Biogeosciences Discuss.,
https://doi.org/10.5194/bg-2017-311-AC1, 2017

Biogeosciences Discussions
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EGU

Interactive comment on "Effects of alkalinity and salinity at low and high light intensity on hydrogen isotope fractionation of long-chain alkenones produced by *Emiliania huxleyi*" *by* Gabriella M. Weiss et al.

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Received and published: 5 October 2017

Anonymous Referee #1 Received and published: 21 September 2017 Weiss et al. (2017) conducted two experiments using batch cultures of the haptophyte Emiliania huxleyii (strain CMP1516) to determine 1) how alkalinity (separate from salinity) might impact hydrogen isotope ratios in alkenones and 2) if high light conditions influence the previously reported salinity-fractionation relationship in alkenones. The results indicate that the alkenone hydrogen isotope salinity-fractionation relationship is robust (and similar to previously reported relationships) regardless of alkalinity and light level,

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which are useful and interesting findings. I recommend this manuscript for publication but request that the authors address the following issues: 1) more in-formation about natural light levels, 2) separate alkalinity as the sole variable as part of the analysis, 3) improve the discussion about mechanisms (if possible include additional lipid isotope and lipid concentration data to support the lack of light effect), plus a handful of technical issues listed below.

We would like to thank the anonymous referee #1 for the constructive review of our manuscript. We will address the comments below as "Response:" following the original comments.

High light growth conditions in the world's oceans: The idea that most alkenones are produced in the high light of surface waters is mentioned in several places (abstract line 5; intro p.3 line19; discussion section 4.2 line 30; conclusion line 26), but without references to support this claim. As satellite imagery clearly illustrates, major coccolithophore blooms occur in highly productive (mainly coastal) areas that are seasonally growth-limited and along the equator, and presumably bloom alkenones are produced in high light conditions near the surface (but surely also deeper in the water column where light is limited due to self-shading from bloom turbidity?). As is stands, the manuscript just states that high light alkenone production likely predominates. But to strengthen your argument and support your experimental design and data interpretation, the predominance of high light alkenone production should be illustrated with references that indicate 1) alkenones are mainly produced in high light at the surface, 2) how ocean surface water light level ranges compare to light ranges in your study, 3) how surface bloom productivity compares to non-bloom conditions/areas, and 4) that these "high light" bloom alkenones are indeed exported to the sediments (more so than "low light" alkenones). Because in vast areas of the ocean, it seems the primary source of alkenones comes from fairly deep in the water column where light levels are a fraction of surface values. For instance, this has been demonstrated at BATS see Fig 1 in Krumhardt et al. 2016 (doi:10.5194/bg-13-1163-2016), at ALOHA Table

3 Prahl et al. 2005 (doi:10.1016/j.dsr.2004.12.001) and further afield Ohkouchi et al. 1999 (DOI:ÂËŸa10.1029/1998GB900024). The vast areas of the ocean with potential deepwater/lowlight alkenone production should be addressed/acknowledged with clarification of how alkenones produced in non-saturating light conditions do or do not affect the alkenone dD paleosalinity proxy in some regions. The same references used in van der Meer (2015) (in quoted text below) are helpful, and should again be used here – and if possible, supplemented with additional references: "E. huxleyi, for instance, is thought to thrive under high light conditions, at mixed layer depths generally <30 meter (Tyrrell and Merico, 2004; Harris et al., 2005). They outcompete other algal species that suffer from photoinhibition under these conditions, a process that is apparently absent in E. huxleyi (Nanninga and Tyrrell, 1996)."

Response: Yes, we agree that this would be a useful addition, and would add that although Krumhardt et al., 2016 do indicate that haptophytes are abundant below surface water layers, they also explain that haptophyte indicative pigments were abundant in the upper 30m, especially during spring as well as the observation that during the mid-90s and over the last 6 years of the data set, that "Chl ahapto was more concentrated especially in the upper 30m of the water column", allowing us to infer that deep-dwelling haptophytes are not the most dominant. We also would like to point out that these blooms are not only occurring along the equator, but also occur in the high latitudes (Holligan et al., 1993, Global Biogeochemical Cycles 7). Furthermore, based on UK'37 core-top calibration, we can be confident that alkenones preserved in the sediments are largely reflecting surface water temperatures during the time of the year that haptophytes are known to bloom (Müller et al., 1998. Calibration of the alkenone paleotemperature index UK'37 based on core-tops from the eastern South Atlantic and the global ocean (60'N-60'S) GCA 62, 1757-1772). Additionally, ocean surface water light levels span a range from zero to over 800 PAR (Frouin and Murakami, 2007, Journal of Oceanography 63), and haptophytes are thought primarily to bloom at light intensities above 500 μmol photons m-2 s-1 (Nanninga and Tyrrell, 1996), so we feel that the light intensity used in our study accurately represents environmental condi-

tions. Most culture studies to date have been performed at light intensities much lower than 600, with Schouten et al., 2006 being one of the higher light intensity studies at 300 μmol photons m-2 s-1. So culture studies already cover the low light range. Van der Meer et al., 2015 suggested that at light intensities above 200 μmol photons m-2 s-1 α responds differently to changes in light intensity than below. This and the observation that haptophytes tend to bloom at light intensities above 500 μmol photons m-2 s-1 warrants the study of hydrogen isotope fractionation in response to salinity at high light intensity.

Alkalinity: Fig 2c clearly shows that there is not a relationship between total alkalinity and fractionation (alpha). However, the discussion of the alkalinity results in the low light treatments is not entirely satisfying (p.6 lines 3-8), and it can be confusing when plots include data with many changing variables that are known to influence the isotopic composition of lipids. It would be useful (even if just in a supplement) to break up the different environmental parameters. Looking only at the low light cultures grown at salinity _35 (12 cultures, salinity range 34.5-35.4, alkalinity range 1.39-4.58, growth rate range 0.69-0.93, alpha range 0.795-0.806) (ie, excluding the cultures that have salinities of _26, _31, _37, or _42, nearly constant alkalinity _2.4, but growth rate range of 0.65-0.93, and alpha range of 0.776-0.824) . . . It is noteworthy that for these 12 cultures there is a correlation between total alkalinity and specific growth rate (=0.05(0.01) * AT + 0.676(0.04), R2 = 0.51, p = 0.0056) in addition to a correlation between specific growth rate and fractionation (= 0.03(0.01) * + 0.776(0.01), R2 = 0.47, p= 0.0081) (figure attached as example). Although this is a very small range in growth rate (_.7 to .9) compare to a previous study of the effect of growth rate on alkenones using a combination of chemostats and continuous cultures (Sachs and Kawka, 2015), it is striking (and probably noteworthy) a) how well they are correlated and b) that it is in the opposite sense of previous 2015 study. In the end, there is still not a significant relationship between alpha and total alkalinity – could it be that the minor growth rate effect here is overwriting any alkalinity effect? It is probable that you already considered all of this and decided it didn't fit in the paper – but it would be useful for the interested

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reader to be able to reference in at least the supplement.

Response: We agree that there appears to be a correlation between specific growth rate and total alkalinity for the batch cultures grown at constant salinity over a range of alkalinity. It cannot entirely be ruled out that the minor growth rate effect could be overwriting a potential alkalinity effect, however, as the reviewer has already remarked, the growth rate-α relationship is a positive one, which is opposite to what has been described previously, meaning that the alkalinity effect would have to be negative to be counter acted by growth rate in this case. Since salinity and alkalinity are usually positively correlated, this would suggest that alkalinity would also counter act the salinity effect on α. We and Sachs et al., 2016 do not observe stronger positive correlation (steeper slope) when alkalinity is removed as a variable between α and salinity, which is what would be expected. Looking at the plots and statistical data generously provided by the reviewer, we feel that the correlations are barely statistically relevant, especially according to Johnson et al., 2013 (PNAS 110) who suggest a cut-off P-value of 0.005 for truly significant findings.

Mechanisms: It is welcome that some effort is put forth to explain the cellular mechanisms of the salinity response at both high and low light. However, this part of the discussion seems confused and needs some work by improving the organization, offering introductory or concluding summaries to help identify your main points, supporting hypotheses with literature, perhaps a schematic outlining your favorite mechanism (which one is most likely here and why?), and the issues below. A little bit of reorganization could help since it seems like the discussion tries to deal with the high light and low light salinity response in both section 4.3 and 4.4. One way to improve this is if section 4.3 can just concentrated on salinity (regardless of light), then address why a different response at high light was expected, and then finally address why there was not a strong light effect here (but more on this below). The first paragraph of section 4.3 is very long, and the main point of it is lost in the length. Maybe at the start let the reader know how many mechanisms you will cover, then at the end summarize which one

seems most likely. Can you rule any out with the results from your experiment? A lot of attention is given to metabolically reduced NADPH, such as that generated through OPP. However, there are a few things to consider. NADPH can't cross organelle membranes – so p.8 lines 17-21 doesn't make sense, unless the complete OPP pathway (and final steps to the alkenone synthesis pathway?) were present in this hypothetical closed compartment.

Response: We will reorganize sections 4.3 and 4.4 to make them clearer and more focused. We were referring to the final steps of the alkenone synthesis pathway, assuming alkenones are synthesized from fatty acids and short chain fatty acids are coming from the chloroplast and are elongated somewhere else with a potential change in NADPH source, possibly the OPP pathway. Since it has been proven difficult to measure hydrogen isotopic composition of some of the key players in biosynthesis with high enough accuracy (intracellular water, NADPH from different sources, etc.), the idea behind this discussion is to try and combine all the information we do have and try to reason what the possible mechanism behind the salinity effect might be.

Alternatively you might invoke the import of alkenone precursors (fatty acids? Pyruvate?) used to build alkenones that reflect proportional changes in photosynthetically vs metabolically reduced NADPH pools (and would have to introduce them around p.7 line18).

Response: Noted, alkenone precursors could be the reason that OPP-derived NADPH might end up in alkenones, meaning that OPP-derived NADPH might be more of an influence for alkenones than photosynthetically derived NADPH.

Lack of light effect: p.9 line3 states "we do not see a clear relationship between hydrogen isotope fractionation and light intensity." But there is no mention of this lack of relationship in the results, where should the reader go to visualize this lack of relationship? Do you mean between the 24 low light cultures compare to the 14 high light cultures in this study? There aren't really cultures in the high light with the same growth

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rate and salinity as the cultures in the low light (although 3 HL cultures at S=35.9 and 12 LL cultures at S=34.5-35.4 actually do show a (very small) significant increase in alpha at the higher light level – however, that could in theory be due to the slightly saltier HL media or the slightly lower HL growth rate rather than the higher light). More importantly, I am not convinced that there is no light effect with only 2 light levels (75 and 600), especially considering the previously observed non-linear relationship between high light in strain RCC1238 and the fact that different strains have shown different responses (van der Meer et al. 2015). Is it possible that both the 75 and 600 light levels are above the range where a response would be detected for this strain? After all, growth rate is lower in the higher light cultures. Even though (it seems) the goal of the paper is not to characterize the light effect (but to test if the salinity effect holds at high light), this part of the discussion should consider the option that if batch cultures were grown at additional light levels, then a light effect in this strain might be apparent.

Response: Based on van der Meer et al., 2015, we expected a significant decrease in fractionation between our high light experiment and those performed at low light intensities. However, the α-salinity relationship at high light intensities plots on top of all of the other experiments. While we do understand that our results cannot necessarily be extrapolated to all strains, we argue that a statistically similar α-salinity relationship is observed independent of all the other parameters that were being tested in previous experiments, specifically with regards to differences between low (this study, M'Boule et al., 2014), intermediate (Schouten et al., 2006; Sachs et al., 2016) and high light (this study) intensities.

p.9 line 4 – "At higher light intensities, we expect a larger pool of photosynthetically derived NADPH inside the cell" – yes of course, but another thing to consider is this doesn't necessarily mean that this NADPH is available for alkenone synthesis.

Response: Yes, we consider and discuss this (p9 lines 12-15)

At high light levels cells might be working hard to dump this extra reductive power (into

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alkenones? or other molecules types – is there literature on algae at different light levels you can turn to?), along with reducing light harvesting capacity, which might be a reason for the lack of light effect (if there is truly a lack of light effect).

Response:There is literature about how other types of algae deal with different light levels, for example, it has been observed in Synechococcus growing in microbial mats that in order to avoid light, they try to decrease the surface area exposed to light by lying on their side (Ramsing et al., 2000. Applied and Environmental Microbiology 66). However, haptophytes, specifically E. huxleyi, are not believed to be photoinhibited organisms, especially not at the light levels we are discussing (Nanninga and Tyrrell, 1996). Van der Meer et al., 2015 did show that there is a light effect on fractionation between the range of 60-600μmol photons m-2 s-1, however, here we are focusing on the α-salinity relationship under high light conditions and how that compares with previous experiments under lower light intensities.

It seems like cells have many different options for dealing with high light situations. Were alkenone (and other lipid) concentrations measured? Were there any large concentration differences at different light levels indicating strategies for dumping excess NADPH? p.9 line 9 – why do you think that transhydrogenase activity is increased at high light?

Response: No, we did not compare the concentrations at high and low light conditions. We think transhydrogenase activity is increased at high light because excess reducing power is harmful to the cell and there is a good chance the algae will try to get rid of it either by biosynthesizing storage products or turning it into a less harmful product.

Can you provide a reference to demonstrate that this is a response to high light in haptophytes or even just eukaryotic (or even prokaryotic) algae? Rokitta et al. (2012) doi:10.1371/journal.pone.0052212 might be helpful here.

Response: Will revise.

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Regarding the last paragraph of section 4.4 – is there any chance hydrogen isotopes were measured on other lipids from these cultures (fatty acids, brassicasterol, phytol. . .except probably not phytol if you didn't saponify)? This information could be extremely helpful for illuminating cellular changes in response to environmental variables, and the interplay between different pools of cellular hydrogen, as it was for Sachs et al. (2017) that found different responses to different fatty acids, phytol, and a sterol, in a diatom grown under constant salinity, temperature, and growth rate at a range of light levels. In other words, just because there wasn't a light effect in the combined C37:2 and C37:3 values doesn't mean that the unique alkenones are not reacting to light differently, or that the hydrogen isotope ratios of other cellular lipids (and lipid precursors) are not reacting to light.

Response: When compared to the results from van der Meer et al., 2015 in which the alkenones were measured in the same manner, less fractionation was expected at highlight than in the other experiments at much lower light intensities. This was not observed in that sense it looks like light intensity does not affect the use of alkenones as a possible paleo salinity indicator. We cannot exclude the possibility the light intensity might affect other compounds, in fact van der Meer at al. 2015 showed that light intensity by itself does affect fractionation in alkenones when E. huxleyi was grown at a constant salinity. This manuscript shows it does not affect the α-salinity relationship.

In this sense, measuring hydrogen isotopes in all possible lipids is a powerful tool for helping to understand these cellular mechanisms and has tremendous value beyond validating paleoproxies.

Response: Yes, we agree that measuring other compounds could be useful in helping to understand cellular mechanisms further, however, we have only measured the hydrogen isotope values of the alkenones. This will be a topic of further study.

In the end – I am not sure you need to devote an entire section of the discussion to explaining the lack of light effect with only two light levels (and only one lipid measure-

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ment) presented. It would be great if this section of the paper could be extended with concentration data or additional lipid isotopes if that is available.

Response: In fact, we are comparing a few different experiments using E. huxleyi, taking into account different strains, temperatures, experimental set-up, nutrients, growth rates, and light intensities. Given this, we find it surprising that the responses of fractionation to salinity are all statistically similar. Thus, we argue that it is not just two light intensities, but indeed a lot more. As mentioned above, we agree that other compounds could be helpful in elucidating intracellular mechanisms, but our main emphasis with this paper is to discuss the α-salinity relationship with regards to reconstructing paleosalinity based on the specific biomarker lipid C37 alkenones, and how there does not appear to be a difference between the α-salinity relationship for high and low light levels.

Technical comments: Abstract: First sentence should read “Over the last decade, hydrogen isotope ratios of long chained. . ..”

Response: Will revise

Line 18. It would be helpful to add “at low light” (or something along those lines) to the end of the sentence “...and independently assess the effects of salinity and alkalinity”.

Response: Will revise.

Intro: p.1 line 28: add the word “and” between UK37 and LDI

Response: Will revise.

p.2 line 30: SPM has not yet been defined.

Response: Will revise as suggested by anonymous referee #3.

p.3 line 2: growth phase should include also the Wolhowe et al. (2009) reference.

Response: Will revise

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p.3 line 7: “created media by evaporation” . . . some culture studies did not use this method: note that salt was added to ultrapure water in Sachs et al. 2017.

Response: Will revise.

p. 3 line 13. How would this work? Please explain why the capacity (of environmental water) to take up H+ would, in theory, impact the hydrogen isotope composition of internal cell water and fractionation during synthesis.

Methods: How was total alkalinity determined? How was salinity determined? What was the equation to calculate growth rate based on how many cell count readings over how many days? What was the size of the growth vessel for batch cultures and how many liters of culture were maintained inside? Were growth chambers swirled to prevent sedimentation? How many grams of Al2O3 were used and how many ml of solvents for purification? What were the FID and IRMS instrument settings (column, oven temp, reactor temp, gas flow, etc)? Were external standards used to correct/calibrate reported Isodat values referenced to H2 for each run? (you could cite a previous paper with this info if the settings were the same).

Response: Different alkalinities were created by adding NaHCO3 and Na2CO3 to increase and concentrated HCl to decrease total alkalinity, which was measured by titration with 0.1 M HCl, and calculated using Gran plots. Salinity was measured using a VWR CO310 Portable Conductivity, Salinity and Temperature Instrument. Growth rate was calculated as the slope of the linear fit of the natural logarithm of cell density (ln[cell density]) in the exponential part of the growth curve. Cells were counted daily over the experimental period of 10-12 days which varied due to differences in growth rates. 600ml of media in triplicate for alkalinity/salinity experiments and 150ml for high light experiments. Smaller volumes were used for the high light experiments because we wanted to ensure that all parts of the culturing vessel would remain under the same constant high irradiance. Yes, growth chambers were swirled to prevent sedimentation. FID and IRMS settings are the same as described in M’Boule et al., 2014. This

information will be incorporated in a revised version of the manuscript.

p.5 line 10: “difference” (not “different”)

Response: Will revise.

Results: Several findings that are brought up in the discussion are not mentioned in the results (but should be) including: constant alpha over a range of alkalinities, the weak negative correlation of growth rate and fractionation for both experiments, the lack of light effect. Why not report the uncertainty on the slope and intercept of the least squares linear regressions? If you are using R for your stats, then that is easy to get using R’s “summary(lm(x_y))”. Although only very slightly biased in this case, it is probably more responsible to report R’s Adjusted R-squared as opposed to the Multiple R-squared.

Response: Noted, will revise.

In the first 2 lines of the results the ranges are reported with different dashes (“25 – 35” and then “26-42”)

Response: Will revise.

p.5 line 20 – “in contrast to previous culture studies” . . . this isn’t true – salt was added to fresh water in Sachs et al. 2017.

Response: Will revise.

Discussion: p.6 line 19 – incorrect reference – Sachs and Kawka 2015 did not find a correlation between growth rate and salinity since growth rate was held constant using continuous cultures – this reference would be more appropriate in the proceeding sentence since they did find a correlation between growth rate and fractionation.

Response: We will revise this in a new draft of the manuscript.

p.7 line 6. – missing the word “to” – after the words “could be due”

Response: Will correct this.

p. 7 lines 15-17 this doesn't make much sense – please rephrase – are you trying to say that cell water is the same as extracellular water isotopically and NADPH is more important?

Response: Original: "Nicotinamide adenine dinucleotide phosphate (NADPH) and intracellular water are the two sources of H available for use in organic compound synthesis, the latter of which is less affected by fractionation, evidenced by a comparably smaller depletion in deuterium (D) compared to that of extracellular water (Schmidt et al., 2003)." NADPH is associated with large isotope fractionation values whereas there is less fractionation between extracellular and intracellular water, but both are used as sources of H for synthesis of organic compounds (Schmidt et al., 2003).

p.7 line 22 – missing important reference (Luo, Y.-H., Sternberg, L., Suda, S., Kumazawa, S., Mitsui, A., 1991. Extremely low D/H ratios of photoproduced hydrogen by cyanobacteria. Plant and Cell Physiology 32, 897–900) after "Photosynthetic production causes NADPH to be depleted by _600‰ in D when compared to intracellular water"

Response: Noted, will add reference to revised manuscript.

p.7 line 24 – missing the letter "s" in "OPP pathway cause_"

Response: Will change.

p.7 line 32 – after the statement "A similar mechanism could be present in E. huxleyi, causing the metabolically reduced NADPH pool to increase relative to other pools, and possibly become a more important source of NADPH for biosynthesis" you might qualify that with something like "if the OPP pathway is present in the same compartment as alkenone production" (since NADPH doesn't cross organelle membranes). p.8 line 2 – the better reference here is Luo et al. 1991 (provided above)

Response: Will add reference to this discussion.

Figures: Please specify what the gray polygons are (95% confidence intervals?). Why no error bars displaying analytical uncertainty on alpha or dD?

Response: Will revise.

Fig.2c is useful for showing the (lack of) relationship between alkalinity and alpha – in this fashion, perhaps an additional figure that displays the (lack of) relationship between light and alpha could also help. In Fig.2c, does it make sense to include the regression line and confidence intervals here? – might be more useful to use a dashed line or some other visual aid to highlight how 2c is different than 2a and 2b rather than (or in addition to) leaving out the regression statistics.

Response: Noted, we will revise the figures.

Fig. 1 might not be necessary, (useful to show that culture water did not have a relationship b/t salinity and dDwater but that is a big figure for a very minor point). Why are a handful of cultures around S=35 15‰ D-enriched relative to the other cultures in the Fig.1a?

Response: Noted. The D-enrichment is likely due to how the media was created, as this was done separately for the alkalinity/salinity and the high light experiments.

References: p.10 line 12: Is there an extra "."? Check this and other references for common mistakes (superscripts missing in some locations, middle initials incorrect in some locations, missing italics for species names in many locations, too many words in title are capitalized in at least one reference).

Response: Noted, will fix them in the revised draft.

Interactive comment on Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-311, 2017.