Thank you for your comments and the helpful feedback. We have responded to each of your points in line with your text below. Our responses begin with the open bullet points. For the most part we agree with your suggestions, and have indicated our plans for a revised manuscript, which we will complete after the discussion period ends. In a few cases, we think that further dialogue would be helpful, either because we are unclear about the intent of your comment, or because we have a different perspective. We have also added some points of clarification to questions you have.

We have highlighted text below in blue where we think additional feedback from you would be helpful as we complete our revisions to the manuscript.

Thanks again for your time and feedback,

Nemiah Ladd

Summary: Ladd et al. describes the lipid composition and hydrogen isotopes of particulate OM through a time series from alpine two lakes and relates observed variability to temperature and nutrient availability. The work is strengthened by concurrent labeled incubations to get at lipid production rates and comparison to recent sediments. This work is robust, the conclusions are generally well supported, and the paper is well written.

I would like to see more data from the sedimentary samples and a revision in terms used to describe fractionation, but otherwise recommend that this manuscript be accepted for publication.

Larger comments:

- Probably my largest criticism of this manuscript is in somewhat confusing usage of terms that descript the magnitude and direction of isotope fractionation. The most common usage in this manuscript is 'increase/decrease in hydrogen isotope fractionation' which is inherently vague because it doesn't indicate direction. Describing alpha values as high and low is equally problematic as a very low alpha describes a very large fractionation (albeit a very large negative fractionation or depleted lipid signature). I would suggest converging on a single nomenclature and describing pools as more or less D-enriched or D-depleted and fractionations as positive, negative, more negative etc, rather than using term like higher and lower and increased and decreased. Section 4.1.1 is a good example of where you switch fluidly between XX permil per degree C to alpha in 0.001 and back with concurrent usage of increase, decrease, etc.
    - Thanks for the feedback. It can often be difficult to tell what will be confusing for other readers when looking at your own writing. You are correct that this could be improved. Based on your comment here and on some of the detailed comments below, it seems like some of the biggest problems stem from the fact that more fractionation means smaller α values when $\alpha<1$, as is typically the case for net fractionation during lipid synthesis by photoautotrophs. We did not adequately account for the possibility of $\alpha>1$ in our usage of this term, and that also caused some points to be unclear. We will revise the

paper carefully with this advice in mind.
    - Another source of confusion is that it is often more intuitive to talk about fractionation in per mil terms (since this is what we report our δ values in). We agree that it is important to make our usage more consistent, and will strive to do so in a revised draft.

- Please differentiate the cyanobacteria from eukaryotic algae. Lumping the two is a vestige from the time prior to DNA sequencing technologies that allowed for easy differentiation. This is imprecise and unnecessary for this manuscript. Please revise.
    - Again, we agree that this is a valid point, and that the original manuscript is not precise enough. We will differentiate cyanobacteria and eukaryotic algae throughout the revised manuscript.

- p10 Section 3.3 – Why do we only get to see data for the c16:0 FA for the sedimentary lipid section? Do the other lipids agree? I would also like to see if the sediment traps and the core top data agree with one another. Given the accumulation rate and sampling procedure the differences might suggest seasonal signals vs. annual integration. Generally the level of data and detail for the sedimentary samples should be as robust as the particulate (given that you are comparing the two and the later is the signal that most people look at).
    - When the sediment samples were originally analyzed, the concentrations were optimized for $nC_{16:0}$. In many cases, the peak areas were not appropriate for other fatty acids, and the standard deviations of those measurements were high. We are running these samples on the GC-IRMS again at a more suitable concentration in order to be able to include sediment data from all of the compounds discussed from the filters, and will include those data and an expanded discussion about the sediment data in our revised manuscript.
    - We will also separate the core top and sediment trap data from each other in our revised version of Figure 4.

- p12-15 – As you have both cell counts and lipid yield data you should be able to normalize for cell counts in this discussion. You note at the end of the paragraph that cell counts agree, but it seems like you can take it a step farther.
    - A problem with doing this is that the cell count samples were collected on different days than the lipid samples. We can linearly extrapolate between the monthly cell counts to come up with an estimate of the number of cells on our sampling dates and normalize the lipid yields to these. Do you think such an analysis would be helpful? Given that the cell counts were collected as part of a separate project that we did coordinate with in advance, is it possible to be more quantitative here, or is a qualitative comparison most appropriate?

- p15-7 – I don't buy this explanation and you definitely haven't demonstrated that this is a general feature of isoprenoids in your samples. Presumably there is plenty of phytol in your extracts, what does that look like?
    - We have phytol in our extracts. It eluted in the first fraction of the silver nitrate columns we performed on our alcohol fraction (third fraction collected during our silica gel columns). We have not

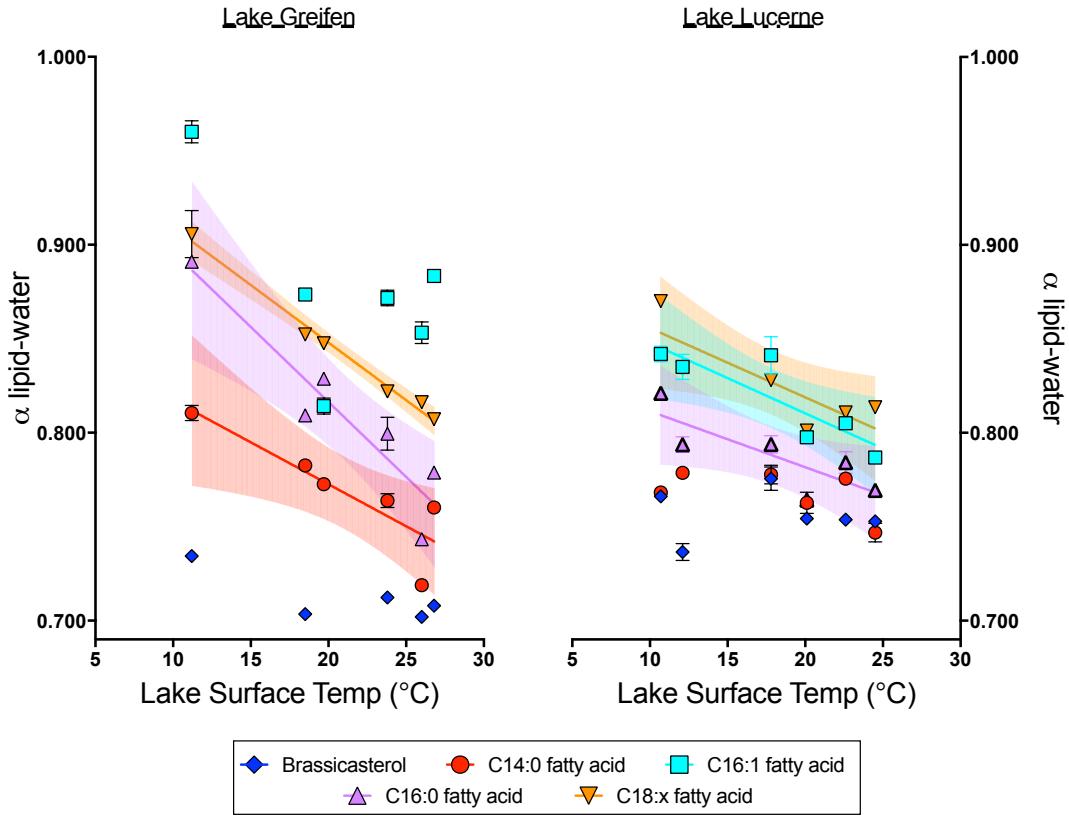
measured phytol δ^2H values from these samples yet, but will try to do so in time to include in the revised manuscript.
    - We agree that we probably overstated our case by referring to all isoprenoids on the basis of brassicasterol measurements alone. We will scale back our claim and revise the text to remove the reference to all isoprenoids.
    - It is likely that phytol will exhibit a different response than sterols, given that it is almost always depleted in ^2H relative to sterols in the same organism. Possible reasons for this depletion are (1) the fact that phytol synthesis occurs in the chloroplast, rather than in the cytosol, as is the case for sterols or (2) phytol can be produced by the deoxy-D-xylulose phosphate (DOXP) pathway, rather than the mevalonic acid (MVA) pathway, which is most commonly used to synthesize sterols in the cytosol.

- p16-1 – Can you calculate and compare a weighted average isotope value (as in Osburn et al., 2016) between these two?
    - We are generally not in favor of calculating weighted average isotope values. The differences in δ^2H values among different lipids are not trivial and can be related to real information about environmental conditions, lipid sources, and physiological state. This information is lost when computing weighted averages, so our preference is to report all compound specific isotope values individually.

- I am generally curious about what other compounds were present in this dataset that can be compared between the particulate and the sediments. In particularly the midchain fatty acids as these are often used to describe paleolake water.
    - In addition to the compounds measured in this study, the filters contained very small amounts of relatively short (~C17) *n*-alkanes, but concentrations were not high enough for H isotope measurements. There was abundant phytol, some cholesterol and stigmasterol, and trace amounts of other sterols at concentrations too low for hydrogen isotopes. The stigmasterol split between silver nitrate fractions and was not measured for H isotopes. The cholesterol was not measured since it was assumed to be mostly produced by heterotrophs. As mentioned in response to a previous comment, it should be straight-forward to measure δ^2H values of the phytol, and we hope to include these data in the revised manuscript.
    - There were only trace amounts of fatty acids longer than C_{18}, and none of the longer chain fatty acids or *n*-alkanes associated with leaf waxes were collected on the filters.

- Figure 3: I am not sure all of these subfigures are useful. A bigger stacked figure showing all compounds from a single lake might be more informative.
    - Here is a modified version of Figure 3 following your suggestions. 95% confidence intervals are only included for the regressions that were significant at the $p < 0.05$ level. Including the statistics in on the stacked figure gets pretty messy, but they are all listed in Table 2. If

you have additional thoughts about the best way to show this data (it is a little busy with all the curves on top of each other), we would appreciate it. We would be inclined to replace Figure 3 with this one in the revised manuscript, and to move the old version of Figure 3 to the supplemental material, in case anyone wants to look at the data in more detail.



- Figure 4: Divide out sediment traps and surface sediment data then report all measured rather than just C16:0
    - We plan to do this for the revised paper, along the lines of our response to your third "larger comment."

Smaller comments

- p4-26-32 – How permeable were the incubating carboys to light and to air? It seems like the experiment is meant to replicate growing conditions, but doesn't really account for change associated with different light conditions or lack of oxygen. We can't really evaluate how comparable the incubations were to normal conditions.
    - The carboys were made from transparent plastic, and we will add this qualifier to the text.

- p5-33 – This is cooler and a shorter time than I have seen before. Is there reference for this? If not, are you sure that this condition was sufficient to quantitatively extract all lipids?
    - When we first bought our microwave extraction system, our lab manager performed a number of tests of different extraction programs using replicates of a large homogenized sediment sample from Lake

Zug in central Switzerland. These included a range of temperatures up to 100°C, and extraction times up to 40 minutes. All samples were extracted twice, and the replicate extractions were analyzed as well in order to assess whether the first extraction had successfully recovered the lipids from the sample. In this test, extracting at higher temperatures than 70°C and for longer times than 5 minutes did not have a significant effect on lipid yields.

- p6-5-8 – You don't report compositional information for other lipids. This is ok, but is there anything noteworthy?
    - Please see above response to 7th bullet point in the larger comments.

- p6-15 – What compound classes are eluting in each of these fractions?
    - We will update this sentence to read: "The first fraction, containing *n*-alkanols and phytol, was eluted with 20 mL of 4:1 hexane/DCM, the second fraction, containing stanols and singly unsaturated sterols (such as cholesterol) with 20 mL of 1:1 hexane/DCM, the third fraction, containing most doubly unsaturated sterols including brassicasterol, with 16 mL of DCM, and the remaining compounds with 4 mL of ethyl acetate."

- p6-22 – Do any of the aforementioned methods need citations or are they all original to this study?
    - The methods reported here reflect minor modifications of long-established protocols. For the most part, they are not unique to this study. For example, the saponification and silica gel protocols are identical to those used in several recent papers about lipids from the Biogeochemistry group at Eawag. However, those methods represent small tweaks on long-standing methods (volume of solvent used, brand of silica gel column, etc.) and do not represent a novel innovation. We observe that in many organic geochemical papers, authors cite their previously published work in the portion of their methods about lipid extraction and purification, even though those papers are not the original example of silica gel chromatography or base hydrolysis. We think it is more useful to provide the specifics of our protocols so that the reader can clearly see what we did, without having to search for additional papers.
    - The specific silver nitrate scheme used to purify brassicasterol in this study is new, but the concept of using silver nitrate impregnated silica gel chromatography to separate organic compounds based on their number of double bonds is long-standing and well-established.

- p7-24 – Please confirm that reported errors include propagated errors from replicate measurements, standards, and derivatization processes.
    - We will add the following text after "mass balance": "and reported errors represented propagated errors from replicate measurements and the uncertainties associated with the added hydrogen."

- p8 section 3.1 – this section could probably be condensed, it is quite repetitive.

- Thanks for the feedback. We will condense this section in the revised manuscript.

- p9-2 – lowest here means fastest right? I would suggest going with time designations when referring to residence times
  - We will change "lowest" to "shortest" and agree that it makes the point clearer

- p11, sec 4.1.2. this has been studied in plants
  - The light effect on hydrogen isotope fractionation in plants has been attributed to changes in transpiration (e.g. Yang et al., 2009, doi:10.1007/s00442-009-1321-1), which is not applicable for algae and cyanobacteria.
  - We are familiar with an experiment by Marc André Cormier and Ansgar Kahmen where biosynthetic fractionation in leaf waxes was measured for plants grown under different light conditions. This work has been presented at conferences and workshops, but is not yet published. Is this what you are referring to? If there is other work about changes in biosynthetic fractionation (not changes in net fractionation due to changes in transpiration) in plants grown at different light levels, we are not familiar with it, and would appreciate it if you could direct us to the appropriate papers.

- p12-29 – lower alpha is confusing here
  - We will modify along the lines of your earlier comment.

- p13-2 – The ultimate explanation should be the same for all FAs unless you are suggesting a different source.
  - Given that the nC16:1 FA concentrations do not covary with the others, and that there are significant changes in the ratio of nC16:0 to nC16:1, it seems possible that there is a different source for them, or that only a limited number of the nC16:0 producers also make nC16:1, and that their relative abundance varied throughout the study period. We can add this point in the revised manuscript.

- p13-11 – This is a bit of an overstatement. Just because we don't understand the governing mechanisms of species specific fraction does not make it random.
  - We will change this line to "…varies by hundreds of per mil among species in ways that are not yet understood."

- p13-27 – Can a statistical analysis help you here? Does any taxa change correlate with changes in fractionation?
  - Unfortunately, the two sample sets were not collected on the same day, since they come from two uncoordinated sampling campaigns. Is it useful to try to apply a statistical analysis to a linear interpolation the changing taxa? We can do a multiple linear regression to see if changes in taxa correlate with changes in fractionation, with that limitation in mind.

- We have also arranged for pigment concentrations to be analyzed on replicate filters that we collected the same day as our lipid samples. While changes in pigment abundance are not as species-specific as the cell counts, they can perhaps help us tell if the cell-count samples missed a notable bloom of specific taxonomic group that occurred closer to our sampling date.

- p13-31&32 – This is a false comparison, there are photoheterotrophs in this paper and they look far more like photoautotrophs than they do heterotrophs. You must at least acknowledge the photoheterotrophs.
    - We will revise this section to make the distinction between photoheterotrophs and heterotrophs more clear, and will also add in references to other work about variability in hydrogen isotope fractionation that is due to metabolic pathways (in particular, papers by Osburn et al. and Heinzelmann et al.).

- p14-2 – This hypothesis is also not consistent with an oligotrophic lake either. many algae and cyanobacteria are capable of mixotrophy. Almost all of them do this at night and some under diverse conditions.
    - Our point was that if mixotrophy (more common in the oligotrophic lake) has a significant effect on ^{2}H-fractionation, one would be expect it to result in relatively more enriched fatty acids. Since fatty acid fractionation factors are similar between the two lakes for most of the summer, and the fatty acids tend to be more enriched in the spring in the eutrophic lake but not the oligotrophic one, we would suggest that seasonal changes in mixotrophy are unlikely to account for the difference in the magnitude of the seasonal change in ^{2}H fractionation between the two lakes. We will try to make this point more clearly in the revised manuscript.

- p14-18 – Be careful, there are plenty of bacteria that make primarily 16:0, 16:1, and 18:1.
    - We acknowledge that heterotrophic bacteria can produce a large amount of 16:0, 16:1, and 18:1. We will modify the text of this section as follows: “If the source of so much $nC_{16:0}$ fatty acids was bacterial, it might be expected to correspond to increased concentrations of lipids associated with heterotrophic bacteria, such as iso- and anteiso-$C_{15:0}$ and $C_{17:0}$ (Perry et al., 1979; Volkmann et al., 1980). Since there are not significant amounts of these short-chain odd-carbon branched fatty acids in the particulate organic matter throughout the time series, including in the early spring, it seems less likely that such a large component of the even-carbon fatty acids is be derived from bacterial sources. However, this does not rule out greater contributions from mixotrophic algae relying on heterotrophy in the spring, nor from heterotrophic bacteria species that primarily produce short-chain, even-carbon fatty acids.”

- p17-14 – Reporting an offset in permil would be helpful to the paleo community
    - We will add this to the revised draft.

- p17-19 – isoprenoid change to sterol, you haven't made the case for isoprenoids more generally.
    - We will change "isoprenoid" to "sterol"

Minor editorial comments:

- p1-9 – isotope → isotopic
    - Changed

- p1-21 – in situ should be italicized here and throughout the manuscript
    - The style guidelines for *Biogeosciences* indicate that common Latin phrases, including "in situ", should not be italicized, so we will leave this unitalicized.

- p1-22 – increased magnitude of fractionation needs a direction term as the fractionation factor for lipid-water can be both above and below 1.
    - We will change this in line with our response to your first comment.

- p2-22-23 – This is stating the obvious, rephrase or combine with the following sentence.
    - We will combine these two sentences into the following revised sentence: The offset between the hydrogen isotopic composition of lipids and source water is described by the apparent fractionation factor, $\alpha_{Lipid\text{-}Water} = ({}^2H/{}^1H_{Lipid})/({}^2H/{}^1H_{Water})$, or in ‰ terms by $\varepsilon_{Lipid\text{-}Water} = ((\delta^2H_{Lipid} + 1000)/(\delta^2H_{Water} + 1000) - 1)*1000$, where $\delta^2H = (({}^2H/{}^1H_{Sample})/({}^2H/{}^1H_{VSMOW}) - 1)*1000$.

- p2-25 – also metabolism see Zhang et al., 2009 or Osburn et al. 2016
    - This is a good point and it is relevant for the subsequent discussion. We will add it to the text and to Table 1, and include two papers from Heinzelmann, 2015 in reference to this point as well.

- p3-1– I think that should be permil per degree
    - Correct, we will modify to "‰ per °C"

- p3-5 – I am not sure what 'more' fractionation means
    - We mean that alpha is further from unity; in this case, since the fractionation is more negative, alpha is smaller and that represents more fractionation. We will revise this sentence to make it clearer, in line with our response to your first comment.

- p3-13 – remove 'the'
    - We will remove it

- p4-10 – What is the depth at this sampling point?
    - The water depth was 96m. This information will be added to this section.

- p5-24&25 – something is wrong with these sentences. Perhaps a ‘was’ after ‘sample’
    - We will add “was” after “sample”

- p7-10 – Initially? Were they subsequently converted to a different scale?
    - We will delete the word “initially,” which is not relevant

- p7-31 13 needs superscript
    - We will change 13 to ^{13}

- p7-32 – lipid
    - We will delete the “s” at the end of “lipids”

- p8-15-16 – these numbers are the same (1.0 and 1.1) but the text suggests that one should be lower.
    - The concentrations were the same as each other for the first sampling date in April (both ~1 μg/L), but subsequently, nC16:1 concentrations were much lower than those of nC16:0 (13 μg/L = maximum value for nC16:1, 55 μg/L for nC16:0)

- p9-19 – add ‘relative’ after depleted
    - We will add this

- p14-26 space before 2010
    - We will add this