

***Interactive comment on* “Sample preservation and pre-treatment in stable isotope analysis: Implications for the study of aquatic food webs” by Marc Jürgen Silberberger et al.**

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We would like to thank you for your insightful comments regarding our manuscript. Based on them and the review of Referee #1, we have decided to make the following major adaptations to our manuscript:

- We will add a paragraph in the introduction about recent Bayesian Stable Isotope mixing models (specifically the MixSIAR framework) to justify our objective of addressing the impact of preservation and pre-treatment effects on such models. Here we will explain that these models utilize mean and variance information for source, consumer, and trophic enrichment factors, point out that Bayesian mixing models are multivari-

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ate analyses and that newer models also incorporate tracer covariance, since carbon and nitrogen isotopic ratios are often coupled (Hopkins & Ferguson, 2012, Parnell et al. 2013, Stock et al. 2018). However, studies that address preservation and pre-treatment effects on stable isotope ratios treat each isotope independently and accordingly there is a potential for biases in mixing models that is not recognized yet.

- Furthermore, we will clarify our interest in the comparison with lipid normalization and formalin correction from literature data. Our main motivation lies in the fact that such general corrections are widely applied to invertebrates, because species-specific or even tissue-specific corrections are typically only available for vertebrates. A recent review of Arostegui et al. (2019) showed that many mixing model studies use lipid corrected data. They demonstrated that estimated diet proportions can be strongly affected by lipid correction in comparison to lipid intact samples. However, since lipid corrections are typically applied when no comparison to lipid extracted samples is possible, we consider it important to identify whether the adjustment translates into a similar model outcome as lipid extracted samples. And accordingly for formalin correction.

- We have re-evaluated the trophic enrichment factors we used in our models. We came to the conclusion that the chosen trophic fractionation for $\delta^{15}\text{N}$ (3.4 ± 1) should be kept. Also we think the trophic fractionation for the first trophic step from POM/SOM to primary consumer for $\delta^{13}\text{C}$ (4.0 ± 1.3) is a good choice. For the second trophic step, however, we have decided to adjust the TEF for $\delta^{13}\text{C}$ to 0.8 ± 0.5 according to Antonio et al. (2011) who reported differences between $\delta^{13}\text{C}$ ratios for *Crangon uritai* and those of a variety of prey species (known from gut contents) in the range of 0.3-1.3‰. So far, we ran mixing models with this new TEF for an initial assessment on short setting and it appears that this change will not affect our results strongly. However, if our results will be affected by this after full model runs, the manuscript will be adapted. Furthermore, we will add a more detailed justification for all the chosen TEF in the method section.

- We will also add a section about TEF in the discussion, where we will compare our observed fractionation to the chosen TEFs and discuss the importance of them in

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mixing models.

- We are currently running all models on extreme setting (this is ongoing, but due to the high number of models this will take some time). So far, we have not seen any considerable differences between the long and the extreme runs, but we will adjust our manuscript accordingly if necessary.

- We will use the Bhattacharyya Coefficient to include pairwise comparisons of the probability distributions of the source contributions.

Please find below our specific answers to your comments:

Comment: This study reports the effects of sample preservation and further treatment on stable isotope values in two marine species (Crustacea and Bivalva) and further consequences on the application of Bayesian mixing models to infer the relative contribution of several food sources. The text “Implications for the study of aquatic food webs” in the title seems to me too ambitious considering that the study only refers to two species.

Response: - We are reconsidering the title. We have not made a final decision about the new title yet, but we are planning to replace the second part of the current title with a mentioning of stable isotope mixing models.

Comment: The first part of the study is well-presented and organized, providing interesting information on SI for the focused species. The authors use different transformations and corrections on original Si values, depending on previous treatment of the samples. So many data manipulations seem rather confusing sometimes and will certainly affect the results achieved in the second part of the manuscript, focused on mixing models.

Response: - We are aware of the complexity of the treatments and corrections in our study and will keep this in mind during the manuscript editing to avoid confusion for the reader as much as possible.

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Comment: I really do not understand the interest of comparing the author's data on SI values with those resulting from mathematical corrections according to Post et al. (2007) and Mc-Connaughey & McRoy (1979).

Response: - Our interest in these two formulas comes essentially from how widely they are applied to benthic invertebrates. For uses of McConnaughey & McRoy see for example Coat et al. 2009. This formula is also recommended in Jardine et al. (2003) a guide for stable isotopes in aquatic systems. The formula according to Post et al. (2007) is probably the most widely used lipid normalization and the authors themselves compared their formula to McConnaughey & McRoy. This wide application of lipid normalization was also pointed out by the review of Arostegui et al. 2019, who then went on to demonstrate that lipid normalization can have a large impact on mixing models. However, their study only showed that mixing models differed between normalized data and data with lipids intact. Accordingly, we think it is quite important to take the step back and compare whether we are able to achieve mixing model results after lipid normalization that resemble the results for samples that had the lipids chemically removed. We will clarify our motivation for this aspect of the paper and expand the mixing model comparison by inclusion of Bhattacharyya Coefficient for easier comparison.

Comment: From my point of view and considering the huge work made on samples treatments, the original data provided in the study is of certain value in itself and will partially contribute to increase the knowledge on stable isotopes in marine organisms. The most interesting part of the study would have been that the authors provide mathematical corrections for the conversion between preservation methods for the objective species, which should be species-specific.

Response: - While we agree that conversion should be species-specific, we are not sure how we could confidently provide these. We mentioned this briefly in the discussion (L. 323-328) but realized that this needs more explanation. The main reason why we feel we cannot propose correction factors is because the only effect we identified

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as significant was the effect of formalin preservation on $\delta^{13}\text{C}$ in comparison to the other preservation methods. All other preservations effects were small and not significant (compare section 3.1.2) and accordingly it seems very problematic to propose a correction. Nonetheless, the mixing model for dried and frozen *Limecola balthica* samples differed consistently and would lead to different interpretation of the species diet. For dried *L. balthica* samples (across all treatments) probability distributions for pPOM and SOM did not overlap considerably (we will highlight this with Bhattacharyya Coefficients) and pPOM would be clearly identified as main diet. For frozen samples, however, there is a huge overlap between pPOM and SOM and we would assume a mixed diet. We consider this a very crucial aspect of our study as it highlights that the non-significant small changes in both stable isotopes that are frequently found in the literature can strongly bias diet estimation. We will also edit the manuscript to connect the first and second part of our analysis more clearly to point out how changes in isotope ratios translate to the mixing models.

Comment: About the second part of the study, there are several constrains. The first one refers to the values applied to trophic enrichment factors (TEF) for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Generalist TEFs are useful when we do not know the actual TEF for a given species, which is the case of this study. However, in some cases those TEFs are unrealistic for a given species, e.g. Planas et al. (2020). (Effect of diet on breeders and inheritance in syngnathids: application of isotopic experimentally derived data to field studies. MEPS <https://doi.org/10.3354/meps13315>).

Response: - We agree that TEF have to be chosen with care and should be chosen as species-specific, stage-specific, and tissue-specific as the literature allows. Unfortunately, this has not been very clear in our manuscript, but we have selected the TEF as consumer-prey pair specific as possible and we will include a justification for each of them in the method section. As mentioned above, we have re-evaluated the TEFs we chose and have come to the conclusion to adjust the TEF for $\delta^{13}\text{C}$ for *Crangon crangon* according to Antonio et al. (2011) who studied *Crangon uritai* and its

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prey. We believe we have chosen adequate TEF for the chosen species (or at least as good as possible). As you correctly pointed out, we cannot know whether these TEFs are correct or not (This is impossible in all uncontrolled field studies). However, we argue that even if they should be somehow wrong, they are equally wrong for all models for each species in our study and accordingly the effect of preservation and pre-treatment is real. We will however discuss the issue of choosing TEF and how our data compares to the TEFs we used. Furthermore, the uncertainty in TEF is accounted for within the MixSIAR framework if an error term that includes residual error is used (Stock and Semmens 2016). According to Stock and Semmens it is essential that mixing model users are faithfully incorporate uncertainty in trophic discrimination factors and expressed that they suspect mixing model users tend to underestimate TDF variance when using borrowed values. We believe we have chosen appropriately large TEF variance considering the uncertainty about our TEFs. Another thing we feel the need to point out in this context is that our study objects are lower trophic level benthic invertebrates. While we are aware of the progress that has been made for vertebrates with regard to TEF, tissue specific lipid correction, and several other methodological uncertainties, this knowledge is not available for the vast majority of benthic invertebrates (and probably never will be). Accordingly, there is no other option than applying rather generalist TEF and also generalist lipid normalization or other mathematical correction in the study of benthic food webs.

Comment: Hence, the use of unspecific TEF values should not be used as reference for checking “improvement” of mixing model outputs. The use of the term “improvement” is another issue in this study. As the authors claim, an objective of the mixing models analysis performed was to assess whether normalization and mathematical corrections should be used to adjust data for the use in such models and to improve modelling results. Achieving an improved model imply a comparison with a control model, which is lacking in the study. The use of generalist TFEs do not ensure that model outputs are actual references for comparisons.

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Response: - We will avoid the use of the word “improved”. However, we think we are able to evaluate whether the correction achieved what we want it to achieve due to the large variety of treatments applied to our samples. For example: We have probability distributions for (a) samples with lipids intact, (b) samples treated the same way as (a) but lipids removed, and (c) data for samples from (a) but lipid normalized. Accordingly, we can assess whether the probability distribution for (c) is closer to (b) than to (a). We will also use the Bhattacharyya Coefficient in this context to make this comparison easier to follow. Since we use the same TEF across all models, this comparison can be made. The chosen TEF might be good or bad, but it is equal across all models.

Comment: Besides, mixing models provide relative contribution estimates of dietary sources. Ideally, overlapping significance of estimates should be analyzed (Bhattacharyya’s coefficients) to assess the accuracy of source contribution estimates (Bhattacharyya, A., 1943. On a measure of divergence between two statistical populations defined by their probability distributions. Bull Calcutta Math Soc 35:99-109).

Response: - We will calculate BC for pairwise comparisons and use the commonly applied $BC > 0.6$ to infer significant overlap between distributions.

Comment: Mixing models were carried out using a “long” run approach (which is not always sufficiently long as expected). Even though diagnostics (Gelman-Rubin and Geweke) might be fine with a long run, important differences in outputs might arise sometimes when the model is submitted to an “extreme” run. Did the authors assayed any extreme run? If so, were there any differences compared to a long run?

Response: - We did some extreme runs initially and there were no differences to the long runs. However, to avoid raising any doubt regarding the length of model runs we decided to run all models on extreme runs. This is ongoing (approximately 40% of model runs are done) and until now we have not observed differences. But we will adjust the manuscript if necessary

Comment: Finally, the use of isotopic “depletion” or “enrichment” is not correct when re-

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ferring to values obtained from sample treatments. The terms “decrease” or “increase” must be used instead.

Response: - We will edit the manuscript accordingly

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