

Genomic potential for mercury biotransformation in marine sediments across marginal slope to hadal zone

Corresponding Author: Professor Yong Wang

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)

The manuscript reports the diversity and abundance in deep coastal to hadal marine zones of a range of genes encoding and expressing for various Hg-transformation pathways. The authors report on novel putative Hg-cycling microorganisms and on the phylogeny of Hg methylators and demethylators, using the latest Hg gene databases to interpret their meta-'omics data sets. They uncover a pathway for Hg methylation in deep waters that contrasts with the more canonically attributed acetyl-coA synthase pathway, and also explore the range of co-metabolic gene pathways available to Hg cycling microorganisms via genome-resolved metagenomics analysis. In general, I find the manuscript to be sound in its approach, results and interpretation, and it contributes usefully to our knowledge of the distribution and diversity of Hg methylators particularly, adding new insight into the effect of marine depth/distance from coastal Hg sources and potential methyl transfer pathways. I recommend for publication following correction/revision of some minor points as follows:

- 1) several times in the manuscript (starting with the abstract), Hg methylation is associated with microbial "metabolism". I understand that the authors probably mean some type of coupling to one-carbon transfer involved in some metabolic pathway, but to claim that Hg is being metabolized, or similar language, might be a step too far for most Hg microbiologists? I would advise toning down this language around "Hg metabolism" to focus more on the chemical "transformation" of Hg to MeHg or vice versa, which is reasonable to say is coupled to metabolism, but not the direct metabolism of Hg (i.e., microbes do not gain energy from Hg methylation, to our current knowledge).
- 2) Similarly to the first point, I think it's probably over-reaching a little to claim that Hg methylation is a detoxification response to Hg(II). The authors cite a single study to support this idea (Schaefer et al. 2011). But it has been more commonly observed that methylation does not seem to be a toxicity response, does not always correlate strongly with Hg(II) or Hg(0). Also, MeHg would be even more toxic to the methylating cell - how would the authors explain the detoxification theory in that context?
- 3) The idea that methylating phyla shift from coastal to hadal waters is an interesting one. Could the authors present some further thoughts as to why? And how this hypothesis might be further tested - noting that the number of coastal shelf cores was less than cores obtained from deeper waters, it seemed? Could there be undersampling?

Reviewer #2

(Remarks to the Author)

I have carefully reviewed the manuscript titled "Biotransformations of Mercury in Marine Sediments Across Marginal Slope to Hadal Zone" by Zhuobo Li, Haifeng Qian, Yong-Guan Zhu, and Yong Wang. This study examines 32 sediment cores, including 101 layers, collected from the deep-sea regions of the South China Sea (SCS) and the Mariana Trench (MT). The authors compare these metagenomes with 41 global reference sediment metagenomes.

While this research provides valuable insights into microbial metagenomic data related to mercury biogeochemical cycling in deep-sea sediment ecosystems, it lacks comprehensive methylmercury analyses. The study only presents Total Mercury (THg) data. Without detailed investigations into mercury speciation, particularly the identification of methylmercury (MeHg) in situ, the results and hypotheses presented in the manuscript are less convincing. It appears that the findings are largely based on metagenomic analyses, with insufficient complementary experimental work, such as qPCR for *hgcA* or *merB*.

genes, or measurements of porewater sulfate and Fe(III) as principal electron acceptors for mercury-methylating microbes.

Additionally, the diversity of merB genes, which encode for MeHg degradation, poses significant challenges for detection due to a lack of conserved regions suitable for primer targeting in Illumina sequencing. The methods used to retrieve merB genes or MerB proteins from the metagenome-assembled genomes (MAGs) are not clearly defined. Without reliable approaches to accurately bin authentic merB or MerB genes, the proposed MeHg demethylators remain questionable. Other concerns are listed below:

1. From the abstract: “we found that the mercury methylation genes hgcAB as detoxification regulators were abundant in marginal seas but negligible in open oceans. Instead of taking the Wood-Ljungdahl pathway as a methyl group donor, some Hg-methylating microorganisms affiliated with Desulfobacterota, Spirochaetota, and Zixibacteria in the deep-sea sediments have the potential to utilize osmolyte-derived trimethylamine for methylation.”, this statement seems to propose a novel hypothesis that lacks experimental support. The assertion that “mercury methylation genes hgcAB act as detoxification regulators” is merely one possible interpretation regarding the evolutionary role of microbial mercury methylation and remains unconfirmed. Similarly, the Wood-Ljungdahl pathway and other proposed pathways are still hypothetical.
2. Regarding lines 411-412 on Page 19: What were the recovery rates for the Total Mercury (THg) analysis?
3. In lines 508-511 on Page 24: Can you provide more details on the approach used for the MerB blasting or binning? How were the MerB genes retrieved from the sediment metagenomes?
4. In line 128, the phrase “mercury metabolism” is not appropriate, as it implies that mercury undergoes metabolic processes, whereas it is only subject to microbial transformation of speciation.
5. Lines 318-330 on Page 15 present assumptions that are largely hypothetical and lack robust supporting evidence. The interpretations and reasoning regarding microbial mercury methylation mechanisms appear superficial.
6. In lines 342-349 on Page 16: The idea of mercury methylation as a detoxification mechanism for MeHg remains one of many hypotheses and has not been experimentally validated. The interpretation of mercury cycling in benthic sea habitats may be biased.

Reviewer #3

(Remarks to the Author)

The study by Li et al. addresses the important issue of microbial metabolic potential for Hg biotransformation in marine sediments. Analysing a large number of sediment genome-resolved metagenomic data, they found that detoxification capacity by Hg methylation was abundant in marginal seas but negligible in open oceans. Conversely, the Hg demethylating taxa exhibits a wider distribution. Their work expands the list of known Hg metabolising microorganism, and their genomic niches.

Overall, the study is relevant and has been conducted with suitable bioinformatics tool. I however have three concerns:

- the study is based on meta-genomics / -transcriptomics data and Hg concentration measurement. The title and several places in the text suggest that results are relevant to biological activity. I rather think that terms such as metabolic potential would be more suitable.
- Authors tried to highlight the high novelty of sediment microbial diversity using MiTags recruited on the SILVA database. I think this is problematic and I suggest an alternative workflow below.
- The metagenomics workflow seems correct, but lacks a more detailed description to be able to fully understand and evaluate the results.

Specific comments:

Title: I find the title not fully representative of the work presented in this study. This study indeed relies on omics data and partly on Hg measurements. The results rather indicate genomic potential for biotransformation, rather than actual biotransformation.

L141: if merT and merR are in the same operon, how can they have different coverage?

L164: could you specify if the 2163 hgcA gene sequence were found assemblies or MAGs?

L332 and elsewhere: several occurrences of « deep sea » instead of « deep-sea »

L 348: I would replace «sources are less available than soils » by « sources can be less available than soils, as organic content in these environments are extremely variable.

L 400: « they were centrifuged » instead of « centrifuge them » ?

L 422 and 441: it would be useful to specify which libraries were built with 350 bp or 550 bp insert size and why, and which samples were sequenced in 2x150 and 2x300 bp and why, unless they were all sequenced twice? Did it have an impact on the mapping results?

L433: I suggest to replace « Universal primers » by « broad-range 16S rRNA gene specific primers » or « bacterial 16S

rRNA gene specific primers »

L458: « categorize the representative MiTags » Could you clarify « categorize », did you mean taxonomic annotation?

L460: The BLASTn search is not very clear: did you mean 16S miTags that had no matching the silva db within a 97% similarity distance where classified as new species? In addition, I think the whole workflow for 16S MiTag analysis is likely to overestimate the microbial novelty and Alpha diversity: sequences from different regions of a 16S that don't have a hit in the Silva database will all be counted as novel species despite the fact that they originate from the same unclassified sequence. It would have been correct in a metabarcoding context, where PCR amplifies the same 16S region. With miTags however, this is not the case. Similarly, I did not understand how the observation matrix used for the PCoA and CCA was built: how did the authors dealt with unassigned (no hit in Silva) miTags? if each appeared has a new ecological unit in the matrix, cf my point above. I realize that this is a minor claim of the study, but account for a significant part of the results (L108-126). A note on this caveat in the text would be very useful for the reader. For this reason MiTags are usually analysed only through taxonomic annotation and grouped by identical taxa (phylotype). As an alternative, I can maybe suggest to use the diversity of a single copy core gene approach from the assemblies, as e.g. in <https://doi.org/10.1038/s41564-019-0449-y>

L472: For sake of reproducibility, could you specify the parameters used to run metaWRAP for the binning? This wrapper has many options a each step. Alternatively, (and this it is my preference as a reader) you can make the code associated with this study available on a git repository. Also, I'm a bit lost at this stage: on line 447, you mention co-assembly of metagenomics from the same cores. Only counting on the cores taken for this study, it should results in 32 co-assemblies, resulting in 32 MAG collections. Did you submit each co-assemblies to metawrap separately? Which reads did you use for the mapping and differential coverage computation for the binners? Then, L475 « using dRep (v.1.4.3) separately for each genome set from the same station »: I understand you dereplicated the 32 MAG collections among core from the same station, normally resulting in 5 new MAG collections (as shown in sup. table 6). 1/ I don't understand the results between table S1 and S6: for instance, SCSB has 3 cores for which you reconstructed 38, 25, and 48 MAGs, so 111 Mags in total for this station. In S6, SCSB station has 111 dereplicated MAGs: does this mean that none of the MAG pairs were within the 99% similarity criterion? 2/ In the absence of MAG dereplication across the entire study, I don't understand how gene and MAG coverages were computed. This can be problematic if nearly identical MAGs are present in the 5 different MAG collections: mapper will randomly assign matching reads to either of these. Overall, I think the metagenomics workflow need clarifications, in the main text and maybe with a workflow diagram.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

It seems that the concerns from my previous MS review have been fairly addressed. I have no further questions as to the revised manuscript.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

I acknowledge that authors have addressed most questions I raised in my first review. I however still have a few remarks that could further improve the comprehension for the readership.

In their response to my comment 11, authors states that "We acknowledge that the original manuscript did not clearly state that our BLASTn analysis and downstream processing were performed exclusively on the V4 hypervariable region of 16S rRNA genes. All microbial novelty identification and alpha diversity assessments were based on strictly filtered V4 region sequences, not full-length 16S sequences. Therefore, the concern regarding overestimation of novel species and alpha diversity due to analyzing different regions of 16S does not apply in our case"

In this case, I do agree that my comments regarding 16S based novelty are irrelevant. In their revised version, authors provide a link to the code used for this analysis (L547). The only python script I could find related to this 16S analysis was the following <https://github.com/ucassee/Challenger-Deep-Microbes/blob/master/Py/miTag.py>. It selects 16S related reads based on their annotations (probably from the rna_hmm3.py analysis, but not clear) and then according to their distance with the nearest Blast results. I however didn't understand how these were filtered for V4 regions. Could the authors clarify this point or specify which script they used for this purpose?

(Remarks on code availability)

the script included in https://github.com/ZhuoboLi/Mercury_metagenomics/blob/master/work_flow.sh seems sound, but i didn't try to run it myself. It would benefit from a rapid presentation in a README file

The code in <https://github.com/ucassee/Challenger-Deep-Microbes/blob/master/> contains multiple scripts, the function of which can only be inferred from their name. This repository would really benefit from a README describing the purpose and use of all these scripts. See also my concern regarding the 16S v4 analysis in my main comments

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Reply to Reviewer #1

Comment 1: The manuscript reports the diversity and abundance in deep coastal to hadal marine zones of a range of genes encoding and expressing for various Hg-transformation pathways. The authors report on novel putative Hg-cycling microorganisms and on the phylogeny of Hg methylators and demethylators, using the latest Hg gene databases to interpret their meta-'omics data sets. They uncover a pathway for Hg methylation in deep waters that contrasts with the more canonically attributed acetyl-coA synthase pathway, and also explore the range of co-metabolic gene pathways available to Hg cycling microorganisms via genome-resolved metagenomics analysis. In general, I find the manuscript to be sound in its approach, results and interpretation, and it contributes usefully to our knowledge of the distribution and diversity of Hg methylators particularly, adding new insight into the effect of marine depth/distance from coastal Hg sources and potential methyl transfer pathways. I recommend for publication following correction/revision of some minor points as follows:

Response 1: Thank you for your positive feedback and constructive evaluation of our work. We are pleased that you find the manuscript sound and its contributions valuable to understanding Hg-cycling microbial ecology. We have carefully addressed all your minor points in the revised manuscript. Your suggestions have significantly strengthened the manuscript's rigor and clarity. Please find the detailed revisions in the text and responses below.

Comment 2: several times in the manuscript (starting with the abstract), Hg methylation is associated with microbial "metabolism". I understand that the authors probably mean some type of coupling to one-carbon transfer involved in some metabolic pathway, but to claim that Hg is being metabolized, or similar language, might be a step too far for most Hg microbiologists? I would advise toning down this language around "Hg metabolism" to focus more on the chemical "transformation" of Hg to MeHg or vice versa, which is reasonable to say is coupled to metabolism, but not the direct metabolism of Hg (i.e., microbes do not gain energy from Hg methylation, to our current knowledge).

Response 2: We sincerely appreciate your professional comments. We agree that claiming Hg is "metabolized" is indeed inappropriate. In response to your suggestion, we have revised all relevant descriptions throughout the manuscript to replace "Hg metabolism" with "Hg transformation." Thank you for your valuable feedback, which has helped improve the accuracy of our manuscript.

Comment 3: Similarly to the first point, I think it's probably over-reaching a little to claim that Hg methylation is a detoxification response to Hg(II). The authors cite a single study to support this idea (Schaefer et al. 2011). But it has been more commonly observed that methylation does not seem to be a toxicity response, does not always

correlate strongly with Hg(II) or Hg(0). Also, MeHg would be even more toxic to the methylating cell - how would the authors explain the detoxification theory in that context?

Response 3: We thank you for your valuable comments. We fully agree that the current evidence is insufficient to support the hypothesis that mercury methylation is an active detoxification response by microorganisms. As you point out, the hypothesis of the methylation process as detoxification is controversial. Although a study by Schaefer et al. (2011) proposed that methylation may reduce intracellular Hg(II) accumulation by altering the chemical form of Hg to facilitate its excretion from cells. It has also been suggested that the ability to produce MeHg is intrinsic and not induced by Hg exposure; however, the methylation rate of microorganisms is highly dependent on the bioavailability of mercury in the culture medium¹. Moreover, the potential toxicity of MeHg to the bacteria themselves still needs to be further investigated. Therefore, we removed this inaccurate statement in the Abstract and expressed this idea more cautiously in the Discussion section (Lines 326-331), describing it as a “possible detoxification strategy” rather than a definitive detoxification strategy.

Comment 4: The idea that methylating phyla shift from coastal to hadal waters is an interesting one. Could the authors present some further thoughts as to why? And how this hypothesis might be further tested - noting that the number of coastal shelf cores was less than cores obtained from deeper waters, it seemed? Could there be undersampling?

Response 4: Thank you for your thoughtful comment and for highlighting the interesting aspect of the shift in methylating phyla from coastal to hadal waters. We agree that this observation deserves further discussion and exploration. In this study, we showed a shift in the diversity of Hg-methylated microorganisms from marginal seas to hadal zone, which might be attributed to various ecological and environmental factors. One possibility is that the availability of mercury required for methylation might vary between marginal seas and hadal zone. In marginal environments, the availability of anthropogenic mercury and organic matter might nourish a variety of Hg-methylating microorganisms² distinct from those in the deeper hadal zone where the environmental conditions such as nutrient availability and extreme pressure could drive the selection of different inhabitants. A study indicates that dissolved organic matter (DOM) greatly inhibited Hg methylation in *Geobacter sulfurreducens* PCA but enhanced Hg methylation in *Desulfovibrio desulfuricans* ND132 with increasing concentrations of DOM³. Another potential influencing factor is oxygen availability⁴. In marginal seas, the finer sediment particle size likely results in limited oxygen penetration, leading to lower oxygen content within sediment layers. In contrast, open ocean regions with coarser sediment particles are presumed to have relatively higher oxygen levels⁵. Under these conditions, Desulfobacterota dominate as potential mercury-methylating microorganisms in marginal seas, while Nitrospirota and Nitrospirata emerge as key mercury methylators in open ocean environments. We have

added the discussion of this issue in the main text (Lines 335-351). We acknowledge that our sampling effort for coastal shelf sediments (13 cores) was smaller than that for deeper waters (19 cores). To mitigate potential undersampling bias and broaden the scope of our findings, we incorporated publicly available sediment metagenomic datasets from additional marginal seas and open oceans (Supplementary Table 2). This expanded dataset reinforces our observations of habitat-specific methylator distributions at a broader scale.

References:

1. Gilmour CC, *et al.* Sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132 as a model for understanding bacterial mercury methylation. *Appl Environ Microbiol* 77, 3938-3951 (2011).
2. Wang Y-L, Ikuma K, Brown AMV, Deonaraine A. Global survey of hgcA-carrying genomes in marine and freshwater sediments: Insights into mercury methylation processes. *Environ Pollut* 352, 124117 (2024).
3. Zhao L, *et al.* Contrasting effects of dissolved organic matter on mercury methylation by *Geobacter sulfurreducens* PCA and *Desulfovibrio desulfuricans* ND132. *Environ Sci Technol* 51, 10468-10475 (2017).
4. Cabrol L, *et al.* Redox gradient shapes the abundance and diversity of mercury-methylating microorganisms along the water column of the Black Sea. *mSystems* 8, e00537-00523 (2023).
5. Li Z, He Y, Zhang H, Qian H, Wang Y. Biotransformations of arsenic in marine sediments across marginal slope to hadal zone. *J Hazard Mater* 480, 135955 (2024).

Reply to Reviewer #2

Comment 1: I have carefully reviewed the manuscript titled “Biotransformations of Mercury in Marine Sediments Across Marginal Slope to Hadal Zone” by Zhuobo Li, Haifeng Qian, Yong-Guan Zhu, and Yong Wang. This study examines 32 sediment cores, including 101 layers, collected from the deep-sea regions of the South China Sea (SCS) and the Mariana Trench (MT). The authors compare these metagenomes with 41 global reference sediment metagenomes.

While this research provides valuable insights into microbial metagenomic data related to mercury biogeochemical cycling in deep-sea sediment ecosystems, it lacks comprehensive methylmercury analyses. The study only presents Total Mercury (THg) data. Without detailed investigations into mercury speciation, particularly the identification of methylmercury (MeHg) in situ, the results and hypotheses presented in the manuscript are less convincing. It appears that the findings are largely based on metagenomic analyses, with insufficient complementary experimental work, such as qPCR for hgcA or merB genes, or measurements of porewater sulfate and Fe(III) as principal electron acceptors for mercury-methylating microbes.

Additionally, the diversity of *merB* genes, which encode for MeHg degradation, poses significant challenges for detection due to a lack of conserved regions suitable for primer targeting in Illumina sequencing. The methods used to retrieve *merB* genes

or MerB proteins from the metagenome-assembled genomes (MAGs) are not clearly defined. Without reliable approaches to accurately bin authentic *merB* or MerB genes, the proposed MeHg demethylators remain questionable. Other concerns are listed below:

Response 1: Thanks for your constructive suggestions. We have supplemented methylmercury (MeHg) concentration measurements in the sediment samples. For qPCR targeting *hgcA* and *merB* genes, we tested primers from prior studies^{1, 2}: Deltaproteobacteria-specific *hgcA* primers yielded specific amplification, while Firmicutes-specific and Archaea-specific *hgcA* primers failed to amplify, likely due to the scarcity of these methylating taxa in our samples. Deltaproteobacteria-associated *hgcA* sequences represented 59.67% of the cumulative *hgcA* abundance (quantified as CPM) across the analyzed 142 metagenomic datasets (Supplementary Fig. 7a). Since the *hgcA* genes were primarily assigned to the Deltaproteobacteria clade, we used primers specific to this clade to quantify their abundance with quantitative PCR assays. The results showed higher *hgcA* abundance in marginal sea samples, compared to open ocean samples (Supplementary Fig. 7b), which is in agreement with the metagenomics results. Notably, the exceptionally low *hgcA* copy numbers detected in the SCS2-2 from the marginal sea group are consistent with our metagenomic findings, potentially linked to its elevated oxygen content³, as most mercury methylators thrive in anoxic environments.

The *merB* primers showed no specific amplification, likely due to the high sequence diversity of *merB* and the absence of sufficiently conserved regions for primer design as reported previously^{2, 4, 5}.

We also analyzed porewater sulfate and Fe(III) concentrations, as detailed in the revised manuscript (Lines 113-116). Additionally, we clarified the workflow for retrieving MerB proteins from MAGs, including stringent validation of conserved catalytic residues to ensure accurate identification of *merB* homologs (Lines 599-602). Please see the responses below for detailed revisions.

We hope these clarifications address your concerns and reinforce the reliability of our results.

Comment 2: From the abstract: “we found that the mercury methylation genes *hgcAB* as detoxification regulators were abundant in marginal seas but negligible in open oceans. Instead of taking the Wood-Ljungdahl pathway as a methyl group donor, some Hg-methylating microorganisms affiliated with Desulfobacterota, Spirochaetota, and Zixibacteria in the deep-sea sediments have the potential to utilize osmolyte-derived trimethylamine for methylation.”, this statement seems to propose a novel hypothesis that lacks experimental support. The assertion that “mercury methylation genes *hgcAB* act as detoxification regulators” is merely one possible interpretation regarding the evolutionary role of microbial mercury methylation and remains unconfirmed. Similarly, the Wood-Ljungdahl pathway and other proposed pathways are still hypothetical.

Response 2: Thank you for your valuable feedback and for pointing out this important concern. We appreciate the opportunity to clarify the points raised in the abstract. We agree that the interpretation of *hgcAB* as detoxification regulators is one possible hypothesis, and we acknowledge that further experimental support is needed to confirm this role. We have discussed this in the context of our findings, and we are also careful to note that this remains a hypothesis based on the observed correlation of gene abundance with mercury levels in marginal seas. To clarify this point, we have revised the manuscript to state that the concept of *hgcAB* as a detoxification regulator is a hypothesis rather than a definitive conclusion at this stage. Please see lines 324-331.

We appreciate your critical assessment of our hypothesis regarding mercury methylation pathways. Our intention in this section was to propose potential novel mechanisms based on genomic evidence, while fully acknowledging the need for experimental validation. As you rightly note, the methyl donor pathways for mercury methylation remain hypothetical, even in the Wood-Ljungdahl framework. We have refined the phrasing in the abstract section (Please see Lines 34-37). Recent work on methanogenic archaea using ¹³C isotopic labeling demonstrated methyl group transfer via Wolfe cycle intermediates, suggesting methyl-tetrahydromethanopterin (CH₃-H₄MPT) as a potential direct methyl donor interacting with HgcA⁶. In our study, we systematically analyzed all known corrinoid-dependent methyltransferases in the genomes of putative deep-sea methylators. Our findings suggest that specific *Desulfobacterota*, *Spirochaetota*, and *Zixibacteria* strains harboring *hgcAB* may utilize MttB methyltransferases to transfer methyl groups to corrinoid cofactors. However, this hypothesis requires rigorous validation through cultivation and isotopic tracing, which remains technically challenging for deep-sea microorganisms. We have therefore added explicit statements in the revised Discussion (Lines 370-396).

Comment 3: Regarding lines 411-412 on Page 19: What were the recovery rates for the Total Mercury (THg) analysis?

Response 3: Thank you for your comment. We also tested the marine sediment standard sample GBW07316, and the recovery rate was 98.31%, which indicates the accuracy of our total mercury analysis. And we have added this statement to the methods section (Please see lines 486-487).

Comment 4: In lines 508-511 on Page 24: Can you provide more details on the approach used for the MerB blasting or binning? How were the MerB genes retrieved from the sediment metagenomes?

Response 4: Thank you for your insightful comments. we have added detailed information on the identification of MerB protein sequences. The coding sequences in the metagenome-assembled genomes (MAGs) were annotated using HMM search within KofamScan, which was aligned against the KEGG Ortholog database. Additionally, we have incorporated a step to check for conserved sites in this revision, as described in lines 599-602.

Comment 5: In line 128, the phrase “mercury metabolism” is not appropriate, as it implies that mercury undergoes metabolic processes, whereas it is only subject to microbial transformation of speciation.

Response 5: Thank you for your insightful comment. We agree that the original phrasing "mercury metabolism" could be misinterpreted as suggesting biological metabolic activity. To address this, we have replaced all occurrences of “mercury metabolism” throughout the manuscript with “mercury transformation”.

Comment 6: Lines 318-330 on Page 15 present assumptions that are largely hypothetical and lack robust supporting evidence. The interpretations and reasoning regarding microbial mercury methylation mechanisms appear superficial.

Response 6: We sincerely appreciate your insightful comments on our proposed mechanism of mercury methylation. In response to your concerns, we have substantially revised the relevant section to clarify the speculative aspects of our hypothesis. We expanded the description of currently recognized methyl donor pathways (Lines 372-375). Our genomic analysis revealed that the deep-sea *Desulfobacterota*, *Spirochaetota*, and *Zixibacteria* MAGs lack genes encoding these currently considered possible methyltransferases but uniquely harbor *mttB*. We provide further evidence for the hypothesized MttB-mediated methylation pathway in terms of the origin of TMA in the deep sea and the reliably docking model of MttB with HgcA. In addition, we have added explicit statements (Lines 395-396) that this mechanism is still not experimentally validated and further experimental validation is needed. We hope that the above revisions have addressed your concerns about the inadequacy of our proposed explanation of the mechanism of mercury methylation.

Comment 7: In lines 342-349 on Page 16: The idea of mercury methylation as a detoxification mechanism for MeHg remains one of many hypotheses and has not been experimentally validated. The interpretation of mercury cycling in benthic sea habitats may be biased.

Response 7: Thank you for your thoughtful comment. As mentioned in Response 2, the hypothesis that Hg methylation serves as a detoxification strategy remains contentious in the field. While early studies speculated that MeHg production might alleviate intracellular Hg(II) toxicity by facilitating its export⁷, subsequent work by Gilmour et al. demonstrated constitutive expression of *hgcAB* genes independent of Hg exposure⁸. We have added a discussion in the manuscript addressing the ongoing controversies surrounding mercury methylation as a detoxification strategy (Lines 324-331).

On the other hand, we have carefully considered your concern about the potential bias in the interpretation of mercury cycling in benthic sea habitats and have made revisions to the manuscript. In the revised version, we have added a discussion about other factors that may influence mercury cycling, such as water mass age and the

composition of dissolved organic matter (DOM), especially protein-like DOM⁹ (Lines 415-419). These additions aim to provide a more comprehensive understanding of the differences in mercury cycling between different sites and to reduce the potential bias in our interpretation. We hope these revisions strengthen the discussion and address the issue you have pointed out. Thank you again for your helpful feedback, which has improved the quality of our manuscript.

References:

1. Christensen GA, *et al.* Development and validation of broad-range qualitative and clade-specific quantitative molecular probes for assessing mercury methylation in the environment. *Appl Environ Microbiol* 82, 6068-6078 (2016).
2. Schaefer JK, *et al.* Role of the bacterial organomercury lyase (MerB) in controlling methylmercury accumulation in mercury-contaminated natural waters. *Environ Sci Technol* 38, 4304-4311 (2004).
3. Li Z, He Y, Zhang H, Qian H, Wang Y. Biotransformations of arsenic in marine sediments across marginal slope to hadal zone. *J Hazard Mater* 480, 135955 (2024).
4. Yang L, *et al.* Seasonal changes in total mercury and methylmercury in subtropical decomposing litter correspond to the abundances of nitrogen-fixing and methylmercury-degrading bacteria. *J Hazard Mater* 442, 130064 (2023).
5. Yin B, Tan S, Wang J, Pan K, Wang W-X, Wang X. Antibiotic application may raise the potential of methylmercury accumulation in fish. *Sci Total Environ* 819, 152946 (2022).
6. Gao J, *et al.* The origin of methyl group in methanogen- mediated mercury methylation: From the Wolfe cycle. *Proc Natl Acad Sci USA* 121, e2416761121 (2024).
7. Schaefer JK, Rocks SS, Zheng W, Liang L, Gu B, Morel FMM. Active transport, substrate specificity, and methylation of Hg(II) in anaerobic bacteria. *Proc Natl Acad Sci USA* 108, 8714-8719 (2011).
8. Gilmour CC, *et al.* Sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132 as a model for understanding bacterial mercury methylation. *Appl Environ Microbiol* 77, 3938-3951 (2011).
9. Sanz-Saez I, *et al.* Microorganisms involved in methylmercury demethylation and mercury reduction are widely distributed and active in the bathypelagic deep ocean waters. *Environ Sci Technol* 58, 13795-13807 (2024).

Reply to Reviewer #3

Comment 1: The study by Li et al. addresses the important issue of microbial metabolic potential for Hg biotransformation in marine sediments. Analysing a large number of sediment genome-resolved metagenomic data, they found that detoxification capacity by Hg methylation was abundant in marginal seas but negligible in open oceans. Conversely, the Hg demethylating taxa exhibits a wider distribution. Their work expands the list of known Hg metabolising microorganism, and their genomic niches.

Overall, the study is relevant and has been conducted with suitable bioinformatics

tool. I however have three concerns:

- the study is based on meta-genomics / -transcriptomics data and Hg concentration measurement. The title and several places in the text suggest that results are relevant to biological activity. I rather think that terms such as metabolic potential would be more suitable.

- Authors tried to highlight the high novelty of sediment microbial diversity using MiTags recruited on the SILVA database. I think this is problematic and I suggest an alternative workflow below.

- The metagenomics workflow seems correct, but lacks a more detailed description to be able to fully understand and evaluate the results.

Response 1: Thank you for your positive feedback and constructive suggestions. Following your recommendations, we have revised the manuscript to use the term metabolic potential to more accurately reflect the genomic insights into microbial mercury cycling. We have also clarified the methodological approach for assessing sediment microbial diversity: while the original analysis using MiTags highlighted novel taxa, we have supplemented this with *rpS3* gene-based diversity analysis (as suggested) for PCoA and CCA to ensure robustness. Additionally, we expanded the description of the metagenomic workflow in the Methods section, including a detailed workflow diagram (Supplementary Fig. 9) to enhance transparency and reproducibility. Please see the response below for details of the revisions.

Comment 2: Title: I find the title not fully representative of the work presented in this study. This study indeed relies on omics data and partly on Hg measurements. The results rather indicate genomic potential for biotransformation, rather than actual biotransformation.

Response 2: Thank you for your insightful feedback regarding the representation of the title to our study. We fully agree that our findings focus on identifying genomic potential for mercury biotransformation. We have revised the title to "Genomic potential for mercury biotransformation in marine sediments across marginal slope to hadal zone ecosystems" as you suggested. This change better reflects the focus of the study on the genetic capabilities inferred from our data.

Comment 3: L141: if *merT* and *merR* are in the same operon, how can they have different coverage?

Response 3: Both *merT* and *merR* belong to the *mer* operon. However, *merT* may have multiple copies in certain microbial genomes due to its critical role as one of the important transporters in the operon^{1, 2}. Additionally, certain microbial strains lack *merR* in the *mer* operon³. These factors could contribute to observed differences in coverage between the two genes. Please see Lines 400-403.

Comment 4: L164: could you specify if the 2163 *hgcA* gene sequence were found

assemblies or MAGs?

Response 4: Thank you for raising this point. We have revised the text to explicitly clarify that the 2163 *hgcA* gene sequences were identified in the assemblies. Please see Line 179.

Comment 5: L332 and elsewhere: several occurrences of « deep sea » instead of « deep-sea »

Response 5: Thank you for your careful review. We have checked all instances of "deep sea" and "deep-sea" in the manuscript and made the following corrections: Changed "deep sea" to "deep-sea" in Lines 141, 381, and 390. Revised "deep-sea" to "deep sea" in Line 294.

Comment 6: L 348: I would replace «sources are less available than soils » by « sources can be less available than soils, as organic content in these environments are extremely variable.

Response 6: Thank you for the suggestion. We have revised the sentence to: "Sources can be less available than soils, as organic content in these environments is extremely variable." Please see Lines 412-413.

Comment 7: L 400: « they were centrifuged » instead of « centrifuge them » ?

Response 7: Thank you for your comment. We have revised the sentence. Please see Line 470.

Comment 8: L 422 and 441: it would be useful to specify which libraries were built with 350 bp or 550 bp insert size and why, and which samples were sequenced in 2x150 and 2x300 bp and why, unless they were all sequenced twice? Did it have an impact on the mapping results?

Response 8: Thank you for your comment. The differences in insert sizes (350 bp with 2×150 bp and 550 bp with 2×300 bp) resulted from distinct sequencing batches during library preparation conducted at different phases. The Miseq sequencing (2×300 bp) was done earlier than the Novaseq sequencing (2×150 bp). Each sample was sequenced only once. This has no effect on the mapping results. The sequencing platform and parameters for each sample are detailed in Supplementary Table 1.

Comment 9: L433: I suggest to replace « Universal primers » by « broad-range 16S rRNA gene specific primers » or « bacterial 16S rRNA gene specific primers »

Response 9: Thank you for the suggestion. We have revised the sentence. Please see Line 512.

Comment 10: L458: « categorize the representative MiTags » Could you clarify « categorize », did you mean taxonomic annotation?

Response 10: Thank you for your suggestion. We confirm that "categorize" in this context refers to taxonomic annotation. The text has been revised to explicitly state "classify" in Line 548 for clarity.

Comment 11: L460: The BLASTn search is not very clear: did you mean 16s miTags that had no matching the silva db within a 97% similarity distance where classified as new species? In addition, I think the whole workflow for 16S MiTag analysis is likely to overestimate the microbial novelty and Alpha diversity: sequences from different regions of a 16S that don't have a hit in the Silva database will all be counted as novel species despite the fact that they originate from the same unclassified sequence. It would have been correct in a metabarcoding context, where PCR amplifies the same 16S region. With miTags however, this is not the case. Similarly, I did not understand how the observation matrix used for the PCoA and CCA was built: how did the authors dealt with unassigned (no hit in Silva) miTags? if each appeared has a new ecological unit in the matrix, cf my point above. I realize that this is a minor claim of the study, but account for a significant part of the results (L108-126). A note on this caveat in the text would be very useful for the reader. For this reason MiTags are usually analysed only through taxonomic annotation and grouped by identical taxa (phylotype). As an alternative, I can maybe suggest to use the diversity of a single copy core gene approach from the assemblies, as e.g. in <https://doi.org/10.1038/s41564-019-0449-y>

Response 11: Thank you for your professional comments. We confirm that we used a 97% similarity threshold to classify sequences without matches in the SILVA database as novel species. We have clarified in the revised manuscript that the previously mentioned 97% refers to sequence similarity (Line 552). We acknowledge that the original manuscript did not clearly state that our BLASTn analysis and downstream processing were performed exclusively on the V4 hypervariable region of 16S rRNA genes. All microbial novelty identification and alpha diversity assessments were based on strictly filtered V4 region sequences, not full-length 16S sequences. Therefore, the concern regarding overestimation of novel species and alpha diversity due to analyzing different regions of 16S does not apply in our case. We have explicitly added a description of the V4 region analysis workflow in the revised manuscript (Lines 545-546).

We acknowledge that the unassigned miTags were not included in the PCoA and CCA analyses, which was indeed not ideal. Following your suggestion, we have now performed diversity analysis using the single-copy core gene *rpS3*. Specifically, we used the relative abundance of contigs containing *rpS3* sequences for microbial community diversity analysis, as outlined in the referenced method. We note that the results based on *rpS3* sequences are consistent with those obtained from 16S miTag analysis, and therefore, no changes were made to the Results section. The detailed

methodology for *rpS3* sequence analysis has been added to the Methods section of the revised manuscript (Lines 557-571).

Comment 12: L472: For sake of reproducibility, could you specify the parameters used to run metaWRAP for the binning? This wrapper has many options at each step. Alternatively, (and this is my preference as a reader) you can make the code associated with this study available on a git repository. Also, I'm a bit lost at this stage: on line 447, you mention co-assembly of metagenomics from the same cores. Only counting on the cores taken for this study, it should result in 32 co-assemblies, resulting in 32 MAG collections. Did you submit each co-assembly to metawrap separately? Which reads did you use for the mapping and differential coverage computation for the binners?

Then, L475 « using dRep (v.1.4.3) separately for each genome set from the same station »: I understand you dereplicated the 32 MAG collections among cores from the same station, normally resulting in 5 new MAG collections (as shown in sup. table 6). 1/ I don't understand the results between table S1 and S6: for instance, SCSB has 3 cores for which you reconstructed 38, 25, and 48 MAGs, so 111 MAGs in total for this station. In S6, SCSB station has 111 dereplicated MAGs: does this mean that none of the MAG pairs were within the 99% similarity criterion? 2/ In the absence of MAG dereplication across the entire study, I don't understand how gene and MAG coverages were computed. This can be problematic if nearly identical MAGs are present in the 5 different MAG collections: mapper will randomly assign matching reads to either of these. Overall, I think the metagenomics workflow needs clarifications, in the main text and maybe with a workflow diagram.

Response 12: Thank you for your suggestions. We have added the parameters used for MetaWRAP binning to the revised manuscript (Lines 580-585) and have shared the analysis code associated with this study on GitHub (https://github.com/ZhuoboLi/Mercury_metagenomics). As you correctly noted, we generated 32 co-assemblies (one per sediment core), and each co-assembly was processed independently through MetaWRAP for binning. The reads from the individual metagenomes were mapped on the selected contigs to calculate coverage level and tetranucleotide frequencies for subsequent MAG binning for a sediment core. These details have now been explicitly clarified in the Methods section (Lines 577-585).

Thank you for your careful review. We acknowledge the need for clarity regarding the MAG counts in Supplementary Tables S1 and S6. In the original submission, both tables listed dereplicated MAG counts, which caused confusion. In the revised version, Supplementary Table S1 now reports the raw MAG counts generated from each individual sediment core prior to dereplication, while Supplementary Table S6 retains the final dereplicated MAGs.

In our original analysis, dereplication was performed separately for each of the five MAG collections to prioritize inter-site comparisons, particularly between marginal seas and open oceans. However, as you rightly noted, this approach could introduce biases during coverage calculations due to potential cross-collection redundancies. To address this, we have now implemented global dereplication across

all MAGs, followed by recalculating gene and MAG coverages. The revised results remain consistent with our original findings (Supplementary Table S7). In addition, we have further clarified the workflow of metagenomics (Lines 577-588) and added a workflow diagram (Supplementary Fig. 9).

We appreciate your attention to methodological rigor, which has strengthened the clarity of our genomic analyses.

References:

1. Zhang J, Zeng Y, Liu B, Deng X. MerP/MerT-mediated mechanism: A different approach to mercury resistance and bioaccumulation by marine bacteria. *J Hazard Mater* 388, 122062 (2020).
2. Hamlett NV, Landale EC, Davis BH, Summers AO. Roles of the Tn21 merT, merP, and merC gene products in mercury resistance and mercury binding. *J Bacteriol* 174, 6377-6385 (1992).
3. Moller AK, *et al.* Mercuric reductase genes (*merA*) and mercury resistance plasmids in High Arctic snow, freshwater and sea-ice brine. *FEMS Microbiol Ecol* 87, 52-63 (2014).

Reply to Reviewer #3

Comment 1: I acknowledge that authors have addressed most questions I raised in my first review. I however still have a few remarks that could further improve the comprehension for the readership.

In their response to my comment 11, authors states that “We acknowledge that the original manuscript did not clearly state that our BLASTn analysis and downstream processing were performed exclusively on the V4 hypervariable region of 16S rRNA genes. All microbial novelty identification and alpha diversity assessments were based on strictly filtered V4 region sequences, not full-length 16S sequences. Therefore, the concern regarding overestimation of novel species and alpha diversity due to analyzing different regions of 16S does not apply in our case” In this case, I do agree that my comments regarding 16S based novelty are irrelevant. In their revised version, authors provide a link to the code used for this analysis (L547). The only python script I could find related to this 16S analysis was the following <https://github.com/ucassee/Challenger-Deep-Microbes/blob/master/Py/miTag.py>. It selects 16S related reads based on their annotations (probably from the rna_hmm3.py analysis, but not clear) and then according to their distance with the nearest Blast results. I however didn't understand how these were filtered for V4 regions. Could the authors clarify this point or specify which script they used for this purpose?

The script included in https://github.com/ZhuoboLi/Mercury_metagenomics/blob/master/work_flow.sh seems sound, but i didn't try to run it myself. It would benefit from a rapid presentation in a README file.

The code in <https://github.com/ucassee/Challenger-Deep-Microbes/blob/master/> contains multiple scripts, the function of which can only be inferred from their name. This repository would really benefit from a README describing the purpose and use of all these scripts. See also my concern regarding the 16S v4 analysis in my main comments

Response 1: Thank you for your valuable feedback regarding the V4 region filtering process. We acknowledge that our originally referenced script (<https://github.com/ucassee/Challenger-Deep-Microbes/blob/master/Py/miTag.py>) did not explicitly detail the V4 extraction steps. To address this gap, we now provide a comprehensive workflow through the dedicated GitHub repository of our research team: https://github.com/heyingshui22/mitag_analysis/blob/main/mitag_analysis-command/running16S.sh. This script documents the complete pipeline for extracting 16S rRNA reads from metagenomic data and isolating V4 hypervariable regions using hmmsearch-based profiling, with a detailed README file available at https://github.com/heyingshui22/mitag_analysis/blob/main/README.md.

Regarding the script at https://github.com/ZhuoboLi/Mercury_metagenomics/blob/master/work_flow.sh, we have added a detailed README file to clarify its structure, dependencies, and execution logic. We appreciate these constructive suggestions which have strengthened the transparency of our computational methods.