

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

Gabriella M. Weiss et al.

gabriella.weiss@nioz.nl

Received and published: 2 July 2019

Anonymous Referee #3

We would like to thank anonymous referee 3 for their feedback on our manuscript. We address their comments following the word ‘RESPONSE’ below the original comment.

The manuscript by Weiss et al. set up quite ambitious goals to address almost all factors affecting D/H fractionation in haptophyte lipids. For that purpose the authors included quite a bit previous published data. However, they were not mentioned until Section 3, Results. Through the Introduction and Method, as well as in figures, one

C1

can hardly tell what are new and what were previously published. In addition, the new and old dataset seem disjointed each other and it seemed to me that such a way of merging data helped to demonstrate a comprehensible story. Though the data presented are interesting and potentially valuable, the manuscript as written suffered many fatal deficiencies. It is very difficult to follow the flow of the manuscript. The arguments in the Discussion section were not well organized and demonstrated. I would suggest resubmission after a complete overhaul.

RESPONSE: We will specify the different experiments in the introduction, and make sure to clarify when we discuss our new results and already published results.

It is a big headache to follow the Method section. I had to list the details of all different cultures on a piece of paper to sort out all different parameters. They were in such a mess: different media, seawater (artificial seawater and filtered seawater), growth temperature (E. huxleyi, E. galbana and R. lamellosa group set up at 15°C, T. lutea strain CCAP 927/14 culture at 20-23°C, and T. lutea strain CCAP 463 and NIES-2590 cultures at 10-35°C), light intensity (60, 100, and 180-220 μmol photons m⁻² s⁻¹), growth phases for collection (linear, exponentially, and stationary), measurements of growth rate (chlorophyll fluorescence, and daily cell counts), and even GC columns (leading to integrated C37 peaks or individual C37:2/C37:3 peaks). . .It would be hard to imagine if anyone else could come up with a more complicated and confusing experiment design than this one. Such awful setup simply made it hard to isolate one single variable and the arguments based on such data less convincing.

RESPONSE: The reason it seems complicated is because these were multiple, separate batch culture experiments conducted at different times. It was not one big experiment. There were five experiments in total. Three separate experiments of E. huxleyi, R. lamellosa and I. galbana respectively grown over a range of salinities, one batch culture of T. lutea grown over a range of temperatures, and one T. lutea batch at two different nutrient concentrations. We will revise the methods section to make it clear that the experiments were separate.

C2

I was curious why the authors did not give any description of methylation of fatty acids or acetylation of sterols, as they are essential to figure out how reliable their reported dD data of fatty acids and sterols. Neither did they present a GC-IRMS trace to demonstrate how well peaks were separated, as sterols often co-eluted. Those are essential to evaluate the data quality.

RESPONSE: Fatty acids, sterol and phytol were all corrected for methylation / acetylation. We will add this information to section 2.2: Fatty acids were derivatized by methylation as described by Heinzelmann et al. (2015), and corrected for the addition of methyl hydrogen ($d_2HME = -171 \pm 1 \text{ ‰}$). The fraction containing brassicasterol and phytol was acetylated using acetic anhydride following Das and Chakraborty (2011), and d_2H of both lipids were corrected for addition of hydrogens (d_2H of acetic anhydride = -126 ‰).

Another fundamental flaw was that not even a single growth curve was presented, given the fact harvest was taken in different growth phases, and temperature would impact the growth rate. In particular, the authors wanted to address the effect of nutrient replete (NR) and nutrient limited (NL) on lipid D/H fractionation. For this purpose, it would be essential to know how growth rate changed daily. When the authors stated NR or NL, only nitrate concentrations were given, but no phosphorous concentrations—this set of experiments were performed using filtered seawater but no information was available regarding N/P ratio in NR and NL cultures. Table 2 did not give the date for the presented division rate, Day 4 or D10 in fact the selection of the date seemed randomly. Indeed no one knows what happened between Day 1 to Day 4, or between Day 4 and Day 10, as far as the status of culture is concerned. As a result we actually don't know when nutrient availability BEGAN to limit the growth rate! Presumably at the given light intensity and initial nitrate concentrations, there shall be no limit on growth rate solely by nitrogen availability at the onset. Then it would be essential to know when the rates in NR and NL cultures began to differ and what could cause the difference. These were batch cultures, not chemostatic cultures (Zhang et al., 2009; Organic Geochem-

C3

istry). As a result, demonstration of growth rate during the log phase truly limited by nitrate availability would be the key. Without growth curves, one was not in a position to address the effect of growth rate. In fact, it would ideal for the authors to give the concentration of individual biomarkers per cell as we can tell if there are any strategic allocation of carbon source or energy during the different growth phases

RESPONSE: We will include growth curves in a revised version. See Figures 1 and 2 below. It is true, growth was not limited by N availability at the onset. Growth rates for the two nutrient experiments started to differ on the second day of growth, as you can see in the figure 2 below. We will provide concentration data for the alkenones from *T. lutea*, but not for the other lipids.

The title set up two goals to address: 1) impact of metabolic pathways, and 2) salinity on the on the hydrogen isotope ratios of haptophyte lipids. However, the Discussion initiated with temperature effect, followed by nutrient effect. The title seemed misleading. I would hesitate to call them nutrient replete (NR) and nutrient-limited (NL) conditions as they merely differed in nitrate concentration by 0.6 mM, and growth rate by barely half. In Zhang et al. (2009, Organic Geochemistry, doi:10.1016/j.orggeochem.2008.11.002), NR and NL chemostatic cultures differed in nitrate concentrations by almost 70 times and cell division rates by 4.5 times. Even among such huge growth rate differences, fatty acids biosynthesized by acetogenic pathway did not show the difference in D/H fractionation.

RESPONSE: We will reorganize the discussion to start with the salinity effect and discuss the *T. lutea* data at the end. For the nutrient experiment, the offsets between exponential and stationary phase are not large, around 6 ‰ which is near machine precision. However, the measured difference in d_2H_{lipid} between the cultures with different nutrient concentrations is larger, 12-13 ‰ and this offset is present for both phases of growth. As you mention, the differences in nutrient concentration are not large, but still we see a depletion at higher nutrient concentration for both growth phases, and this depletion is larger than machine error, so we think this is a real signal. While Zhang et

C4

al. (2009) did not observe a difference for fatty acids at the different nutrient concentrations, there was a difference observed for sterols, an enrichment of approximately 30 ‰ at lower nitrate concentrations, and we note the same trend for long-chain alkenones. It might be that the effects of nutrients and growth rate are also related to biosynthesis and cellular compartment.

In fact the data supporting for argument of temperature and growth rate effects seemed farfetched. Judged by Table 2, I would say the difference was rather small. If we choose dD of C37:3 as an example, the first line for growth rate at 0.14 could give -121-3‰ - 124 ‰ the last line growth rate at 0.21 gave -130+6‰ -124‰ almost the same. At least such differences were rather small. The same could be found in Table 1 regarding the temperature effect. At least for the batch culture of *T. lutea* NIES 2590 with salinity varying from 15 to 30, all dD values of C37:3 could be rounded to -140‰ within standard deviation they were simply the same. It seemed to me that those data would hardly support the argument for the significant positive correlation with temperature, though the phenomena observed differed from reported in Zhang et al. (2009, OG). Maybe D/H fractionation in alkenones much less sensitive to temperature than fatty acids?

RESPONSE: For the temperature experiment, it is true that the differences are not large between the different temperatures. However, the overall trend is enrichment, which is different from previous results. There is a strong, positive correlation between d2H and temperature ($r = 0.80$, $p < 0.001$), and the relationship is represented by the linear regression equation: $d2H = 0.7 * T - 154$ ($R^2 = 0.63$, $p < 0.001$, $n = 28$). It is definitely possible that alkenones are less sensitive to temperature than fatty acids, we are not ruling that out. We will make these statements more nuanced in the manuscript.

The data on salinity effect seemed more robust, at least for *R. lamellosa* and *I. galbana* (Fig. 1), but it is questionable to say “The $\delta^{2}H_{C37}$ ratios from *T. lutea* (temperature and nutrient experiments) fit well with values noted for other Group II species *I. galbana* and *R. lamellosa* (Fig.3).” (Page 9, Lines 6-7). Such data varied a wide range at a given

C5

salinity which could be considered a substantially large standard deviation. Again, there are too many variables influencing D/H fractionation. As a result, such data should not be plotted in Fig. 3. On the other hand the authors should provide full linear equations for *R. lamellosa* and *I. galbana* under different salinities as the relationship between slope and intercept could help reveal more information.

RESPONSE: The variation seen for the *T. lutea* data at a salinity of 30 (Figure 3a in the manuscript) is caused by the temperature experiment, so it is actually the variation in temperature that you see there. There are a lot of factors influencing fractionation, however, species and salinity seem to be the two most robust effects. There is approximately a 100 ‰ difference between alkenones from Group II and Group III species, with Group II species being more enriched. The fact that *T. lutea* data falls in the range of other Group II species is important because it adds further evidence to support the observation that Group II and III species fractionate differently. We shall add the linear regression information for *R. lamellosa* and *I. galbana* to Figure 3.

Section 4.4– Discussion This section needs an overhaul as it is very hard to follow the argument. I would strongly suggest add a schematic figure to demonstrate how biosynthetic pathways affect biomarker D/H fractionation. However, I don't think there were new discoveries here which deserves more than two full pages to elaborate already well known hypothesis. It is well known from previous algal culture experiments that different classes of biomarkers were characterized by substantially different D/H fractionation, in particular, among acetogenic, MVA and DOXP/MEP pathways. On the other hand, the current dataset could not provide sufficient evidence about the OPP pathway supplies a larger portion of NADPH for biosynthesis, as light intensities in the experiments were not low enough.

RESPONSE: Light intensity in the *E. huxleyi*, *I. galbana* and *R. lamellosa* experiments was low at 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, which we think is low enough to provide information about OPP supplying more NADPH for biosynthesis. Previous experiments and field studies have shown larger variation in fractionation at light intensities below 200

C6

(van der Meer et al., 2015; Wolhowe et al., 2015), which might be caused by larger input of OPP derived NADPH. We will make this section more concise in a revised version.

There are quite a few different families (species) of halophytes. Just wonder if alkenones might be biosynthesized in different organelle among different species. The authors cited (Rontani et al., 2006) to suggest that alkenones are synthesized from these shorter chain fatty acids by elongation and subsequent decarboxylation in the chloroplast (Page 10, Lines 10-12), but then claimed “Chain elongation leading to longchain alkenones does take place in the cytosol” (Page 14, Line 4). Previous studies did show different D/H fractionation in biomarkers biosynthesized among different organelle. Would it be possible for difference in alkenone D/H fraction among different families due to different organelle for synthesis of alkenones?

RESPONSE: Fatty acids are formed and initially elongated in the plastid. Subsequent elongation occurs in the endoplasmic reticulum and utilizes a cytosolic pool of acyl-CoA (Huerlimann and Heimann, 2013). We will rephrase these two statements to emphasize that elongation takes place in the E.R., not the chloroplast, and relies on cytosolic intermediates. We agree strongly that different organelles impart a different biosynthetic signature / effect of fractionation and that is one of the main arguments of this manuscript (see page 11 lines 15-16, final line of page 11 to lines 1-2 of page 12, page 14, lines 7 – 12). It could be that different species, or species from the different Groups II and III, use different organelles for synthesis, but instead, a more plausible explanation for the species offset might be due to their osmotic regulation capacities.

Technical corrections 1. Replace “metabolic” with “biosynthetic” as the paper only address about the biosynthesis of biomarkers.

RESPONSE: We will fix this by changing the title of our manuscript to, “Impact of lipid biosynthesis pathways and growth parameters on hydrogen isotope ratios of haptophyte lipids”.

C7

2. Page 5, lines 8 and 12: “n-alkanes”, here “n” should be italic

RESPONSE: We will fix this.

3. Page 6, Line 22 – There was no Fig. 2c

RESPONSE: We will fix the figure notations for this section. Figure 2 shows fractionation plotted against nutrient concentration (a) and growth rate (b).

Interactive comment on Biogeosciences Discuss., <https://doi.org/10.5194/bg-2019-147>, 2019.

C8

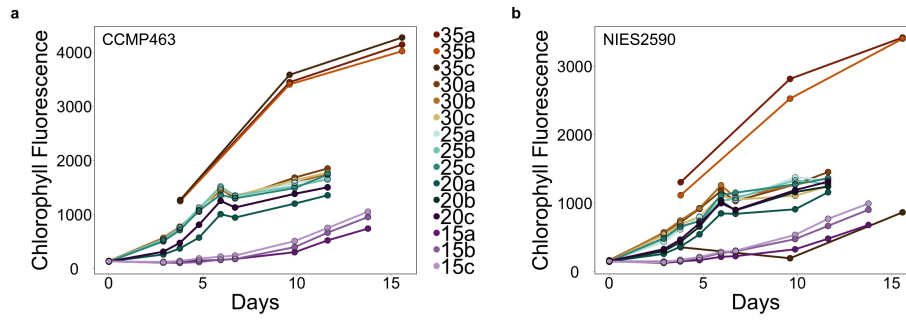


Fig. 1. Growth curves for the Temperature experiment based on chlorophyll fluorescence.

C9

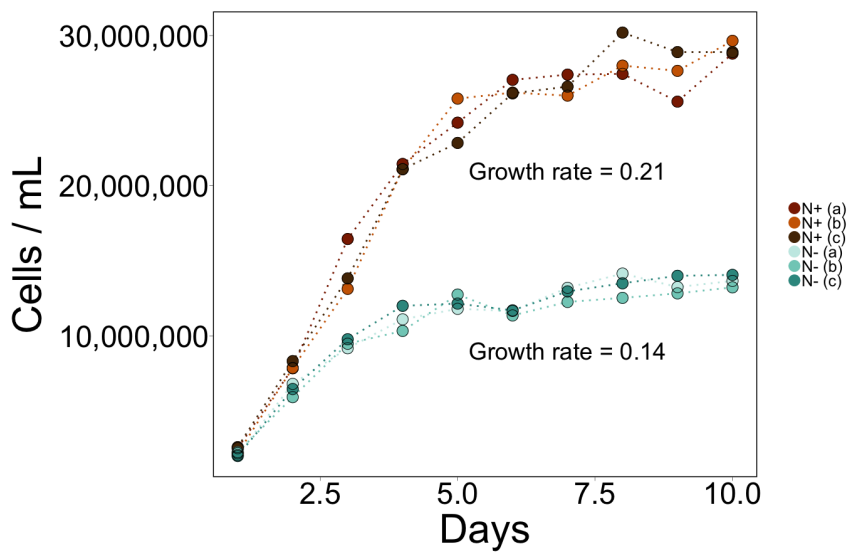


Fig. 2. Cell counts and growth rates for the nutrient experiment.

C10