Summary

Turba et al. (bioRxiv 2022.06.17.495388, revision submitted to PCI Ecology) made extensive revisions to their previous manuscript, clarifying points and sections as needed and emphasizing the main contributions of this project. Below I reply to the authors' response to my comments on the first version. I thoroughly enjoyed reading and reviewing this paper and have no further comments to add or revisions to suggest.

Response to Reviewers (Turba et al.)

I have only included the first line for each comment for brevity; the detailed comments are still provided in the initial review and the full response to the reviewers by Turba et al.

1. Clarification about methods.

Author Response: I have included a flowchart (Figure 2) to illustrate the study design with the different protocols and also clarify which parts are relevant to the main paper and which is found in the supplemental material. I have expanded the description of each method in the Materials and Methods section as well (lines 148-165).

DMS Response: The inclusion of the flowchart and the changes in the text have clarified the methods. I think the revisions will make it a lot easier to replicate the methods as well.

2. Implement a consensus approach for differential abundances.

Author Response: As a consensus approach, I have included the ALDEx2 analysis in the study and compared results with DESEq2 and the output for the beta-diversity analyses (Material and Methods – Differential abundance section).

DMS Response: I am glad to see this complement the DESEq2 results, and I think it adds some important ecological context to the results.

3. Interpretation of results.

Author Responses: I have included the estimates and lower/upper limits of the confidence intervals in Table 2.

We have modified the focus of our Results and Discussion away from p-values and instead focused on model fit with R2. Results for both are still presented in the text (when appropriate) and in the tables.

We kept the FDR results in the table with the p-values.

DMS Response: Thank you for the revised results section, as I found it really clear to understand the importance of the results. I think the authors have made any necessary revisions.

4. Structure of the results section.

Author Responses: I find it easier to center the discussions related to the biases introduced by each protocol when focusing on each primer separately, since they capture distinct biota and the protocols are compared in a three- pairwise way. Otherwise, I think the text would be very

repetitive and add more confusion. I expect that with the new edits both Results and Discussion sections are clearer.

Modifications were done accordingly.
DMS Response: I think the rationale provided by the authors is completely valid, and the revised results and discussion sections are very clear.

Minor Comments

1. Lines 68-69: Could the authors clarify what is meant by "driven"? My interpretation is the organic and inorganic matter form the foundations of the food web, as driven suggests an active role, but I also know their presence in the water column is what leads to problems with filtration.

Author Response: Changed to "leading to an accumulation of organic and inorganic matter" (lines 71-72).
DMS Response: Thank you!

2. Lines 120-121: As I noted in my summary, I think these results can extend into rivers, streams, and ponds. No response is necessary, but I wanted to offer this to the authors.

Author Response: Changed to "We expect these results will be of interest relative to eDNA sampling in other aquatic systems as well, such as rivers, streams, and ponds, especially those with turbid waters" (lines 122-124).
DMS Response: Thank you!

3. Lines 190-191: I think having shared sequencing runs adds a lot of value and merit to the method. Sequencing can be very expensive and you will likely be sharing a sequencing run with at least 1 other researcher. I think this realism is a benefit to this method proposal/comparison, because it is done under completely realistic and not idealistic conditions. No response is necessary, but I wanted to offer this to the authors.

Author Response: Thank you!

4. Lines 283-292: Great work by the authors interrogating their data and taxonomy assignments. No response is necessary, but I wanted to offer this to the authors.

Author Response: Thank you!

5. Lines 341-342: Please correct me if I am wrong, but I was confused seeing tidewater goby presented with the 16S results when I think it should be with 12S.

Author Response: 16s rRNA has been used as target for fish communities and in fact is the second most used marker in fish diversity analyses (Shu, Ludwig & Peng, 2021: https://doi.org/10.3390/genes11030296). It is not surprising that this primer was able to capture other biota besides bacteria and archaea, but it is nice to see it is able to identify the tidewater goby as well. I added some comments on that in the Discussion with references (lines 584-586).
DMS Response: Thank you for letting me know! I am most familiar with 16S with bacteria (including getting contaminants in isolate cultures), but the clarification by the authors has helped. The quick remark in the discussion could also help readers like me who might not know

that 16S is commonly used for fish, as I think the bacterial microbiome field has really dominated 16S.

6. Lines 393-394: Reference databases are one of the biggest limitations to any barcoding work, particularly in aquatic systems. I think this point might be worth further elaboration, particularly for systems that do not or can not use 12S or 16S (e.g., freshwater macroinvertebrates). I know this is not the main point of the present paper, but I think this method comparison will be useful to aquatic researchers across ecosystems and emphasizing reference database limitations is a major fact. No response or changes are necessary unless the authors choose to make revisions.

Author Response: Thank you!

7. Lines 400-402: Very good and transparent interpretation of the methods and the inherent trades. As a single water sample was taken and this is a common method, I do not think this is a 'limitation' of the present study. I would suggest that water samples could potentially be collected from multiple points within a coastal lagoon (or any ecosystem), pooled as a single sample, and homogenized before processing. This would allow researchers to get a representative sample of the whole habitat but not increase the processing and sequencing costs (except for time and some labour).

Author Response: I've changed this section to include this discussion briefly, since this approach was not the focus of the current study but it might be of interest to others when designing their methods (lines 454-458).

DMS Response: Thank you! It would be interesting to know what, if any, differences there are between individual and composite samples, but that is beyond the scope of the current project.

8. Lines 409-421: Great discussion here by the authors regarding the sediment samples and the seemingly unusual lower read counts. No response is necessary, but I wanted to offer this to the authors.

Author Response: Thank you!

9. Note on the CAP Analysis: I do not know if the CAP analysis is really needed, with a focus on the PERMANOVA and the contrasts providing the most relevant information in as simple a piece of evidence. The PERMANOVA will tell you if the protocols resulted in different community composition, with further pairwise contrasts performed to determine which individual treatments differed. Moreover, an ordination plot with either convex hulls or ellipses for each community type would be easier to interpret for the readers and quickly show if the protocols do result in different compositions.

Author Response: While the PERMANOVA analysis helps to see if there are discernible community differences, the CAP allows us to see which species is driving most of the difference between protocols. Since this lagoon is an area of conservation interest, I thought it relevant to discuss this difference at the species level as well.

DMS Response: I think the justification by the authors is completely fair and accurate.

Reviewed by:

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Please do not hesitate to contact me directly via electronic mail if any of my comments were not clear or require further clarification during the review and revision process.