Authors' Response to Reviewers:

Suggestions for revision or reasons for rejection

(visible to the public if the article is accepted and published)

Jabinski and co-authors have thoroughly revised their manuscript to accommodate the suggestions Reviewer 1 and I made on the previous version. I think the clarity of the text and figures is significantly improved, and especially appreciate the change in terminology from alpha-w to aw, and the additional context provided for how this was calculated – I obviously misunderstood this when I read the original manuscript, which led to some confusion. I realize now that the intention of the study was not really to investigate H isotope fractionation, however I still think that there are some nice results in this regard and that these could be highlighted more clearly. At the moment, the revised version includes some changes that were made in response to my previous comments that get at this a bit, but don't really integrate the H isotope fractionation results with the rest of the manuscript. In some places it is still confusing whether water H assimilation efficiency or H isotope fractionation is being discussed. I guess I also still don't really see why water H assimilation efficiency is so important to focus on in and of itself – the Zhang et al 2009 paper assessed this to provide context for studying H isotope fractionation and the Kopf et al 2015 paper did it in the context of using 2H as a tracer for microbial growth rates. In both cases it is a background calculation buried in the supplement, but here it becomes the focus of the H isotope discussion. The introduction doesn't set up clearly for me why this is important or interesting. However, substrate induced changes to metabolism clearly affect net H isotope fractionation in bacteria (Wijker et al., 2019) and it seems like this study shows similar patterns in fungi. I think this is an interesting finding that could be highlighted more clearly, and better linked to the existing literature on H isotope fractionation – the important papers are cited, but the links and the context are not really clear. I think that with an additional round of minor revisions to the text, this paper will be a very useful contribution and I look forward to seeing it published.

Best wishes,

Nemiah Ladd

We thank Prof. Ladd for improvig this manuscript. She has identified a critical distinction in stable isotope investigations of H isotope fractionation (i.e. ε), which primarily applies to natural systems and paleo records, and water H assimilation efficiency (a_W), which mostly applies to stable isotope probing studies. These parallel concepts are now directly addressed in revised Introduction, where we highlight the significance of a_W for SIP applications. These points were also addressed in a recent review [Pilecky, M. et al. 2025, TrAC Trends in Analytical Chemistry, 118194. doi: 10.1016/j.trac.2025.118194], which is cited in the revised manuscript.

The a_W concept was introduced by Zhang et al (2009, PNAS), who presented two figures in the main text to describe the trends, and later empirically determined by Kopf et al. (2015, PNAS), who applied it to estimate microbial growth rates. The importance of the water H assimilation efficiency factor, as questioned above, is underscored by how it contrasts with $\varepsilon_{18:2/water}$ values. As demonstrated in this study (cf. Table 1) and by Zhang et al. (2009), a single species growing on the same substrate under the same conditions can exhibit a broad range of $\varepsilon_{18:2/water}$ values (Figure 1 in this reply), owing, in part, to $^{2/1}$H kinetic and equilibrium isotope fractionation. Importantly, the δ^2H value of a lipid or any biomolecule is also determined by the mass balance

of the H that derives from water versus other sources of H; this is especially true for heterotrophs, who incorporate a non-negligible portion of H from their carbon source into lipids. As shown in the figure below, $\varepsilon_{18:2/water}$ estimates converge on two different values, depending on whether the incubation was performed under natural or $^{2}H_2O$-labeled conditions; hence, neither is an accurate approximation of the true isotope fractionation factor ε, which is independent of $\delta^{2}H_{H2O}$ in both open and closed systems (Hayes 2004). In contrast to $\varepsilon_{18:2/water}$ values, the water H assimilation efficiency accounts for both $^{2/1}H$ fractionation AND the proportion of H deriving from water, and can therefore be interpreted more robustly as a constant for each species-substrate combination (see Methods). While $\varepsilon_{18:2/water}$ values may remain relevant for natural abundance applications for which there is relatively small variation in $\delta^{2}H$ of ambient water, the upshot of a_W is that it can be applied to (1) upscale rates of water-H assimilation into fatty acids in SIP studies to achieve a more accurate estimate of total microbial production (e.g. Kopf et al., 2015; Wegener et al., 2012), and (2) assess the metabolic mode of the organism that has produced the biomarker fatty acid, as shown for methanogens, for example (Wu et al., 2020). Similarly to methanogens, fungi are able to metabolize a wide range of substrates. The motivation for this study was to determine a_W values and inorganic C incorporation values for heterotrophic fungi, which will help to inform applications (1) and (2) described above. The corresponding $\varepsilon_{18:2/water}$ values contribute to the mounting database of $^{2/1}H$ fractionation factors described for organisms across all domains of life. While this was not the primary aim of this study, we agree with Prof. Ladd that this estimate is informative and have added text to discuss these findings. We note however that, whereas we performed at least n = 4 independent incubation experiments to determine a_W for each species-substrate combination, n=2 for estimates of $\varepsilon_{18:2/water}$ under natural $^{2/1}H$ abundance conditions.

Please find additional responses to all comments below; the listed line numbers refer to the resubmitted manuscript including track changes.

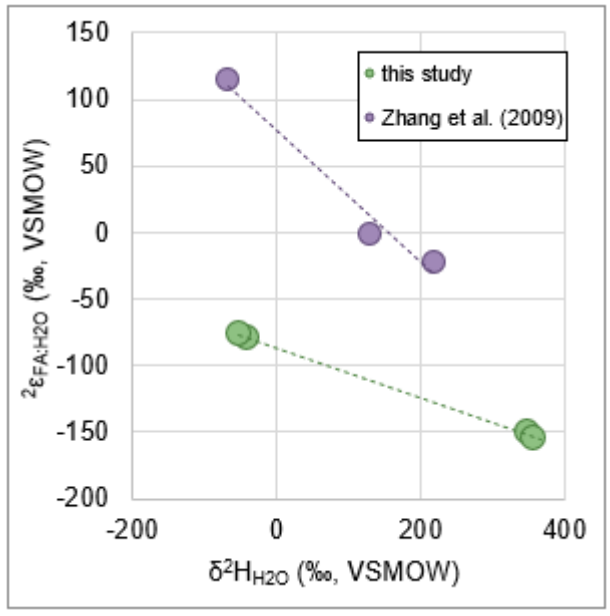

Fig. 1. Variability in ε values calculated between $^{2/1}H$ compositions of fatty acids and environmental water ($^{2}\varepsilon_{FA:H2O}$) are inherently coupled to $\delta^{2}H_{H2O}$. Shown are data for $C_{18:2}$ fatty acid of *Mortierella* grown on glucose (this study; Table S1) and $C_{16:1}$ fatty acid of *C. oxalaticus* grown on acetate (Zhang et al., 2009). Increases in $^{2}\varepsilon$ values with $\delta^{2}H_{H2O}$ are a consequence of the changing end-member value (i.e., $\delta^{2}H_{H2O}$) in the isotopic mass balance, because water-H is not the only source of H to fatty acids. In contrast to $^{2}\varepsilon$, the water H assimilation efficiency factor (a_W) accounts for not only kinetic and equilibrium isotope fractionation between water and fatty acid, but also the relative proportion of H derived from water, and is thus constant for the plotted growth experiments (a_W = 0.67±0.01 and 0.52±0.04, respectively).

Specific suggestions:

Q1: Lines 60-62: This sentence doesn't fit with the flow of this paragraph or make it clear how this is relevant. If I understand correctly, you are mainly using the 2H labeled water to look at how much of the H in lipids comes from water, and not really discussing the differences in 2H/1H

fractionation? But we know that that there are big differences in 2H/1H fractionation with NADPH metabolism, which makes it hard to isolate these two processes. Is that what you are aiming to do here?

R1: This section of the Introduction has been revised to provide more clarity (Lines 60-71). Our aim was to define ^{2}H incorporation into lipids from water, which involves multiple factors. These include, but are not limited to the net $^{2/1}H$ fractionation ($\varepsilon_{lipid/water}$; e.g., via NADPH metabolism, biosynthesis enzymes, etc.) AND the proportion of H (f_H) that is derived from water versus alternative H sources (e.g., substrate). These individual terms ($\varepsilon_{lipid/water}$ and f_H) are difficult to disentangle, and to our knowledge, cannot be empirically determined for heterotrophs. However, the combined effects of $\varepsilon_{lipid/water}$ and f_H (a.k.a., a_W) be identified by the SIP approach applied in this study as described by the equations presented in Kopf et al. (2015). Similarly to H, we aimed to define trends in the inorganic C incorporation for each substrate and species.

Q2: Lines 155-156: The TMSH achieves this by methylating the fatty acids, right? Maybe good to specify this since you switch to describing FAMEs in the next sentence and this way it would be clear where they come from. Also, how do you correct for the isotopic effect of the added C/H from the methyl group?

R2: Thank you for the suggestions to clarify this switch between fatty acids and fatty acid methyl esters; we have revised the sentences accordingly. There was no correction performed for the added methyl group, as the primary objective of this study was the determination of a_W and inorganic C incorporation, for which we compare relative changes in $^{2/1}H$ and $^{13/12}C$, such that knowledge of the stable H and C isotope composition of the additional methyl group is irrelevant (as it is included in FAMEs from both labeled and natural treatments). We have added text to clarify that this correction would improve the accuracy of the reported ε values; however, the high variability ε values for individual growth experiments (~100 ‰; Fig 5, main text) likely far exceeds any shifts in $\delta^{2}H_{18:2}$ values that may result from the correction.

Line 164-165: "…trimethylsulfonium hydroxide (TMSH) was added on the sample to increase the volatization by transforming the fatty acids into fatty acid methyl esters (FAMEs) and improve measurement sensitivity."

Line 199-201: "Notably, calculation of %IC and aW consider the relative increases in 13/12C or 2/1H composition, and thus do not require a correction for the methyl group added during derivatization of the fatty acid. The εC18:2/water values reported in this study were also not corrected for the additional methyl group and add to the uncertainty of the reported values, as further discussed below."

Q3: Lines 158-159: Did you analyze two separate aliquots of sample biomass (once for H, once for C)? Not clear as written

R3: Yes, this is now clarified in the revised manuscript (Line 167). The samples were analyzed separately for carbon and hydrogen isotopic composition of the same biomass. The conversion to CO_2 or H_2 for IRMS is stated in the subsequent lines.

Q4: Line 182: Kopf et al based their calculations based on approach used by Zhang et al., 2009, so it would make sense to cite that paper here as well (already cited several other places in the manuscript).

R4: The citation has been added.

Q5: Lines 267-272: This is a different set of results than the water assimilation efficiency, and I think it would make sense to report it with a different sub-heading. I realize this wasn't in the originally manuscript or something that you were focused on before my previous comments. I do think there are some cool results here, showing that the same patterns observed in bacteria are found in fungi, and would be consistent with NADPH metabolism affecting H isotope fractionation during fatty acid synthesis by fungi

R5: A new sub-heading has been added to the revised manuscript.

Q6: Lines 270: It seems like there would be significant differences in epsilon C18:2/water if you compared values for different substrates within single species. There is a lot of differences in fractionation among species, so this gets lost when you look at the average fractionation for each substrate for all species. Within a species, we would expect there to be higher epsilon values for cultures grown on succinate than on glucose for example, which does seem to be the case in your data (it is a little hard to tell from the overlapping symbols in figure 5)

R6: All ε values were reported in Table S1. We now provide statistical comparison (homoscedastic t-tests) only for the non-labeled growth experiments (Lines 287-296), which excludes variability introduced from labeling experiments (discussed in our response above) and is more similar to traditional H isotope fractionation approaches. The significant differences observed between substrates or within species are now reported in the new sub-heading of the Results section; we interpret these with caution given that the number of replicates for each natural incubation are low (n = 2 or 3 for each species-substrate pair).

Q7: Lines 325-342: I still find this paragraph confusing as it is not clear how the discussion of the water hydrogen incorporation relates to the discussion of the fractionation factors

R7: Please refer to our response above. The important point is that NADPH may contribute only "around half of all lipid hydrogen". We have added the following text to the first sentence to provide further clarification:

Line 351: "…and importantly for heterotrophs, a non-negligible proportion of hydrogen may derive from sources other than ambient water."

Q8: Lines 349-350: I think this is a misrepresentation of the Wijker et al paper, which is focused on how changes in NADPH production and turnover within cells affect H isotope fractionation, not net water-H incorporation into lipids. Wijker et al only assessed water incorporation into lipids for growth on glucose (figure S2 of their paper) and found similar rates among the three species of bacteria they tested. There is no discussion of NADPH related to the water assimilation rates that I could find, but quite a lot about how NADPH metabolism impacts net 2H/1H fractionation factors

R8: We have removed this sentence and citation. The revised text now describes the coupling of CUE and a_W as they both relate to the proportion of substrate C and H (i.e., mass balance) that is incorporated into biomass (see Section 4.3.1).

Q9: Line 370: Zhang et al did not grow E. coli on succinate in this study (not listed among the cultures in their Table 1, no data shown for this species/substrate pair)

R9: Thank you for this correction. *C. oxalaticus* was not grown on succinate, it was and it was corrected.

Line 370: "… reported for *C. oxalaticus* when grown on succinate…"

Q10: Conclusions don't say anything about H isotope fractionation during lipid synthesis, even though you have discussed this some throughout the manuscript. I realize this wasn't an original focus of the manuscript, but I think you could make this thread clearer through the intro/results/discussion/conclusion and have some very useful results in this regard.

R10: We have added text to Discussion (Lines 429-439) and Conclusion sections (Line 478-479) to interpret the $\varepsilon_{18:2/H2O}$ values, which are now reported in the Results of the revised manuscript as suggested by this reviewer (see comment above).

Minor comments and typos:

Q11: Line 86: I would use 2H instead of D to be consistent with the rest of the manuscript

R11: Line 86: … and deuterated water (^2H_2O).

Q12: Line 121: delete "a" before "12 mL"

R12: …platinum catalyst to 12 mL exetainer vials…

Q13: Figure 4: The alphas in the figure legends should be changed to "a"

R13: The legends in the figure 4 were changed to "a"

References Cited

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Zhang, X., Gillespie, A. L., & Sessions, A. L. Large D/H variations in bacterial lipids reflect central metabolic pathways. Proceedings of the National Academy of Sciences, 106(31), 12580-12586. doi:10.1073/pnas.0903030106, 2009.