysis while minimizing the risk of confounding factors. We have clarified these points in lines 381-384.

(7) Line 369: How was the fractionation done? Is there a risk of cross-contamination between fractions?

11. The stratified DNA-CsCl solution was fractionated into 18 aliquots following the published protocol[9, 10]. Briefly, the tube was fixed in a clamp, and the bottom was

carefully pierced with a needle in a smooth and controlled manner, after which the needle was discarded. A syringe pump was used to inject sterile deionized water into the top of the tube through the needle, and drops were collected from the bottom into sterile 1.5 mL tubes. The pump rate was set to yield approximately 18 × 280 µL fractions within 12 minutes (425 µL min^{-1}, ~ 40s per fraction). The density of all fractions was checked using a refractometer to confirm that the gradients were consistent across all tubes. Importantly, the tubes were carefully and immediately retrieved after centrifugation to avoid disturbing the gradients. To minimize diffusion and cross-contamination between fractions, the gradients were processed as quickly as possible. New sterile equipment (e.g., syringe, needle, and 1.5-mL tubes) was used for each fraction to further reduce the risk of contamination. Additionally, fractions were collected in a consistent manner to avoid mixing between them, and the process was performed rapidly to prevent gradient mixing. We have supplemented these details in lines 416-428.

(8) Line 376-378: The indicated reference is not the original one for the indicated primers. Please use the original reference and mention something about the taxonomic coverage since this is an important parameter.

12. We have provided the original reference and supplemented the taxonomic coverage about the primers in lines 430-434 "qPCR was performed using a CFX Connect real-time system (BioRad, CA, USA) with the 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) paired primers (Herlemann et al., 2011, ISME J)[11], which match approximately 90% of all bacterial sequences and cover all phyla in the Ribosomal Database Project release 10.25."

(9) Line 387-388: These are very old primers known to miss several groups (even entire phyla). A cautionary discussion about the potential to overlook some active MeHg demethylators is warranted.

13. We appreciate the constructive comments. We acknowledge that the primers 27F and 1492R may miss some active CH_3Hg^+ demethylators, though they are widely used for full-length 16S rRNA gene amplification due to their broad coverage across a wide range of bacterial phyla. We have included a cautionary discussion of this potential limitation in the revised manuscript (lines 261-264). Moreover, we performed shotgun metagenomic sequencing, which provides additional species-level annotations and enables the identification of microbes involved in CH_3Hg^+ degradation. Results showed that the dominant and differential microbial taxa identified through metagenomics were generally consistent with those detected by 16S rRNA gene amplicon sequencing (Fig. 2; Supplementary Figs. 3 and 9). This complementary approach allows for a more comprehensive identification of microbial groups involved in CH_3Hg^+ degradation (lines 261-270).

(10) Line 499-501: There are plenty of easy access and persistent data archives available for depositing sequence data. This contribute to reproducible science and I

would rplease ask the authors to deposit their data in such repositories before publication can ber considered. There is really now excuse for not doing so.

14. We have now deposited the full-length 16S rRNA gene amplicon sequences and metagenomic sequences generated in this study to the NCBI SRA database under the BioProject IDs of PRJNA1183541 and PRJNA1183649 (lines 586-589).

(11) The reference list is very long and for sure it can be trimmed to remove redundancies without compromising the rigor of the article.

15. We have checked the reference list and removed redundant references without compromising the rigor of the article.

(12) Figure 5 is hard to interpret and can be removed while the numbers can be presented in running text.

16. Thank you for your suggestion. We have removed the figure and presented the data as a new table (Supplementary Table 3).

Minor comments:

Title (line 1): "unraveling" is a vague term and is not really needed in the title.

17. Revised to "Microbial potential to mitigate neurotoxic methylmercury accumulation in farmlands and rice".

Line 32-33: Strange sentence. I would write "are known to degrade CH_3Hg^+ in pure culture".

18. Sorry for the strange sentence. The ability of these candidate taxa (i.e., *Dechloromonas denitrificans* and *Methylovorus menthalis*) to degrade CH_3Hg^+ was confirmed by this study. Therefore, we have revised the sentence to "Some of which (i.e., *Dechloromonas denitrificans* and *Methylovorus menthalis*) are subsequently confirmed to degrade CH_3Hg^+ through pure culture assays" (lines 34-36).

Line 34: The metagenome assembled genomes are fictional population genomes and as such they are not involved in any biological process (the organisms they were assembled from are on the other hand).

19. We have made the necessary revisions to clarify this point in lines 36-37 "Metagenomic analysis further reveals that most of these candidate $^{13}CH_3Hg^+$ degraders……."

Line 36+89: This is not an assessment, but an estimate or prediction (depending on how you see it).

20. We have changed "assess" to "estimate" in lines 39 and 95.

Line 136: "enrichment" should be "^{13}C enrichment"

21. Revised as suggested (line 149).

Line 146: "dominated both in the" is redundant and can be deleted.

22. Deleted.

Line 156: “putative microbial taxa” is awkward and pointless. Do you mean Candidate taxa?

23. Yes, we have changed “putative microbial taxa” to “candidate taxa”.

Line 160: Clarify that you mean “metagenome sequencing and reconstruction of metagenome assembled genomes”.

24. Clarification as suggested (lines 176-177).

Line 163: What are these requirements? Is it a fixed limit or depending on service provider?

25. The requirements here refer to the typical DNA quantity needed for shotgun metagenomic sequencing. These are not fixed limits but depend on the sequencing platform required. For example, using the Illumina NovaSeq 6000 platform (2 × 150 bp), 1-5 μg of DNA is generally needed for sequencing[12, 13]. Due to the low recovery of ^{13}C-labeled DNA in DNA-SIP, we pooled DNA from multiple replicates to meet these requirements. We have clarified this point in lines 472-480.

Line 225-226: This is an incorrect statement. Please specify that this relates to degradation of organic substrates for energy acquisition.

26. Sorry for this incorrect statement. The primary biodegradation pathway for compounds by *Methylophilaceae* is related to energy acquisition. We have now removed this sentence to refocus the discussion on the microbial taxa identified from both 16S rRNA gene amplification and metagenomic sequencing analyses according to Reviewer 3’s comments.

Line 233: Remove “the”, since many more taxa may be shared beyond the most abundant.

27. Done.

Line 239: “especially” rather than “specifically”.

28. Revised as suggested (line 248).

Line 283: “to decreases of” does not fit here.

29. Revised as “to decrease CH_3Hg^+ accumulation” (lines 306-307)

Line 287: Reading the sentence one can interpret this as if you actually were able to enhance the in situ activities of these putative demethylators, but I think this is only a suggestion, right?

30. Yes, this section is an extended discussion on how to enhance the activity of candidate CH_3Hg^+ degraders in situ to mitigate health risks. We suggest promoting the in situ microbial activity for degrading CH_3Hg^+, thereby reducing health risks. We have rewritten this sentence in line 311.

Line 341: “accurate” is not the right word.

31. We have removed this word.

Line 403: What is “optimized” sequence? You mean “trimmed or quality controlled?

32. We are referring to “the process of obtaining clean reads by removing chimeric sequences with UCHIME” in this context. We have revised this in lines 465-467 “chimeric reads were removed with UCHIME to acquire quality controlled sequence.”

Line 415: Remove “proceeded”.

33. Done.

Reviewer #3 (Remarks to the Author):

The authors attempted to elucidate the microbial communities and their functions involved in degrading highly toxic methylmercury (CH_3Hg^+) through SIP and metagenomic analyses, using DNA extracted from soils treated with CH_3Hg^+. Additionally, they calculated the potential for CH_3Hg^+ degradation in various soils across China.

The key flow of this manuscript is summarized as follows:

(1) CH_3Hg^+ was added to High-Hg contaminated paddy soil, High-Hg contaminated upland soil, Low-Hg contaminated paddy soil, and Low-Hg contaminated upland soil. The CH_3Hg^+ degradation rates were determined (Fig. 1, Supplementary Fig. 1).

(2) A SIP analysis was conducted using DNA extracted from soils treated with CH_3Hg^+, focusing on High-Hg contaminated paddy soil and Low-Hg contaminated upland soil. Quantitative PCR targeting 16S rRNA was performed, and the community structures of the peak fractions were analyzed via amplicon sequencing. *Arenimonas*, *Dechloromonas*, and *MM2* predominantly assimilated ^{13}C in High-Hg contaminated paddy soil, while *Ramlibacter* and *Sumerlaea* were identified in Low-Hg contaminated soil, suggesting their involvement in CH_3Hg^+ degradation (Fig. 2, 3, Supplementary Fig. 2, 3).

(3) Among these, isolates of *Dechloromonas* (the second most abundant) and *Methylovorus* (possibly related to the fifth most abundant *Methylotenera*) were obtained from DSMZ or another source, and their ability to degrade CH_3Hg^+ was experimentally demonstrated (Supplementary Fig. 4).

(4) Shotgun metagenomics was performed on DNA extracted from soils ($^{13}CH_3Hg^+$-treated?), and MAGs were constructed. More than half of the constructed MAGs were derived from Actinobacteria, while bacteria identified as dominant in (2) were absent. The authors examined the CH_3Hg^+ degradation pathways and other metabolic pathways encoded in the genomes of these MAGs (Fig. 4, Supplementary Fig. 5).

(5) Using metagenomic analysis, the relative abundance of various metabolic pathways was compared between soils treated with $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ (Supplementary Fig. 6-9).

34. We appreciate that the reviewer highlighted the key flow of our work. Shotgun metagenomics was performed on the fraction with the highest 16S rRNA gene copies from the $^{13}CH_3Hg^+$ treatment, as well as the corresponding fraction from the $^{12}CH_3Hg^+$ treatment (lines 472-480). We now provide detailed responses to all your concerns below.

Major Comments:

(2) Analysis of High-Hg contaminated upland soil and Low-Hg contaminated paddy soil

There is no mention about High-Hg contaminated upland soil and Low-Hg contaminated paddy soil in this manuscript. Why were these soil types not selected for subsequent analysis? This information is missing throughout the manuscript.

35. We appreciate your concerns. In this study, degradation efficiencies of $^{13}CH_3Hg^+$ varied among soil types. The paddy soil from the high Hg-contaminated site (High_P) and upland soil from the low Hg-contaminated site (Low_U) exhibited higher microbial degradation potential, with degradation efficiencies of 88% and 65%, respectively. Considering the relatively high cost of DNA-SIP experiments and the need for sufficient ^{13}C incorporation to ensure accurate identification of key microbial taxa, we selected the High_P and Low_U soils for subsequent analyses. We have now clarified these important points in lines 124-130 and 132-134 of the revised manuscript.

The Discussion states that similar bacteria contributed to CH_3Hg^+ degradation in soils located 500 km apart. Does this mean that upland and paddy soils were being compared? Given that the potential for CH_3Hg^+ degradation across different Hg contamination levels within China was calculated, wouldn't it be more appropriate to compare to each other among paddy soils? If upland soils were analyzed, the Introduction should provide a clear rationale, and the comparisons should extend to upland soils as well.

36. Yes, we initially compared the microbial taxa from upland and paddy soils to highlight their shared role in the $^{13}CH_3Hg^+$ degradation process, demonstrating their potential ubiquity across different environments. We acknowledge that comparing paddy soils with varying Hg contamination levels would indeed be more appropriate for the specific focus of this study. However, to ensure sufficient ^{13}C enrichment and accurate identification of key microbial taxa and metabolic pathways, we selected High_P and Low_U soils for the $^{13}CH_3Hg^+$-DNA-SIP experiments, which exhibited higher microbial $^{13}CH_3Hg^+$ degradation potentials. We also recognize that water-dry rotation is a common agricultural practice in China, where uplands are often converted to paddy fields seasonally, and this contributes to our initial inclusion of both soil types. We have supplemented the

rationale in the Introduction section and comparisons between the paddy and upland soils in the result section (lines 54-57 and 153-174).

(3) Selection of *Dechloromonas* and *Methylovorus*

Why were *Dechloromonas* and *Methylovorus* selected? *Dechloromonas* is the second most abundant, and *Methylovorus*, possibly related to *Methylotenera*, ranks fifth. Is it unnecessary to examine other bacteria?

37. We selected *Dechloromonas denitrificans* and *Methylovorus menthalis* for further pure culture assays based on their broad distribution patterns observed in both light and heavy fractions of the $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ treatment. While most biomarkers were only enriched in the ^{13}C-DNA heavy fraction (the fraction 11), suggesting their potential as CH_3Hg^+ degraders, the two genera exhibited significant enrichment in both light and heavy fractions (the fractions 9-14) from the $^{13}CH_3Hg^+$ treatment (Fig. 3; Supplementary Fig. 4). This broad distribution prompted us to validate whether they are involved in CH_3Hg^+ degradation. Pure culture assays with *Dechloromonas denitrificans* and *Methylovorus menthalis* confirmed their ability to degrade CH_3Hg^+, thus supporting their potential role in CH_3Hg^+ degradation. While other biomarkers were also enriched in the $^{13}CH_3Hg^+$ treatment, the two genera were prioritized for further investigation due to their distinct distribution patterns. Future studies, such as isolating these specific taxa in pure culture, are warranted to reinforce and consolidate the results of this study. The rationale for selecting these two genera for pure culture assays has now been provided in the manuscript (lines 166-172).

(4) Issues with the constructed MAGs.

This is considered the most critical issue.

More than half of the constructed MAGs were derived from *Actinobacteria*, and none of the bacteria identified in the SIP analysis as contributors to CH_3Hg^+ degradation were present.

38. We appreciate the reviewer's concerns. In the previous manuscript, we compared microbiota in the fractions of 9-14 in the $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ treatments, including both heavy and light fractions, which may have masked differences between the active microbial taxa in treatments. To address this, we have now focused on analyzing the 16S rRNA gene amplicon sequences from the fraction with the highest abundance of 16S rRNA gene copies in the $^{13}CH_3Hg^+$ treatment and the corresponding fraction in the $^{12}CH_3Hg^+$ treatment[14]. This targeted analysis better represents the active microbial taxa directly involved in $^{13}CH_3Hg^+$ degradation. The results revealed that genera such as *Pseudarthrobacter*, *Dechloromonas* and *MM2* were significantly enriched in the $^{13}CH_3Hg^+$ treatment (Fig. 3C), suggesting that these taxa are key microorganisms involved in CH_3Hg^+ degradation (see lines 161-172).

We further re-analyzed the $^{13}CH_3Hg^+$-SIP-metagenomics in the fraction with the highest abundance of 16S rRNA gene copies in the $^{13}CH_3Hg^+$ treatment and its corresponding fraction in the $^{13}CH_3Hg^+$ treatment to investigate the role of these taxa in CH_3Hg^+ degradation. Among the five MAGs identified, three were annotated as g_*Arthrobacter_I* in the GTDB database (Supplementary Table 2), where the "I" typically indicates an inadequately classified genus. Phylogenetic analysis revealed that MAG1, MAG2, and MAG5 clustered with *Pseudarthrobacter*, indicating a strong phylogenetic relationship (Supplementary Fig. 7). Metabolic potential and pathway analyses of these MAGs further support their potential role in CH_3Hg^+ degradation, providing functional evidence to link these MAGs to the key taxa identified in the ^{13}C-DNA fraction (lines 205-220).

Moreover, the dominance of *Actinobacteria* in the reconstructed MAGs is not surprising, as these taxa are abundant in many soil environments, particularly in polluted soils, due to their metabolic versatility and stress adaptation. Their high GC content likely led to overrepresentation in metagenomic assembly. To assess whether the enrichment of *Actinobacteria* in the ^{13}C-DNA heavy fraction was solely due to GC content, we compared their relative abundance in the fraction with the highest abundance of 16S rRNA gene copies from the $^{13}CH_3Hg^+$ treatment and its corresponding fraction in the $^{12}CH_3Hg^+$ treatment. Interestingly, *Actinobacteria*-associated taxa showed higher relative abundances in the $^{13}CH_3Hg^+$ treatment compared to the $^{12}CH_3Hg^+$ treatment (Supplementary Fig. 9), supporting their functional role in CH_3Hg^+ degradation rather than simply reflecting a GC content bias (lines 289-304).

In summary, we re-analyzed both the 16S rRNA gene amplicon and shotgun metagenomic sequences in the fraction with the highest abundance of 16S rRNA gene copies in the $^{13}CH_3Hg^+$ treatment and its corresponding fraction in the $^{12}CH_3Hg^+$ treatment. The results highlighted *Actinobacteria*-associated genera (i.e. *Pseudarthrobacter*) as candidate microbial taxa involved in $^{13}CH_3Hg^+$ degradation, further improving the alignment between SIP-derived taxa and MAG annotations. We have updated these analyses in the revised manuscript (lines 161-172, 205-220 and 289-304).

Actinobacteria are known to have high GC content, as does the second most abundant *Gemmatimonas*. Since the MAGs were constructed from metagenomic data of the heavy fractions, it is possible that the bacteria identified are not contributors to $^{13}CH_3Hg^+$ degradation but were enriched due to their high GC content. The authors should evaluate whether genes from *Actinobacteria* or *Gemmatimonas* are significantly more abundant in soils treated with $^{13}CH_3Hg^+$ than in soils treated with $^{12}CH_3Hg^+$. If discrepancies arise between the metagenomic and SIP analyses, the reasons for these differences should be addressed.

39. We sincerely thank the reviewer for the thoughtful comments and for highlighting the potential role of *Actinobacteria* and *Gemmatimonas* (i.e., *Gemmatimonadota*) in CH_3Hg^+ degradation, as well as the possible influence of high GC content on

their enrichment in the ^{13}C-DNA heavy fractions. To assess whether the enrichment of *Actinobacteria* and *Gemmatimonadota* in the ^{13}C-DNA heavy fractions was driven solely by GC content, we compared their relative abundance in the $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ treatments. If GC content were the primary factor, similar relative abundances would be expected in both $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ treatments. Our results revealed consistently higher relative abundances of both *Actinobacteria* and *Gemmatimonadota* in the fraction with the highest abundance of 16S rRNA gene copies from the $^{13}CH_3Hg^+$ treatment compared with the corresponding fraction in the $^{12}CH_3Hg^+$ treatment (Supplementary Fig. 9), suggesting their functional role in CH_3Hg^+ degradation rather than the enrichment due to GC content bias (lines 289-293).

Additionally, we analyzed the functional genes involved in key biochemistry pathways (e.g., mercury reduction, H_4F pathway and carbon metabolism) within *Actinobacteria* and *Gemmatimonadota*. Results showed no significant differences in the relative abundance of *mer* operon genes (e.g., *merA* and *merB*) between the $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ treatments (Supplementary Fig. 10). In contrast, genes associated with one-carbon (C1) compound metabolisms, such as *fdoG* (formate dehydrogenase) and *gcv* (glycine cleavage system protein), were significantly more abundant in the $^{13}CH_3Hg^+$ treatment compared with the $^{12}CH_3Hg^+$ treatment. These C1 metabolism-related genes, enriched in both *Actinobacteria* and *Gemmatimonas*, suggest that other pathways, rather than *mer*-mediated reductive demethylation, may be the predominant CH_3Hg^+ degradation mechanism in the paddy soil. This was further supported by the lack of significant enrichment of *mer* genes and the notable enrichment of genes associated with carbon and nitrogen metabolism at the community level (lines 293-304).

As detailed in our response to #38, we re-analyzed the 16S rRNA gene amplification and shotgun metagenomics in the fraction with the highest abundance of 16S rRNA gene copies in the $^{13}CH_3Hg^+$ treatment and its corresponding fraction in the $^{12}CH_3Hg^+$ treatment. The results improve the alignment between SIP-derived taxa and MAG annotations, further emphasizing the functional role of *Actinobacteria*-associated genera (i.e. *Pseudarthrobacter*), particularly in metabolism pathways. These clarifications have been incorporated into the revised manuscript (lines 161-172, 205-220 and 271-304).

If the study aims to discuss the microbial communities and their functions involved in CH_3Hg^+ degradation, greater emphasis should be placed on bacteria identified through SIP analysis as $^{13}CH_3Hg^+$ degraders. The metabolic pathways associated with functional genes from these bacteria should be evaluated via metagenomics.

Specifically, the microbial origins of functional genes obtained through metagenomics should first be identified. Functional genes derived from microbes identified as $^{13}CH_3Hg^+$ degraders in the SIP analysis should be prioritized for discussion.

40. We appreciate the constructive suggestions on the improvement of our manuscript. We have now re-analyzed the 16S rRNA gene amplification and metagenomic data

from the fraction with the highest abundance of 16S rRNA gene copies in the $^{13}CH_3Hg^+$ treatment. This fraction more accurately represents the active microbial taxa contributing to CH_3Hg^+ degradation. As shown in Fig. 3C, genera such as *Pseudarthrobacter*, *Dechloromonas*, and *MM2* were significantly enriched in the $^{13}CH_3Hg^+$ treatment, suggesting that these taxa may be key microorganisms involved in $^{13}CH_3Hg^+$ degradation. Furthermore, we reconstructed a total of 5 MAGs, three of which were associated with *Pseudarthrobacter* (Fig. 4B; Supplementary Fig. 7). In particular, these *Pseudarthrobacter*-associated MAGs were subsequently analyzed to investigate the metabolic pathways related to CH_3Hg degradation. In the revised manuscript, we identified functional genes associated with pathways such as reductive degradation mediated by the *mer* operon, oxidative degradation related to one-carbon (C1) metabolism, as well as sulfur metabolism and nitrate reduction from these MAGs related to the identified candidate CH_3Hg^+ degraders through long-read sequencing analysis and metagenomics. These functional genes from microbes identified as $^{13}CH_3Hg^+$ degraders in the SIP analysis have now been prioritized for discussion (lines 161-220 and 252-288).

Materials and Methods of Shotgun Metagenomic Sequencing

Was shotgun metagenomics conducted only for High-Hg contaminated paddy soil?

41. Yes, shotgun metagenomics was conducted exclusively on the High-Hg contaminated paddy soil (High_P), which was selected due to its highest potential of microbial $^{13}CH_3Hg^+$ degradation. The health risks associated with CH_3Hg^+ are most prominent in paddy fields, where the flooded conditions create an anoxic environment favorable for CH_3Hg^+ production. Exploring the key taxa and metabolic potential of CH_3Hg^+ degraders in paddy soils is essential for understanding and mitigating CH_3Hg^+ risks in agroecosystems. Moreover, given that soil microorganisms assimilated only small amounts of ^{13}C from $^{13}CH_3Hg^+$, we monitored ^{13}C assimilation at multiple time points to ensure sufficient ^{13}C enrichment for accurate DNA-SIP analysis. For the paddy soil from the high Hg-contaminated region, we performed three rounds of ultracentrifugation and 48 DNA-SIP fractionations, followed by quantitative analysis of about 800 DNA samples from the fractions. The choice to conduct shotgun metagenomics on the High_P soil was made after considering the relatively high cost and time consuming of both DNA-SIP experiments and metagenomic sequencing, as well as the need for sufficient ^{13}C markers with $^{13}CH_3Hg^+$ for accurate analysis. Based on the $^{13}CH_3Hg^+$-DNA-SIP methodology we developed, future studies on additional soil samples will deepen our understanding of CH_3Hg^+-degrading microorganisms and their metabolic pathways. We have added this detail to the revised manuscript (lines 181-184 and 472-474).

The authors should specify which fraction from which day was used.

42. The fraction with the highest 16S rRNA gene copies from the $^{13}CH_3Hg^+$ treatment of the High_P soil on day 28, was selected for shotgun metagenomic sequencing. We have added this detail to the revised manuscript (lines 182-184).

(5) Discussion of metabolic pathways from metagenomic analysis

The abundance of various pathways obtained from metagenomic analysis of soils treated with $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ is discussed, but no significant differences were observed between ^{12}C and ^{13}C.

43. We acknowledge this point. As addressed in response to comment #38, we initially compared the differential microbiota in the 9-14 fractions of the $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ treatments in the previous manuscript. However, since these fractions included both heavy and light fractions, this approach may have masked the differences between the active microbial populations associated with CH_3Hg^+ degradation. In the revised manuscript, we have now focused on comparing the fraction with the highest 16S rRNA gene copies from the $^{13}CH_3Hg^+$ treatment and the corresponding fraction from the $^{12}CH_3Hg^+$ treatment. The results showed that the relative abundances of genes related to sulfate metabolism (e.g., *cysH*, *cysD* and *cysN*), the H_4F pathway (e.g., *metH* and *mtgA*), the TCA cycle (e.g., *acnA* and s*dhA*), nitrate reduction, and denitrification (e.g., *glnA*, *nirB* and *napAB*) were significantly higher in the $^{13}CH_3Hg^+$ treatment compared with the $^{12}CH_3Hg^+$ treatment (Fig. 4; Supplementary Fig. 6). Please see the revisions highlighted in lines 184-204.

Based on these results, it is difficult to discuss microbial functions associated with $^{13}CH_3Hg^+$ degradation.

44. We appreciate the reviewer's comment. In earlier version of the manuscript, the comparison of the $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ treatments did not show significant differences, which may have made it difficult to link microbial functions with CH_3Hg^+ degradation. However, as detailed in the revised manuscript, we have refined our analysis by focusing on the fraction with the highest abundance of 16S rRNA gene copies in the $^{13}CH_3Hg^+$ treatment and the corresponding fraction in the $^{12}CH_3Hg^+$ treatment. This refined approach revealed significant differences between the two treatments. Specifically, genes related to sulfate metabolism (e.g., *cysH*, *cysD* and *cysN*), the H_4F pathway (e.g., *metH* and *mtgA*), the TCA cycle (e.g., *acnA* and s*dhA*), nitrate reduction, and denitrification (e.g., *glnA*, *nirB* and *napAB*) were significantly more abundant in the $^{13}CH_3Hg^+$ treatment compared with the $^{12}CH_3Hg^+$ treatment, while no significant differences were observed for *mer* genes between the $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ treatments (Fig. 4; Supplementary Fig. 6). These findings combined with the analyses of 16S rRNA gene amplification and MAGs suggest that these microbial functions could be associated with CH_3Hg^+ degradation, while *mer*-mediated reductive demethylation is unlikely to be the dominant CH_3Hg^+ degradation pathway in the paddy soil. We have discussed these results in the revised manuscript (lines 271-288) to better link microbial functions with $^{13}CH_3Hg^+$ degradation.

General Impression

Overall, the analysis and discussion of the samples possibly with high degradation potential (as demonstrated in (1)) seems to be insufficient.

45. We appreciate the reviewer's comments. In the revised manuscript, we have expanded the analyses based on both 16S rRNA gene amplification and shotgun metagenomic sequencing. Specifically, we compared the relative abundance of microorganisms in the fraction with the highest abundance of 16S rRNA gene copies from the $^{13}CH_3Hg^+$ treatment with the corresponding fraction from the $^{12}CH_3Hg^+$ treatment. Linear discriminant analysis effect size (LEfSe) analysis identified genera such as *Pseudarthrobacter* and *Dechloromonas* as biomarkers in the $^{13}CH_3Hg^+$ treatment (Fig. 3C), suggesting that these taxa may be key taxa involved in $^{13}CH_3Hg^+$ degradation.

Our SIP-metagenomic results further indicated that multiple metabolic pathways are involved in $^{13}CH_3Hg^+$ degradation (Fig. 4). We reconstructed a total of five fairly complete MAGs linked to $^{13}CH_3Hg^+$ degradation, predominantly affiliated with *Actinobacteria* and *Gemmatimonadota*. Among these MAGs, 3 were identified as novel species associated with the genus *Pseudarthrobacter* (Supplementary Fig. 7). Notably, these MAGs did not contain the *merA* and *merB* genes, which are pivotal for reductive demethylation via the *mer* operon, suggesting that *mer*-mediated reductive demethylation is unlikely to be the dominant CH_3Hg^+ degradation pathway in these taxa. This was further supported by the no significant difference in the relative abundances of *mer* genes between the $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ treatments at the community level (Supplementary Fig. 6). In contrast, significant enrichments of genes associated with carbon and sulfur metabolism were observed in both MAGs and metagenomic analyses, particularly those involved in the H_4F pathway, which plays a critical role in one-carbon metabolism. This finding indicated that oxidative degradation involved the co-metabolism of CH_3Hg^+ resembling the metabolism of other small organic substrates (e.g., C1 compounds) by heterotrophic bacteria, including sulfate-reducing bacteria and methanogens, could be the primary pathway for CH_3Hg^+ degradation in the paddy soil.

Overall, these updated analyses provide a more comprehensive understanding of the microbial functions and pathways potentially involved in CH_3Hg^+ degradation, shedding light on the underlying mechanisms in paddy soil. Please see our revision in Figs. 3-4 and lines 161-174, 205-220 and 271-304.

We sincerely thank all the comments/suggestions of reviewers, which substantially improve our manuscript.

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Response letter

Responses to reviewer's comments:

Reviewer #2 (Remarks to the Author):

The authors have dealt diligently and effectively with my previous comments (and those from the other reviewers). The manuscript is now much more clear and less speculative. I have only a few remaining questions and suggested edits as outlined below.

1. We appreciate the positive comments on our responses and modifications to the previous concerns. We now address your remaining concerns as follows.

(1) The way the manuscript is written it's still hard to decipher the actual methyl mercury concentrations in the microcosms.

It is stated that the systems were spiked with ca 45 microgram/kg (line 364) but it's also stated that the final concentration was 50 microgram/kg (line 360), and looking at the original features of the soils, the in situ methylmercury concentration seems to vary quite a bit from less than one to a few micrograms per kg (Supplementary table 1). I also don't see how this agrees with results presented in figure 1 and supplementary figure 1 where the measured concentration of methylmercury varies quite a lot and typically is way below 50 microgram/kg. How was in situ methyl mercury was handled in estimating the degradation efficiency? Please clarify this. How did you analytically distinguish between ^{13}C-methylmercury and ^{12}C-methylmercury? (fig 1A,B)? Many questions left unanswered here.

2. We are sorry for the unclear statements. We now explain the concentration of methylmercury (CH_3Hg^+) used in the microcosms. All soils were spiked with CH_3Hg^+ at a fixed concentration of 50 μg kg^{-1}. As a result, the actual CH_3Hg^+ concentrations in the microcosms reflected the sum of the background CH_3Hg^+ (ranging from 0.45 to 3.48 μg kg^{-1}; Supplementary Table 1) and the spiked CH_3Hg^+ (50 μg kg^{-1}). We acknowledge that the CH_3Hg^+ concentrations measured at day 0 (around 45 μg kg^{-1}) did not always match the theoretical values (Fig. 1A-B; Supplementary Fig. 1), likely due to inevitable experimental errors, including variations in extraction efficiency and analytical recovery. According to previous studies, acceptable recoveries of CH_3Hg^+ from reference materials typically range from 75% to 115%, depending on soil properties[1-3]. To ensure the accuracy of degradation efficiency calculations, we used the measured CH_3Hg^+ concentrations at day 0 (including the background CH_3Hg^+) as the baseline, rather than relying on the theoretical concentration. These clarifications have been provided in lines 368-378 and lines 410-419.

   Additionally, CH_3Hg^+ concentration was measured at multiple time points in both the $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ treatments during the incubation (Fig. 1A-B; Supplementary Fig. 1); however, we did not analytically distinguish the two

isotopologues. We have clarified this point in lines 419-425, and revised Fig. 1 and Supplementary Fig. 1, accordingly. While the individual degradation efficiencies of $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ were not directly quantified, the observed decline in total CH_3Hg^+ concentration, along with the significant ^{13}C enrichment of microbial taxa in the heavy DNA fractions from $^{13}CH_3Hg^+$-DNA-SIP analyses, provide strong evidence for microbial demethylation of $^{13}CH_3Hg^+$ during the incubation. This approach effectively supports our goal of identifying the active microbial taxa and associated metabolic pathways involved in CH_3Hg^+ degradation.

Line 185-187, 300-304: The absence of *merAB* genes in the "heavy" fraction is interpreted as if another methylmercury pathway is in operation. But can't it just be that the methyl group that is produced in the demethylation is not assimilated into DNA to any major extent? In such a case the demethylating microbes would still be in the isotopically "light" fractional. Assimilation into biomass is a critical assumption in this work and it is not a law of nature, so alternative explanations should at least be mentioned.

3. We appreciate the reviewer's insightful comments. We agree that not all microbial taxa incorporate methyl-derived carbon into DNA, and some demethylating microbes may remain in the light fraction due to the limited amount of ^{13}C-methyl assimilation. We have now discussed this possibility in the revised manuscript (lines 308-310) as "We acknowledge that some *merAB*-mediated demethylators may have remained in the light fraction and were not detected due to the limited amount of ^{13}C-methyl assimilation.".

   However, our $^{13}CH_3Hg^+$-DNA-SIP analyses provide direct evidence that at least a subset of the microbial community actively assimilated ^{13}C from $^{13}CH_3Hg^+$. Specifically, if ^{13}C from $^{13}CH_3Hg^+$ were not significantly incorporated into DNA, the DNA fractionation patterns between the $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ treatments would be similar. Instead, we observed a gradual shift of the highest 16S rRNA gene abundance from the light DNA fractions (at day 7) to heavier fractions (at days 14 and 28) in the $^{13}CH_3Hg^+$ treatment (Fig. 2A-F), indicating that certain soil microorganisms had incorporated ^{13}C from $^{13}CH_3Hg^+$. Moreover, 16S rRNA gene and metagenomic analyses revealed significant ^{13}C enrichments of microbial taxa (e.g., *Pseudarthrobacter*, *MM2* and *Dechloromonas*), along with the absence of *merA* and *merB* genes in the reconstruction MAGs from the $^{13}CH_3Hg^+$ treatment (Figs. 3-4). Consistently, *merA* and *merB* genes did not exhibit significant enrichment in the $^{13}CH_3Hg^+$ treatment at the community level compared with the $^{12}CH_3Hg^+$ treatment (Fig. 4A).

   Taken together, these results suggest that *mer*-independent pathways may dominate CH_3Hg^+ degradation in the actively ^{13}C-labeled taxa. Nonetheless, we acknowledge that some *merAB*-mediated demethylators could have not been detected due to the limited amount of ^{13}C assimilation, and emphasize the need for complementary methods to further elucidate the underlying metabolic mechanisms involved in CH_3Hg^+ degradation (lines 308-314).

Line 155/156: should read "the $^{12}CH_3Hg^+$ treatment"

4. Revised as suggested (lines 155-156).

Line 176: What is "combination analyses"? Should this read "Combined analyses"?

5. Revised as "Combined analyses of metagenome sequencing and……" (line 177).

Line 279: "The no significant difference" is awkward and should be revised. "similar"?

6. Revised as suggested (line 289).

Line 334: "enormously" should be deleted. Unclear what it means.

7. Deleted.

Line 467: "sequence" should read "sequences" and "sequences were"

8. Done.

Reviewer #3 (Remarks to the Author):

While the authors have made meaningful progress in refining their analysis and the overall quality of the paper, several issues still need to be addressed, as outlined below.

9. Thank you for your comprehensive review and the positive comments on our efforts to refine the analyses and improve the manuscript. We have carefully considered your comments and made substantial revisions to address your concerns. Please see our point-by-point responses below.

L184–204: Most of the genes discussed in the main text are either absent from Supplementary Fig. 6 or not indicated as significantly enriched. This discrepancy suggests that either the figure needs to be replaced with a more representative version, or the corresponding descriptions in the main text should be substantially revised to align with the data shown.

10. We appreciate the reviewer's concerns. We would like to clarify that the genes discussed in the results section are primarily represented in Fig. 4A, which highlights key genes involved in Hg reduction and some biochemical pathways (e.g., sulfate, carbon and nitrogen metabolism)[4-6]. The data presented in Fig. 4A and Supplementary Fig. 6 were integrated to provide a comprehensive overview of the multifunctional metabolic characteristics related to the $^{13}CH_3Hg^+$ degradation at the community level. To improve consistency between the main text and the figures, we have provided detailed statements of Supplementary Fig. 6 in lines 197-214. For example, "genes encoding isocitrate dehydrogenase (*IDH3*), 2-oxoglutarate ferredoxin oxidoreductase (*korAB*), tetrahydromethanopterin S-methyltransferase subunit B (*mtrB*), phosphoribosylaminoimidazole-succinocarboxamide synthase (*purC*)… exhibited significant enrichment in the $^{13}CH_3Hg^+$ treatment compared with the $^{12}CH_3Hg^+$ treatment (lines 200-206)."

L187–189: References should be added here.

11. We have provided references in lines 190-192 as “genes involved in sulfate reduction, carbon metabolism (e.g., methane metabolism) and nitrogen metabolism (e.g., nitrate reduction and denitrification) were searched within the metagenomic contigs[4-6]”.

L297–304: Why were *fdoG* (*Gemmatimonadota*) and *gcvP* (*Actinobacteria*) highlighted as significantly enriched among the numerous genes related to carbon metabolism? While the shift in focus toward carbon metabolism is understandable due to the absence of significant differences in *merA* and *merB*, the rationale behind this focus should be more explicitly justified (as described above: L187-189’s reference). Additionally, the biological or ecological significance of these specific genes must be elaborated upon in greater detail.

Currently, the discussion risks appearing selective or post hoc—as if only the genes that support a convenient narrative were chosen from Supplementary Fig. 6.

12. We thank the reviewer for the thoughtful comments regarding our discussion of *fdoG* (encoding formate dehydrogenase) and *gcvP* (encoding glycine dehydrogenase). As explained, both genes are involved in carbon metabolism, particularly one-carbon (C_1) transformations. *fdoG* is involved in the oxidation of formate, a key step in C_1 metabolism that contributes to the generation of C_1 units used in various biosynthetic pathways[7], while *gcvP* is a critical component of the glycine cleavage system, contributing to the production of methylene-tetrahydrofolate in the C_1 metabolic network[8]. Given that oxidative demethylation of CH_3Hg^+ has been hypothesized to mediate via co-metabolic pathways involving C_1 compounds[9], the significant enrichment of *fdoG* and *gcvP* genes in the $^{13}CH_3Hg^+$ treatment suggests their potential role in *mer*-independent CH_3Hg^+ degradation. We highlighted these genes as representative examples enriched in *Actinobacteria* and *Gemmatimonadota* in the previous manuscript, in support of the hypothesis that *mer*-independent oxidative demethylation may be the predominant degradation pathway in the paddy soil.

    To address the concern regarding potential selectivity or post hoc bias, we have now removed the gene-specific discussion to avoid overinterpretation. Instead, we emphasize broader functional trends across multiple analytical levels. At the MAG level, all reconstructed ^{13}C-labeled MAGs lacked both *merA* and *merB*, but contained genes involved in carbon and nitrogen metabolism (Fig. 4B). At the phylum (i.e., *Actinobacteria* and *Gemmatimonadota*) and community levels, the comparable relative abundances of *mer* operon genes were observed between the $^{13}CH_3Hg^+$ and $^{12}CH_3Hg^+$ treatments, while genes associated with carbon and nitrogen metabolism were significantly enriched in the $^{13}CH_3Hg^+$ treatment compared with the $^{12}CH_3Hg^+$ treatment (Fig. 4A; Supplementary Fig. 6; Supplementary Fig. 10). These consistent trends across MAG, phylum, and community levels strongly support that alternative pathways, such as oxidative demethylation, rather than *mer*-mediated reductive demethylation, may dominate

CH_3Hg^+ degradation in the actively ^{13}C-labeled microbial communities. We also highlight the need for complementary methods to further elucidate the underlying metabolic mechanisms involved in CH_3Hg^+ degradation. Overall, we now modify the discussion to provide a more balanced and integrative interpretation of the metagenomic data (see revised lines 286-296 and 301-314).

To strengthen the discussion, it is essential to clarify the reasoning behind the observed enrichment of bacteria lacking *merA* and *merB* under the ^{13}C-Hg treatment and to ensure that all descriptions accurately reflect the underlying data.

13. We appreciate the reviewer's insightful comment. In response, we have substantially revised the discussion to clarify the potential reasoning behind the observed enrichment of bacteria lacking *merA* and *merB* in the $^{13}CH_3Hg^+$ treatment, and to ensure that all descriptions are precisely aligned with the underlying data. At the MAG level, all reconstructed MAGs in the $^{13}CH_3Hg^+$ treatment lacked both *merA* and *merB* genes (Fig. 4B), suggesting that *mer*-mediated reductive demethylation was not accountable for the dominant $^{13}CH_3Hg^+$ degradation pathway in these taxa. This was further supported by community- and phylum-level (i.e., *Actinobacteria* and *Gemmatimonadota*) metagenomic analyses, which showed comparable relative abundances of *mer* operon genes between the $^{12}CH_3Hg^+$ and $^{13}CH_3Hg^+$ treatments (Fig. 4A; Supplementary Fig. 6; Supplementary Fig. 10). Instead, MAG-based analysis indicated that genes involved in carbon and nitrogen metabolism were present in the reconstructed genomes, while both community- and phylum-level analyses showed significant enrichment of these genes in the $^{13}CH_3Hg^+$ treatment. These findings suggest the involvement of *mer*-independent CH_3Hg^+ degradation, such as oxidative demethylation mediated via co-metabolic processes involving simple carbon sources (i.e., C_1 compounds)[9]. While our results provide functional and genomic evidence supporting the existence of *mer*-independent degradation pathways, the underlying biochemical mechanisms remain to be experimentally validated. To avoid overinterpretation, we have now modified the discussion to present a more cautious and integrative narrative that highlights the potential role of alternative pathways (e.g., oxidative demethylation), while also acknowledging the need for complementary methods to further elucidate the underlying metabolic mechanisms involved in CH_3Hg^+ degradation. All relevant statements and discussions have been carefully updated to ensure they are supported by the data from this study (lines 286-296 and 301-314).

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4. Lu, X. et al., Methylmercury uptake and degradation by methanotrophs. *Sci Adv* **3**, e1700041 (2017).

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7. Matheus Carnevali, P. B. et al., Meanders as a scaling motif for understanding of floodplain soil microbiome and biogeochemical potential at the watershed scale. *Microbiome* **9**, 121 (2021).

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9. Barkay, T. & Gu, B., Demethylation-the other side of the mercury methylation coin: A critical review. *ACS Environ Au* **2**, 77-97 (2022).