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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 #2, 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-

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 δ15N (3.4$\pm$1) should be kept. Also we think the trophic fractionation for the first trophic step from POM/SOM to primary consumer for δ13C (4.0$\pm$1.3) is a good choice. For the second trophic step, however, we have decided to adjust the TEF for δ 13C to 0.8$\pm$0.5 according to Antonio et al. (2011) who reported differences between δ13C 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

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: The ms by Silberberger et al. investigated the effect of different preservation methods (drying, freezing, formalin) on C and N stable isotope ratios in two marine invertebrate species, and the effect of acidification and lipid removal. The authors then apply Bayesian mixing models to determine the extent to which these sample prep methods affect the outcome of the mixing models. While there are some valuable data in the manuscript, I fail to see the overall relevance of explicitly investigating how these sample prep differences translate into mixing model output. The point is that this will depend strongly on the 'absolute' values of the sources and consumers, and I don't see how general conclusions can be drawn.

Response: - We have realized that our introduction did not introduce Bayesian SIMMs and accordingly the justification of objectives 3 and 4 is not given. Of course, absolute values of sources and consumers together with the chosen trophic fractionation determine the mixing model results. However, the fact that such models are multivariate models but studies that address preservation or pretreatment effects consider isotopes individually, makes it difficult to predict how any effect translates to mixing models. Furthermore, models like IsotopeR or MixSIAR framework incorporate variance of sources and mixtures but also that tracer covary in source/mixture (Hopkins & Ferguson, 2012, Parnell et al. 2013, Stock et al. 2018). This is in contrast to our understanding of preservation and pre-treatment effects, which is treating different isotopes

independently from each other and mostly worry about average absolute changes of the isotope ratios. Consequently, we think it is important to compare how sampling and sample processing affect stable isotope ratios and how this translates to mixing models. We will clarify this in the introduction prior to the objectives to justify the motivation for the mixing models.

Comment: The authors mention that they also tested whether lipid and formalin corrections 'improved' the model results but there is no objective way to claim whether the output is better or worse then the original. "improved" implies closer to reality. Hence I do not see how we should interpret the authors' conclusions that (L408): 'the model outcomes are only rarely improved and equally often worsened' – the authors remain vague on how they interpret this.

Response: - With regard to lipid and formalin correction, the word 'improved' was used to describe that the modeling result was closer to the modeling results of the treatment for which the correction was applied. The purpose of these corrections is quite clear, e.g. lipid normalization is applied to account for not removing lipids from samples and the desired result after lipid normalization would resemble the result for samples that had lipid removed. We will adjust the ms to clarify what is the desired outcome after corrections and we will avoid using the term "improved". Also, we will use Bhattacharyya Coefficient to make pairwise comparisons of probability distributions, which will help to clarify the results section and discussion as it provides an objective way of identifying significant overlap in diet contribution between models (in addition to the visual representation of the credible intervals).

Comment: If dried samples (and acidification for d13C if carbonates could be present) are the reference, then indeed all of the pre-treatment and preservation methods may or may not results in shifts in measured d13C or d15N values. A wealth of studies have assessed such changes, often with variable outcomes – but we have a good idea of the range of changes that can be expected. When subsequently applying a (Bayesian or not) mixing model, the key assumptions that need to be made are regarding the trophic

shifts for d13C and d15N.

Response: - We agree that an important assumption for mixing models are the TEFs. This issue is rather well recognized (e.g. Bond & Diamond 2011). Any mixing model basically requires the user to make an educated guess based on the literature or experimentally determine new TEF. Recent Bayesian mixing models (in contrast to earlier mixing models), however, include uncertainty in TEFs into Bayesian mixing models (Stock and Semmens 2016) and accordingly are somehow accounting for this general difficulty in mixing models, if appropriate model parameters are selected. Stock and Semmens (2016) have pointed out that they "suspect mixing model users tend to underestimate TDF variance when using borrowed values". We believe we decided for reasonable TEFs and use appropriate variance for them (please see detailed justification for the chosen TEF below). Nonetheless, we acknowledge that there remains always some uncertainty about TEFs and will give this aspect some space in the manuscript. However, we argue that the choice of the TEFs is not as essential for the comparison of different models in our study since our focus is on the effects of preservation and pre-treatment. We used the same TEFs across all models and accordingly all the models are equally right or wrong and the differences among the models are caused by the preservation and treatment. Since trophic enrichment is a natural process and has happened prior to sample collection, it is appropriate to use the same trophic fractionation across all models. Differences between them were introduced by our methodological choices.

Comment: In my view, it would make sense to think about how these trophic shifts were determined – if those literature estimates were based on measurements with standard sample prep (no lipid extraction etc) then they need to be applied to similar data, if not then a correction to your sample data is needed in order to account for that.

Response: - We agree that it would make sense to think about this, but: o this requires prior knowledge about how preservation and pre-treatment effects on individual isotopes translate into the results of MixSIAR. (objective 3) o this also requires prior

knowledge about whether the applied corrections will give us the same modelling results as for the treatment for which it corrects for (objective 4) If we were able to get model results after a data correction that resembles the model result for samples that were treated accordingly, only then it would make sense to think about such an approach. - We will discuss the aspect of how trophic fractionation is determined in the literature, and specifically for the TEFs we used. However, since our corrections did not consistently achieve the desired outcome, we will advise against this approach (At least for benthic invertebrates for which species-specific correction methods are virtually not existing)

Comment: If I consider the objectives of this manuscript: (i) quantify how sample preservation and pre-treatment affect carbon and nitrogen SI ratios (ii) identify potential interaction effects between preservation and pre-treatment methods (iii) study how preservation and pre-treatment affect the results of Bayesian mixing models (iv) assess whether lipid normalization and mathematical formalin correction should be used to adjust data for the use in such models. then for me (i) and (ii) are fine, but (iii) and (iv) are not. I feel the ms should either forus on objectives (i) and (ii) but in that case it becomes a small dataset that is perhaps not sufficiently novel compared to the existing literature, or alternative think on expanding the scope – but I'm not sure that is feasible with the data at hand.

Response: - As mentioned above, we have realized that our introduction failed to give a clear justification for objectives 3 and 4 and we will edit the manuscript as mentioned above. In addition, our results highlight the importance of addressing these objectives. For example, in the first part of our study, we did not detect a significant difference between dried and frozen Limecola balthica for both isotopes. Nonetheless, the mixing models differed very strongly between dried (predominantly pPOM diet) and frozen (mixed diet of pPOM and SOM). Consequently, we would not apply any correction based on the effects on the isotope ratios, but it alters the estimation of the diet considerably. We will edit the manuscript to highlight these discrepancies between the

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two parts (individual isotope ratios vs. mixing models) as it is from our point of view a crucial aspect of this paper.

Specific suggestions/comments: Comment: The authors apply a 'scaled trophic fractionation' for d13C, whereby this is assumed to be large for the first trophic transfer (4 +/- 1.3 per mil) and smaller (0.4 +/- 1.3 per mil) for subsequent trophic transfers. Frankly, it is the first time I come across this, and if I follow the references this is based on, this all comes from a single paper (Hobson et al. 1995). I do not see much confirmation of the validity of this assumption in later reviews on trophic fractionation, e.g. Caut et al. (2009) Caut et al (2009) Variation in discrimination factors (Δ15N and Δ13C): the effect of diet isotopic values and applications for diet reconstruction. Journal of Applied Ecology 46: 443-453. https://doi.org/10.1111/j.1365-2664.2009.01620.x

Response: - Unfortunately, the trophic transfer from POM/SOM to benthic primary consumers is typically excluded from reviews (like Caut et. al (2009) or McCutchan et al. (2003)) because the diet is a mixture. We provided Hobson et al. (1995) as a reference for the trophic fractionation of 4$\pm$1.3, since we are not aware of a review that could be used for this strong trophic enrichment in 13C. Our choice is, however, not based only on this study but on multiple studies that observed this trophic enrichment for POM/SOM and benthic consumers globally. To our knowledge Fry and Sherr (1984) were the first to recognize such a global pattern for POC to benthic filter feeder's trophic enrichment ~4‰ for 13C and since then this is omnipresent in marine benthic food web studies. For example, Nerot et al. (2012) reports similar trophic enrichment of benthic filter feeders in comparison to POM along a depth gradient in the northern Bay of Biscay. Iken et al. (2010) found a similar strong enrichment from POM and SOM to benthic consumers in the Pacific Arctic under 4 different water masses with differing 13C baseline. Furthermore, a similar trophic enrichment can be deduced for the Gulf of Gdansk from the data presented in Sokołowski et al. (2012). Also, we observed the same in our own studies from the North Sea and northern Norway (Silberberger et al. 2018) or Svalbard (Kedra et al. 2012). While the reasons for

this strong fractionation seem to remain unclear, it is a general pattern throughout the literature and applying a smaller general TEF according to one of the highly cited reviews would be not appropriate here, especially with regard to our data structure that clearly shows a strong 13C enrichment from the OM sources to Limecola balthica (compare fig. 2 and 3 in our ms). We have, however, re-evaluated all our chosen fractionations and have come to the conclusion to make a slight adjustment to the TEF for δ13C for Crangon crangon according to Antonio et al. (2011) who studied Crangon uritai and its prey (0.8±0.5). A widely applied trophic fractionation of 3.4±1 for nitrogen was chosen according to Post (2002). He developed this general value largely on filter-feeding bivalves (pelagic baseline) and grazing snails (littoral baseline) and accordingly we assume it a suitable trophic fractionation for Limecola balthica. The same trophic fractionation was applied for the second trophic step, since we could not find a more suitable fractionation. The applied fractionation compares well with the fractionation of 3.6‰ that has been assumed by Fry (1988) in a study that included also Crangon sp. Furthermore, it is quite close to experimentally determined trophic enrichment of a Mysid that was fed an Artemia diet (3.55) (Gorokhova & Hanssen 1999). We also considered calculating TEF from formulas for invertebrates from Caut et al. (2009). However, we decided against it for 3 reasons: (1) the calculated fractionations would be unrealistically low with regard to our data, (2) the formula only gives a mean trophic fractionation without any variance term, and (3) Caut et al. (2009) reports R2 of 0.09 for the invertebrate formula for carbon and accordingly we don't think this specific formula should be applied.

Comment: In particular for datasets such as the one the authors have, where C and N isotope ratios for most sources (pPOM, sSOM, ssSOM) are very similar and one is quite different (rPOM), the choice of the frationation factor has a very strong influence on the results, likely much more then the relatively small shifts induced by sample pre-treatment or storage.

Response: - We agree that the choice of fractionation is crucial for Bayesian stable

isotope mixing models. However, this is a well-recognized limitation (e.g. Parnell et al. 2013, Bond and Diamond 2011) and requires an educated choice based on the literature. As described above, we have given this long consideration and believe we have chosen adequate TEF. As mentioned before, since we apply the same trophic fractionation for all samples from one species, all our models are equally right or wrong and accordingly our observed differences are caused by preservation and pre-treatment. We will discuss the trophic fractionation we selected in comparison to our data and also the importance of the applied trophic fractionation in models.

Comment: There is also a large body of additional literature looking into the effects of lipid extraction and lipid corrections, e.g. the recent one by Cloyed et al. (2020) and references therein. Cloyed et al. (2020) The effects of lipid extraction on δ13C and δ15N values and use of lipidâĂŸ Rcorrection models across tissues, taxa and trophic groups. Methods in Ecology & Evolution 11: 751-762. https://doi.org/10.1111/2041-210X.13386

Response: - Thank you for pointing out the literature. We have looked into the paper and the references in it and have found some interesting literature we will address. However, that paper also highlights that there are big differences between the knowledge available for vertebrates and invertebrates, which we will address as well.

Terminology: Comment: -avoid the use of 'stable isotope concentrations' (L38), you're looking at stable isotope ratios

Response: - We will edit the ms accordingly

Comment: -d13C values of different samples are higher or lower compared to each other, or they increase or decrease. Samples are enriched or depleted in 13C relative to each other. Avoid the use of mixed terminology such as 'a depletion in d13C' (L43 and throughout the ms), an 'enrichment in d13C' (L43 and throughout the ms).

Response: - We will edit the ms accordingly

Comment: -data corrections: in the methods (L131-133) the authors mention that data were corrected using pure reference gases calibrated against IAEA standards. This is not correct: the reference gases should not be used to calibrate the data, they merely serve as a monitoring gas, and data should be corrected using results of certified (or in-house calibrated) standards during the run. Perhaps this was done (I hope so) but it is merely not formulated properly.

Response: - Thank you for pointing this out. This was done. We will clarify the text in the revised ms.

Comment: -C/N ratios (L163 and further): to avoid confusion, please mention if these are weight/weight or molar ratios.

Response: - We will edit the ms accordingly

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