Review of: "An efficient early-pooling protocol for environmental DNA metabarcoding"

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Potential competing interests: The author(s) declared that no potential competing interests exist.

Detailed methodology of DNA metabarcoding is variable among researchers and not optimized necessarily based on experimental evidences.

The authors performed a comparison between 1st-PCR indexing and 2nd-PCR indexing methods in DNA metabarcoding analysis and provided technically important conclusions. These included a fact showing that the effect of reaction scale (number of PCR replicates and volume of template DNA) is significant regarding the number of detected animal OTUs.

The logic and description of the author is clear, and the analysis and figure making are well-done. I think the manuscript is well-written, and has no critical problems for publication.

Although I have gone through the manuscript, I have only a few minor comments. I am sorry for my little contribution to finalization of the manuscript. I hope that the followings are of help to the authors:

Minor points:

Figure 5 legend: "Ellipses indicate 95% confidential intervals for each study site."
I think that the statement of ellipses indicating intervals are geometrically inaccurate. For example, "the colored circles indicate 95% concentration ellipses" (or 95% confidence ellipses) may be better.

References: "Ficetola, G.F., Miaud, C., Pompanon, F. & Taberlet, P. (2008). Species detection using environmental DNA from water samples. Biology letters, 4, 423–5."
Pages may be 423–425.

"Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C. & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular ecology, 21, 2045–50."
Pages may be 2045–2050.

"Yamahara, K.M., Preston, C.M., Birch, J., Walz, K., Marin, R.I., Jensen, S., et al. (2019). In situ autonomous acquisition and preservation of marine environmental DNA using an autonomous underwater vehicle. Frontiers in Marine Science." Volumes and pages may be missing (6, 373).