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Interactive comment on "Impact of metabolic pathways and salinity on the hydrogen isotope ratios of haptophyte lipids" *by* Gabriella M. Weiss et al.

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Anonymous Referee #2

We would like to thank anonymous referee 2 for their feedback on our manuscript. We propose to fix the grammatical errors in a revised version of the manuscript, and address the more major comments following the word 'RESPONSE' below the original comment.

This paper combined hydrogen isotope salinity data from alkenones (previously published) with hydrogen isotope salinity data from additional lipids (some previously pub-

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lished), also new temperature and nitrogen culture data for alkenones (C37). The data suggest that increased temperature may cause C37 2H-enrichment, confirms that higher growth rates (achieved through different media N levels) leads to increased C37 fractionation, and confirms that other lipid classes (not just C37) in 2 haptophyte groups also become 2H-enriched at higher salinity (but not phytol in 2 species). I especially appreciate the measurement of several different lipid classes. Alkenones have been the sole objective of many previous studies – but ignoring the other lipid classes restricts the potential for understanding the fractionation mechanisms (in haptophytes and other species). The isotopic responses of non-alkenone lipids to environmental variations in culture are inherently fascinating in their own right and add valuable insight into the innerworkings of microbes and their isotopes. Please, tell all your friends, measure the other lipids too – it is worth the instrument time. With that said, it would be great if the nutrient and temperature part could include other lipids besides just C37.

Despite the potential of the paper and the quality of the data, the flow of the paper is currently difficult to follow, and the discussion arguments seem like they are not fully thought out. I offer specific comments below that should hopefully help improve the manuscript, but suggest a major re-working of the structure and perhaps framing of the manuscript. I don't see why the authors want to combine the new temp/nutrient experiments with salinity data (maybe they are not enough for a stand-alone manuscript?) but as is, these aspects don't do a good job supporting one another in a comprehensible story. They seem disjointed and unrelated. One suggestion is to tell the reader why these two findings are combined in a single manuscript – how do they support each other, and what new insight can be gained from putting them both here? If it just doesn't work – maybe they should be separate. Finally, since the time this paper was submitted, a new D/H NADPH paper has been published. It might help streamline or motivate the discussion: www.pnas.org/cgi/doi/10.1073/pnas.1818372116

RESPONSE: Indeed, the new paper from Wijker et al. (2019) would be useful to add. They showed that different pathways greatly controlled isotope ratios of NADPH and

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therefore lipid 2H ratios, which is similar to what we show here. Genetically, T. lutea is a Group II species, and we wanted to determine whether the 2H ratios were also characteristic of Group II or perhaps showed a more marine 2H signal. We agree that other lipids would add to the interpretation, but at this moment, we do not have that data. The offset between species is important and can potentially help elucidate the salinity mechanism because different pathways might be preferred under salt stress. We will make this clearer in a revised version.

Title - “metabolic pathways” should be replaced with “lipid biosynthesis pathways” or at least “lipid metabolism” because there are so many things associated with metabolism (but not directly related to lipid biosynthesis) that could potentially impact lipid isotope ratios (or not affect them at all). As it stands, your title doesn’t capture the added contribution of various lipid classes that this paper has to offer, it would be great if it could. Additionally, why ignore the temp and nutrient data in the title?

RESPONSE: Yes, that is a good point. We propose a new title: Impact of lipid biosynthesis pathways and growth parameters on hydrogen isotope ratios of haptophyte lipids.

Abstract - Line 27: Again, the word metabolism is too vague here. I think “location of lipid synthesis” would be more specific and thus more helpful for readers to follow your meaning. While the abstract successfully and clearly explains the results, it ends abruptly and the opportunity to add the “so what” part to your paper is lost. Are you excited about knowing a little bit more about the mechanism? Is it important that not all lipids respond equally to salinity. . ..what are the implications to the biogeosciences? I would pick a motivating point and wrap up the abstract with something that will make the reader want to read more.

RESPONSE: The important role of specific biosynthetic pathways as a determiner of species-related differences in 2H fractionation is exciting. Understanding these metabolic fluxes and sources of NADPH for specific lipids is therefore very important.

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Recent work pointed out above (Wijker et al., 2019) has highlighted the importance of metabolic pathways, specifically for NADPH, on d2H, and our study also suggests that biosynthetic differences in lipid synthesis are important for understanding 2H ratios. We will add a final sentence to the abstract that states: “These findings suggest that not all lipids retain a correlation with salinity, and this appears to be governed by differences in biosynthetic pathways and cellular compartments. Use of lipids relying on a cytosolic connection are more appropriate for applications of 2H ratios to reconstruct salinity in the geologic record.”

Introduction - Line 12: Sorry if I am wrong about this, but would be worth checking if C. tobin is in Group 1. DOI:10.1371/journal.pgen.1005469

RESPONSE: To our knowledge, C. tobin does not synthesize alkenones.

Page 2 Line 15: Sachs and Kawka 2015 is not an appropriate reference here as they don’t experiment with salinity. Since you are including field studies (sachse et al. 2012) you might also mention studies that came out after 2012 (ie http://dx.doi.org/10.1016/j.gca.2014.03.007).

RESPONSE: Yes, you are correct. We will remove the Sachs and Kawka, 2015 reference and add newer references.

Methods - It isn’t mentioned anywhere that fatty acids were corrected for added H from methylation or sterol/phytol corrected for acetylation. I am assuming this was done? If it wasn’t, please do so and update data/tables/graphs as necessary.

RESPONSE: Yes, fatty acids, sterol and phytol were all corrected for methylation / acetylation. We will clarify this in the methods section.

Page 3 Line 21: Why are you calling the sterol/phytol fraction the polar fraction?

RESPONSE: We separated the TLE into three fractions which we refer to as apolar, ketone and polar fractions.

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Page 4 Line 6: Were the fatty acids extracted from the other half of the TLE? This is unclear

RESPONSE: Yes, our traditional separation method using aluminum oxide removes fatty acids, so we separated the TLE into two aliquots before this step. One was used for fatty acids and the other for the alkenones, sterol and phytol.

Page 4 Line 9: Please provide the nutrient recipe(s) Page 5 Line 10-11. H2 gas was only used to monitor machine accuracy? H2 gas at beginning and end of sequence needs to be used to tie the Isodat software calculations as well, how else are you getting Isodat to correct?

RESPONSE: H2 gas was used for Isodat calculations. We will clarify this.

Results - It isn't clear until the Results section that all of the C37 data built up in the introduction is actually from other studies. Maybe earlier you can clarify what exactly you are adding to previous C37 data. The supplement table really helps to do this, perhaps is should be in the main part of the paper.

RESPONSE: We will make it more apparent in the introduction that the alkenone and I. galbana fatty acid data is already published, and move the supplementary table into the main manuscript.

Page 5 Line 26: since you used artificial seawater, can't you just measure your lab's water and estimate alpha with some reasonably big error bars - if you don't know the month it was collected, analyze samples from each month

RESPONSE: since we used extracts from an experiment conducted a few years ago in a different lab, measuring the lab water to calculate alpha for this experiment would be difficult

Page 6 Line 14: by "nutrients" don't you just mean "the effect of nitrogen limitation"? Please use more specific language

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RESPONSE: Yes, we will change this.

Page 7 Line 4. I think this is supposed to be section 3.2 (not 3.1)

RESPONSE: Yes, thank you for catching this mistake.

Discussion - 4.1 – There are 3 issues. Firstly, it was claimed that this temperature part was of secondary interest earlier in the paper, and yet it is the leading discussion point. Either move this down or change the framing of the paper. Secondly, a tremendous amount of text was devoted to invoking abundance shifts in alkenone type to explain the temp trend but no graph (either data or schematic) is offered to support this interpretation. (Along those lines, it is always interesting to show how UK37 does in temperature experiments, even if just supplementary. It would be worth reporting how well this strain does at reconstructing temperature when grown in controlled temperature conditions.) Thirdly, the final sentence is confusing – how is invariable alkenone concentration evidence that growth rate didn't impact 2H/1H ratios? And do you mean total alkenone concentration? B/c most of this section eludes to alkenone abundance changes. Page 7 Line 20-21. How does it compare to the other microbe temp-D/H studies? (Dirghangi and Pagani 2013 http://dx.doi.org/10.1016/j.orggeochem.2013.09.007 & http://dx.doi.org/10.1016/j.gca.2013.05.023 and Zhang et al. 2009 doi:10.1016/j.orggeochem.2008.11.002)

RESPONSE: We propose to restructure the discussion section, and will move the temperature part to later in the discussion. We focused on the temperature effect noted for haptophytes, and therefore did not include other lipids. We will add a supplementary figure showing the correlation between UK'37 and temperature for T. lutea. The final sentence should be revised to: "Total alkenone concentrations did not vary substantially over the temperature range, but faster growth was noted (measured as chlorophyll fluorescence) at higher temperatures. It is possible that growth rate had an effect on δ2HC37 ratios, but since we do not have daily cell counts, we cannot easily compare this effect with previously noted growth rate effects. In previous studies, faster growth

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resulted in isotopic depletion, but we note isotopic enrichment for the higher temperatures and faster growing cultures. This isotopic enrichment could be governed by different isoenzymes, which can be associated with different fractionation, operating at higher or lower temperatures, as suggested by Zhang et al. (2009) and Jahnke et al. (1999)."

Page 7 Line 23: Please report somewhere the entire significant positive correlation (with slope, intercept, and their standard errors) for this and other relationships reported in this paper. Maybe just a table or on the graph would be fine if it fits.

RESPONSE: We were discussing the correlation between the alkenone 2H and temperature, not the linear regression equation. We can add the linear regression equation to the graph.

4.2 – Line 25 a reference is missing here (Sachs and Kawka 2015) Same comment about section 4.1 apply regarding the framing of the paper. Both sections never really get around to the "so what" part and neither does the conclusion. Please, tell us what is the purpose of these sections – how do they add to the story and why are they important? It would make a little more sense if section 4.1 and 4.2 also included non alkenone data, but as is they really stick out. 4.3 – if you really want this to be the main point of your paper, you should address it first in your discussion

RESPONSE: We agree that the discussion of the temperature and nutrient experiments should be moved to the end of the discussion. Since we discuss how the effect of 2H fractionation is different lipids for different haptophyte species, we felt it was valuable to include the alkenone data from T. lutea here since there has been no previous characterization of d2H ratios for this alkenone-producing strain. We agree that measurement of other lipids from this strain would be interesting and useful, but at this moment, we do not have that data. Understanding the effect of different biosynthetic pathways on the 2H-salinity relationship is not only interesting, but is important for paleo applications because one major issue for the alkenone paleosalinity proxy is our

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lack of understanding of the salinity effect on 2H ratios. Additionally, the fact that this salinity signal is present for other lipids is intriguing and may potentially shed light on how this relationship with salinity actually works. Of course, a more in-depth look into these biosynthetic mechanisms would be better, but some insight can still be gained from our approach here. In sediment samples from the geologic record, an integrated signal incorporating effects from a number of variables in addition to salinity (growth related effects as a result of temperature and nutrient concentrations, light intensity, etc.) and it is important to constrain the impact of these variables on d2H ratios as well, which is why we decided to include T. lutea data in this manuscript. We will add a better comparison of our temperature dataset with previously published data.

Line 11 – "in" not "Impact of salinity "of" haptophyte lipids" 4.3.1 - Page 9 Line 21 – somewhere around here would be a good place to compare the lack of C16:0 EHUX correlation with the strong relationship found in Sachs et al. 2016

RESPONSE: We will add this.

Page 10 Line 1 – "values" is misspelled Page 10 – Lines 12-14. This is extremely misleading. Plenty of pyruvate is also made in the chloroplast (as the paper mentions later on). Furthermore, Acetyl-CoA is not known to pass organelle walls according to several plant biochem text books. DeNiro and Epstein is not an appropriate reference for this – instead you should check Lohr et al. 2012 (10.1016/j.plantsci.2011.07.018) and Hemmerlin et al. 2012 (10.1016/j.plipres.2011.12.001) even though they focus on sterols, it is clear that pyruvate can be made in the cholorplast. Certainly under some conditions algal pyruvate seems to be imported into the chloroplast (DOI:10.1371/journal.pgen.1006490) but it is incorrect to leave your statement as is.

RESPONSE: Thank you for these references. Pyruvate can be imported and exported from the chloroplast, and therefore used in both compartments for formation of acetyl-CoA. We are not suggesting that acetyl-CoA is passing organelle walls. Our thinking here was that if pyruvate originally formed in the cytosol entered the chloroplast and

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Discussion paper

was used in fatty acid synthesis, this might explain why some fatty acids show a correlation to salinity despite being (mostly) synthesized in the chloroplast. We will rephrase this sentence.

Page 11 Line 7. Incorrect information, actually the diatom sterol was highly affected by light intensity, strikingly in the opposite manner as phytol and the C14:0 fatty acid. This mistake, and the interpretation that depends on it needs to be fixed.

RESPONSE: Yes, thank you for catching this. We will revise this discussion to address this.

4.4 - This section would greatly benefit from some rearrangement and reworking to help the reader. It is difficult to follow. One way to improve this is add a brief outline of the points you want to make in the first paragraph before hitting on all of them. A schematic would also help. Are you suggesting anything new here or just reporting all the previously suggested hypotheses? There is no need to devote so much text to explaining these previous hypotheses, a short summary sentence for each should do. Isn't there something more unique you can add now that you have this extra data from the other lipids? Isn't it significant that several studies now have seen only a weak (or no) relationship with phytol? One of the main issues with the NADPH (OPP vs PS1) hypothesis is that NADPH isn't known to cross organelle walls. Is there an OPP pathway inside the chloroplast in haptophytes? If you want to rely so heavily on this explanation, some evidence (in the form of a citation) for 1) NADPH crossing the membrane or 2) OPP in the chloroplast is really needed here.

RESPONSE: Cormier et al. (2018) showed the OPP in both the chloroplast and cytosol (Fig. 4), and Sachs et al. (2016) discuss presence of OPP in the cytosol. It is well known for diatoms that OPP is located in the cytosol and the same is thought to be the case for haptophytes. We think our hypothesis is still sound with respect to OPP vs PS1. We think it is significant that phytol generally has a weak correlation with salinity. This is what led us to the separation of lipid synthesis compartments with respect

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to the salinity correlation. Phytol is different than these other lipid biomarkers being assessed because it is entirely synthesized in the chloroplast and is a component of chlorophyll, and strongly correlated to light intensity (Sachs et al., 2017). We agree that numbering the hypotheses for salinity mechanisms would be beneficial and help make the discussion more succinct. We see why you might be confused about the OPP vs PS1 hypothesis, and we are not proposing the NADPH would cross organelle walls. What we propose is that under certain conditions, OPP or PS1 might be more or less active. If isoprenoid precursors are synthesized by either MVA or MEP, these pathways are located in separate compartments and would derive NADPH from either OPP or PS1 respectively, and thus lipids would be influenced by this difference in activity of OPP or PS1.

FIGURES - While the figures indicate in the caption where previous data is coming from, it would be helpful if this info was more visually accessible in the key, either next to species names if no regression is given (Fig 3 and 4), or, next to regressions that should be provided (full equations) (Fig 4). Some figures have regression lines some don't. Is there a purpose to this? Fig. 4 - If a relationship isn't significant (phytol) don't add a regression line. . .or do something like make regression lines for significant regressions solid lines and not signification regressions dotted. C16:0 symbol colors and shape are too similar to phytol's.

RESPONSE: We did not include regression lines for Figure 2 because of the low number of data points. We will add regression lines to Figure 3 and take your suggestion to make different regression lines when the relationship is significant. We will add publication data into the figure.

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