Author responses to Reviewer 1 – please find our responses in the blue font below each reviewer comment below.

In this work, Jabinski and colleagues quantify the water assimilation factors and heterotrophic incorporation of inorganic carbon into biomass, using fungal cultures. This "dual-SIP" approach is useful for calibrating environmental SIP approaches. I have no major comments on this work and recommend it for publication. I have detailed some minor comments below that should be addressed prior to publication.

Thank you for your attention and recommendations to improve the manuscript. We have implemented all suggestions in the revised version of the manuscript.

C1: Section 3.2.2 (and throughout): the water assimilation factor should be noted as a_w, not alpha_w, to avoid confusion with alpha fractionation factor notation.

R1: Thank you for the comment clarifying the text; this is an important issue also raised by the second reviewer. As suggested, we changed the water assimilation factor from alpha to a_W from Section 3.2.2 and throughout the manuscript.

C2: Figure 3: the units of the x and y axes should be cleaned up. For these values I would recommend ppm.

R2: Thank you for the suggestion. We have changed the units to ppm for Figure 3 (Fig. 4 in revised manuscript).

C3: Line 58: Please add the following references here:

Warren 2022 "D2O labelling reveals synthesis of small, water-soluble metabolites in soil."

Caro et al. 2023 "Hydrogen stable isotope probing of lipids demonstrates ..."

Canarini et al. 2024 "Soil fungi remain active and invest in storage compounds during drought independent of future climate conditions"

R3: Thank you for the suggested references, they were added to the corresponding line "a useful tracer of microbial activity in a diverse range of environments (Canarini et al., 2024; Caro et al., 2023; Fischer et al., 2013; Kellermann et al., 2012; Wegener et al., 2016; Warren 2022; Wu et al., 2018)." These references were also added to the revised list of references; Caro et al. was already in the reference list:

Canarini, A., Fuchslueger, L., Schnecker, J., Metze, D., Nelson, D. B., Kahmen, A., ... & Richter, A. (2024). Soil fungi remain active and invest in storage compounds during drought independent of future climate conditions. *Nature Communications*, *15*(1), 10410.

Warren, C. R. (2022). D2O labelling reveals synthesis of small, water-soluble metabolites in soil. *Soil Biology and Biochemistry*, *165*, 108543.

C4: Line 180: Make sure to subscript CO_2.

R4: The error was corrected as suggested.

C5: Line 287: An a_w of 1.2 is nonsensical, no? I was curious and looked at the figure I believe you are referencing, and the histogram bins only go to 1.0. It would be more accurate here to say something along the lines of "A_w can theoretically vary between 0 and 1, representing conditions where an organism acquires none or all of its lipid H from water. The consensus from previous studies is that microbial heterotrophs exhibit a_w values typically around 0.71 +/- 0.17."

R5: Briefly, we agree that it's a very rare occurrence that a_W would exceed value of 1, and have modified the text to cite the typical range. However, there are examples of the literature where a_W was reported to exceed 1 (see below), and we have noted this in the text as well (Line 304).

Regarding theory, although water H assimilation efficiency (a_W) can be conceptualized as the proportion of lipid-H derived from water-H (a value ranging from 0 to 1), the term is more complex. In principle, it is not impossible for a_W to exceed 1, as the term integrates several isotope effects during the incorporation of water-H into lipid-H. In any of these steps, the intermediate product may become enriched in the heavy isotope relative to the substrate (inverse isotope effect; e.g., enrichment in 2H of TCA intermediates, [Wijkers et al. 2019; www.pnas.org/cgi/doi/10.1073/pnas.1818372116]; or NADPH residence time, [Torres-Romero et al., 2024, https://doi.org/10.1073/pnas.2318570121]). This signal could be perpetuated into lipid d^2H signals, depending also on the proportion of lipid-H that is derived from NADPH, for example. Post-synthesis modification of fatty acids provides another node for ^2H enrichment (e.g., Chikaraishi et al., 2004 (Phytochemistry 65, 2293-2300), where some fatty acids may serve as the substrate in desaturation reactions. An a_W of higher than 1 is rare and has not yet been reported before for bacterial or archaeal organisms, but was already previously reported for eukaryotic organisms such as a ciliate called *T. thermophila*, where the fatty acids as well as another biomolecule demonstrate a_W values exciding 1 [Dirghangi, S. S., & Pagani, M. (2013). Hydrogen isotope fractionation during lipid biosynthesis by Tetrahymena thermophila. *Organic geochemistry*, *64*, 105-111]. A higher a_W value was also reported for fungi [Jabinski, S., d. M. Rangel, W., Kopáček, M., Jílková, V., Jansa, J., & Meador, T. B. (2024). Constraining activity and growth substrate of fungal decomposers via assimilation patterns of inorganic carbon and water into lipid biomarkers. Applied and Environmental Microbiology, 90(4), e02065-23].

C6: Fig. 5: It is unusual to have a figure included in the conclusion section. I would recommend switching the position of this figure to the main text. Furthermore, it is currently unclear what this figure adds to the manuscript. The color and shape scheme is very difficult to parse and should be cleaned up, or the figure should be removed.

R6: The figure has been moved to a new Discussion section in the revised manuscript, section 4.4 Dual-SIP. The shape extends beyond the points displayed as it incorporates the error of the measurements; this information has been added to the figure caption. Text has been added to the figure to clarify the shapes corresponding to each substrate. The color scheme was chosen based on journal requirements for color vision difficiencies, and applies distiguishablecolors feature of Matlab.

Author responses to Reviewer 2 - please find our responses in the blue font below each reviewer comment below.

Jabinski et al. cultured fungi from several species with 2H-labeled water and 13C-labeled DIC. The fungi took up very little inorganic carbon, indicating that they were primarily heterotrophs and ate the unlabeled organic carbon provided in the cultures. Apparent $^{2}H/^{1}H$ fractionation factors varied among species and substrates, but I don't think the alpha values reported for these were calculated correctly (although what the authors have done is consistent with some other publications, I don't think it is appropriate when the product and the substrate are separated by multiple ^{2}H-fractionating reactions, as explained below). The discussion was also a bit shallow in terms of integrating these new results to other publications that have investigated the relationship between metabolism and $^{2}H/^{1}H$ fractionation. The emphasis is more on the utility of the dual-SIP approach, and it seems like there is a bit of a missed opportunity to discuss how these results can inform our understanding of fungal metabolism. I think the data are unique and I appreciate that the authors have put in a large amount of work to generate them. Very little is known about 2H/1H fractionation by fungi, and this has the potential to be a useful tool to understand fungal metabolism in soils. I think with some rewriting, this manuscript will be a helpful contribution to this field.

Best wishes,

Nemiah Ladd

We thank the reviewer for her attention and comments to improve our manuscript. We have addressed the discrepancy between the terms a_W and the terms $\alpha_{L/W}$ and $eps_{L/W}$ that are traditionally used to describe kinetic and equilibrium isotope effects, all of which are reported in the revised manuscript and a figure was added to display corresponding eps values (Fig. 5). We expected the fungi cultured for this study to grow heterotrophically and hypothesized that heterotrophic inorganic C uptake (e.g., via anaplerotic reactions rather than explicit autotrophy) would vary depending on the organic C substrate provided for growth; this was not observed however. The revised manuscript further clarifies this hypothesis and finding and provides recommendations for future applications. Additional text and figures take the opportunity to further explore the relationship between $^{2/1}H$ fractionation and fungal metabolism and CUE, in the context of what is known for bacterial heterotrophs.

Specific comments:

C1: Line 27: this fractionation factor is actually much lower than the values typically seen for bacterial heterotrophs, and I think is due to the way you have calculated alpha (see comment below)

R1: Thanks for the comment and highlighting this confusion. The water assimilation factor a_W is not the alpha normally reported for isotope effects, but the net water-H incorporation efficiency, which includes a mass balance of water-H versus other H sources as well as all the different isotope effects happening between the water-H incorporation into fatty acids during lipid biosynthesis. It can be considered as $a_W = x_W * \alpha_{fa/w}$; with x_W being the mole fraction of water derived hydrogen and $\alpha_{fa/w}$ being the traditional isotope effect describing net hydrogen isotope fraction during lipid biosynthesis (Kopf et al., 2015). This equation now appears in the Methods section of the revised manuscript. In the current study, a_W was determined according to Kopf et al., 2015, by culturing fungi in media having four different water isotopic compositions. This allowed us to determine a_W values for the biomarker from the linear regression of $^{2}F_{biomarker}$ versus $^{2}F_{water}$. As mentioned in the Methods Section

"The 'net' contribution of water hydrogen to lipid H is reported as the water hydrogen assimilation factor a_W (Kopf et al., 2015), and was estimated based on the slope of the linear regression line between H isotopic composition of lipid versus growth medium water (Fig. 3)". We discuss this topic again in a comment below.

C2: Lines 107, 117, 128, 139 "analytical error": specify if you mean accuracy (measured offset from known value) or "precision" (standard deviation or standard error of replicate standard measurements)

R2: Thank you for the comment, we mean the precision of our measurements and we have changed the term analytical error to precision.

Line 107 "The analytical precision was below 1‰."

Line 117 "Analytical precision of δ^2H was <1.5‰."

Line 128 "The analytical precision was around 1‰."

Line 139 "The analytical precision was <0.04‰."

C3: Line 166: would be good to specify both precision and accuracy for these measurements. Were samples measured more than once (in duplicate or triplicate)? If so, can you report average standard deviation of replicate sample measurements?

R3: The error reported here is the precision calculated from replicate measurements of the international reference standards USGS 70 and USGS 72 using the same sample introduction technique. Each fungal biomass sample was measured once, but replication was achieved in the form of replicate cultivation experiments (at least two natural and two with ^{2}H-enriched medium). With regard to accuracy, we have normalized the data according to the responses of USGS international standards; we note however that even if there is an offset in accuracy, this should apply to all samples such that the slope (a_W value) will not be affected by consistent inaccuracies.

C4: Section 3.2.2, Figure 3: I've seen several instances where ^{2}H/^{1}H fractionation factors between lipids and water are calculated from the slope in this way, but I don't think it works to calculate alpha from the slope if there is more than one reaction separating the product from the substrate, as is the case for fatty acids synthesized from water. If there was a single fractionating step, the alpha value calculated from the slope would correspond to the epsilon value calculated from the y-intercept. This doesn't seem to be the case for your data. See Sessions, A.L., Hayes, J.M., 2005. Calculation of hydrogen isotopic fractionations in biogeochemical systems. Geochim. Cosmochim. Acta 69, 593–597. It would be better to calculate alpha from each lipid-water pair and report average apparent fractionation factors per species. At a minimum, you should provide the full equations for each linear regression that is shown, including the y-intercept and not just the slope. There is a statement that the data set will be published, but it is not available for reviewers to see, so I'm not able to check this myself.

R4: We believe that there is confusion among the terms a_W defined by Kopf et al. (2015) and the isotope effect described by alpha, which is an alternative notation of the epsilon value [eps = (1-alpha)*1000]; this was also highlighted by Reviewer 1. Over the last decades, researchers have reported the isotope effect between water-H and lipid-H ($eps_{L/W}$) as the combined isotope effects of

the many enzymes and equilibration reactions that ultimately determine δ^2H-lipid during biosynthesis and subsequent modifications (c.f. Sachse et al., 2012), with the understanding that there is no single fractionating step for the incorporation of water H into lipids during biosynthesis; these are now reported in the revised manucript. The term a_W is similar, but represents the product of the traditional alpha and the proportion of lipid-H that is derived from environmental water (Kopf et al. 2015). The latter is not described by the traditional isotope effect alone, at least in theory, as presented in the equations in Sessions and Hayes (2005). a_W is not inherently connected to the $eps_{L/W}$ value via a reversal of the equation noted above, nor does the y-intercept of the regressions of $^{2/1}H_{water}$ vs $^{2/1}H_{lipid}$ represent the $eps_{L/W}$ value; it would also be influenced by $^{2/1}$H of other H substrates, for example. We have added the equations described by Kopf et al. (2015) and Hayes (2004) to distinguish these terms in the revised Methods section (2.3).

The revised manuscript includes the full regression equation in the figure. The delta values and regression data are now available as a SM table. We have added a figure displaying the corresponding eps values (Fig. 5).

C5: Section 4.1, Figure 4: shouldn't the CUE results be presented already in the results section, not in the discussion as they are here?

R5: The figure and the CUE results are condensed from the results presented in the results section (Fig. 1) and were introduced in the discussion section as supportive evidence. The revised manuscript now presents the CUE values and this figure (now Fig. 2) in the Results section, and further explores these data in the discussion section.

C6: Line 282: see also a recent paper about H isotope fractionation by yeast by Ashley Maloney et al. (PNAS, 2024), which is relevant here since it is one of the few studies to look at H isotope fractionation in fungi

R6: Thank you for bringing this paper to our attention. We included the reference "… H incorporation is suggested to be a function of transporters and electron acceptors (NADPH and NADH), with contributions accounting for around half of all lipid hydrogen (Maloney et al., 2024)."
It has also been added the reference to the reference list:
"Maloney, A. E., Kopf, S. H., Zhang, Z., McFarlin, J., Nelson, D. B., Masterson, A. L., & Zhang, X. (2024). Large enrichments in fatty acid 2H/1H ratios distinguish respiration from aerobic fermentation in yeast Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences*, *121*(20), e2310771121."

C7: Figure 1, subsequent figures: It would be nice if all six species were indicated in the legend, rather than the three genera. It is a little confusing how half of the colors in the figure are not shown in the legend, and you have to read the caption to make sense of them. Then for the other figures that use the same color scheme, you always have to refer back to the figure 1 caption to figure out what they mean.

R7: We have added the abbreviation for each species in corresponding color to Fig 1,2, 4, and 5. Fig. 3 already contains a lot of text in the figure panel, where a_W values are reported; therefore, the species description appears only in the figure caption.

Technical corrections:

There are several places where articles (e.g., the) are missing or where they are used when they should not be. I've noted some examples of this below, but I suggest that someone read through the manuscript carefully and correct this throughout.

C8: There are also many cases were subscripts or superscripts are missing (e.g., CO2, 13C)

R8: subscripts and superscripts were checked throughout the manuscript.

C9: Line 39: Add "the" before "atmosphere"

R9: The text has been revised as suggested by the reviewer.

C10: Line 53: Awkward phrasing, I suggest editing to "now allow microbial taxa to be linked to specific processes..."

R10: The text has been revised as suggested by the reviewer.

C11: Line 10: missing articles (need "a" before "standard" and "laboratory"

R11: The text has been revised as suggested by the reviewer.

C12: Line 108: Here, I think you don't need "the"

R12: The text has been revised as suggested by the reviewer.

C13: Line 151: Add "The" before "transfer"; ion source should not be capitalized

R13: The text has been revised as suggested by the reviewer.

C14: Lines 201-208: I would move the equations and the descriptions of them to the methods

R 14: The Lines 201 – 208 were moved into the method section (Lines ~167 – 177)

C15: Line 276: I think you mean to refer to figure 4 here?

R15: Thank you for the correction; yes, the former figure 4 is referred to here. This is now Figure 2 in the revised manuscript.