Reviewer 3 Summary:

The objectives of this study were to assess the impacts of time following permafrost thaw stage on methane emissions and methanogenic community composition. To do this, the authors identified two bog sites with permafrost that thawed 30 and 200 years ago. Analyses conducted at these sites included (1) metagenomic assessments, (2) dissolved gas concentrations (CO2 and CH4), (3) surface emissions,(4) d13C signatures for both CH4 and CO2 (used to assess the relative contribution of acetoclastic methanogenesis to total methanogenesis).

Overall, this paper effectively approaches that goal.

I have two primary concerns:
(1) My main concern with this paper is centered around the use of isotope d13C signatures. The alpha value is an accepted method for discerning the relative contributions of acetoclastic vs. hydrogenotrophic methanogenesis. While the authors used alpha values for dissolved gas analysis, they limited their assessments of acetoclastic input to 13C signatures of methane (without concomitant 13C-CO2 signatures). Is the 13C-CH4 signature on its own a sufficient indicator of acetoclastic contribution? If so, please provide citations that indicate so.

We agree that the presentation of the isotope data can be clearer and more consistent throughout. We do not calculate alpha values for our fluxes as a significant proportion of the $\delta^{13}C\text{-}CO_2$ signature will be heavily influenced by autotrophic respiration. This would significantly influence the alpha values and lead to a bias towards acetoclastic methanogenesis, thus we do not calculate it. In the results section and throughout the manuscript we will change how we present the isotope data. The new format we will follow will be to first present the alpha vale with the delta values in parentheses. We will keep Figure 3c the same, showing results from the Keeling plot method as this is a commonly used approach to assess the processes involved in determining the isotopic composition of atmospheric CH_4. (Keeling, 1958). During methanogenesis, fractionation by hydrogenotrophic and acetoclastic methanogens produces CH_4 with a $\delta^{13}C\text{-}CH_4$ of -110‰ to -60‰ and -65‰ to -50‰, respectively (Hornibrook et al., 1997, 2000).
Many studies have used these plots, along with the known range of $\delta^{13}C\text{-}CH_4$ signatures associated with the methanogenic pathways, to identify the source signature, some of these are listed below.

References

- *Keeling, C. D. (1958). The concentration and isotopic abundances of atmospheric carbon dioxide in rural areas. Geochimica et Cosmochimica Acta, 13(4). https://doi.org/10.1016/0016-7037(58)90033-4*
- *Hornibrook, E. R. C., Longstaffe, F. J., & Fyfe, W. S. (1997). Spatial distribution of microbial methane production pathways in temperate zone wetland soils: Stable carbon and hydrogen isotope evidence. Geochimica et Cosmochimica Acta, 61(4), 745–753. https://doi.org/https://doi.org/10.1016/S0016-7037(96)00368-7*
- *Hornibrook, E. R. C., Longstaffe, F. J., & Fyfe, W. S. (2000). Evolution of stable carbon isotope compositions for methane and carbon dioxide in freshwater wetlands and other anaerobic environments. Geochimica et Cosmochimica Acta, 64(6). https://doi.org/10.1016/S0016-7037(99)00321-X*

Studies using the keeling plot method to identify CH_4 source

- *Fisher, R. E., et al. (2017), Measurement of the 13C isotopic signature of methane emissions from northern European wetlands, Global Biogeochem. Cycles, 31, 605– 623, doi:10.1002/2016GB005504*
- *Marushchak, M. E., Friborg, T., Biasi, C., Herbst, M., Johansson, T., Kiepe, I., Liimatainen, M., Lind, S. E., Martikainen, P. J., Virtanen, T., Soegaard, H., and Shurpali, N. J.: Methane dynamics in the subarctic tundra: combining stable isotope analyses, plot- and ecosystem-scale flux measurements, Biogeosciences, 13, 597–608, https://doi.org/10.5194/bg-13-597-2016, 2016.*

- *Santoni, G. W., Lee, B. H., Goodrich, J. P., Varner, R. K., Crill, P. M., McManus, J. B., Nelson, D. D., Zahniser, M. S., and Wofsy, S. C. (2012), Mass fluxes and isofluxes of methane (CH4) at a New Hampshire fen measured by a continuous wave quantum cascade laser spectrometer, J. Geophys. Res., 117, D10301, doi:10.1029/2011JD016960.*
- *McCalley, C., Woodcroft, B., Hodgkins, S. et al. Methane dynamics regulated by microbial community response to permafrost thaw. Nature 514, 478–481 (2014). https://doi.org/10.1038/nature13798*
- *S. Sriskantharajah, R. E. Fisher, D. Lowry, T. Aalto, J. Hatakka, M. Aurela, T. Laurila, A. Lohila, E. Kuitunen & E. G. Nisbet (2012) Stable carbon isotope signatures of methane from a Finnish subarctic wetland, Tellus B: Chemical and Physical Meteorology, 64:1, DOI: 10.3402/tellusb.v64i0.18818*

Furthermore—I was not clear on whether the apparent difference in alpha values between mature vs. young sites was indeed statistically significant. This comparison needs to be made explicit. If significant differences between sites can only be found at specific depth intervals, then that should also be stated explicitly.

We agree, this has not been made clear in the text and will rectify it. The depth profiles of dissolved CH_4, dissolved CO_2, $\delta^{13}C$-CH_4, and α_C are significantly different between the young and mature bog. We will clearly state that these depth profiles in the young and mature bog are distinct from one another. Within our analysis, we control for depth as we are not interested in differences at specific depths, but rather how the depth profiles overall differ between the thawed sites. Direct comparison of specific depths between the two sites can be complicated and misleading as depths in the young and mature bog do not correspond to one another with regard to depth from the water table, age, time spent since thaw occurred, and peat composition. Thus, we are interested in how these entire depth profiles differ between thaw sites. We can split the depth profiles into peat that accumulated before and after the most recent thaw event at the site (the depths these are found at are indicated by arrows in Figure 1a). Similar to the entire depth profile, dissolved CO_2, $\delta^{13}C$-CH_4, and α_C are significantly different between the young and mature bog for peat that accumulated before and after the most recent thaw event. This additional information and statistical analysis will be added to the text.

(2) Your goal was to examine the effects of thaw stage on methane fluxes/methanogenic community composition. It is difficult to wrap my head around this goal since thaw succession causes shifts in so many different environmental factors (soil temperature, thickness of the unsaturated peat column, availability of labile organics). This makes your results difficult to build off of/apply to other settings. Perhaps you could perform an ordinary least squares regression analysis (OLS) to try to tease apart the relative influence of these numerous factors on a dependent variable of interest (perhaps total methane emissions, or acetoclastic methane emissions).
We agree that peatland succession following permafrost thaw presents a dynamic, complex landscape that may influence microbial community composition and its activity in a myriad of ways. Here, we present data from two areas that have thawed 30 and 200 years ago. Each site has a distinct water table position, vegetation community, soil temperatures, and volume of peat accumulated at the surface following thaw. Each site does however have identical histories in the peat layers that accumulated prior to permafrost thaw (Heffernan et al., 2020). The objective of this study was to address how the combined effect of the ecological conditions (exposure of previously frozen peat, water table position, vegetation community, soil temperatures) found in the decades following thaw (young bog) and those found centuries following thaw (mature bog) influences the soil methanogen community, its activity, and the resulting CH_4 fluxes to the atmosphere. We found that no single factor drives differences, but rather it is the overall ecological conditions and interactions between these and microbial community members that influences the microbial community structure, its activity, pathways of methanogenesis, and CH_4 surface fluxes. .

We explored potential relationships between environmental factors and methanogen community composition using a distance-based redundancy analysis (RDA; Figure 6). Included in our RDA was an initial backward stepwise regression (L453-457) to determine what environmental factors were significantly influencing the methanogenic community and should be included in our RDA. These included dissolved concentrations of

CO_2, CH_4, DOC, temperature, enzymatic activity estimate, thaw stage, depth, and distance to water table. This stepwise regression serves a similar purpose to the prosed ordinary least squares and allows us use redundancy analysis once significant variables have been identified.

While we could use the environmental data at each site (water table depth, soil temperatures, time since thaw) to model our total CH_4 emissions, and in doing so determine the main drivers of our CH_4 emissions over a growing season, this was not the objective of this study. This study aimed to relate the methanogen community to surface CH_4 fluxes, and to comment on how long elevated CH_4 emissions may persist following thaw. The 2018 growing season data presented in this study is being prepared, along with multiple other years, in a separate study to achieve this.

References

- *Heffernan, L., Estop-Aragonés, C., Knorr, K.-H., Talbot, J., & Olefeldt, D. (2020). Long-term impacts of permafrost thaw on carbon storage in peatlands: deep losses offset by surficial accumulation. Journal of Geophysical Research: Biogeosciences, 2011(2865), e2019JG005501. https://doi.org/10.1029/2019JG005501*

Specific Questions/Recommendations

(1) I am unclear on what is mean by the term "ecological" in the context of this manuscript (e.g. L27-28). I get the impression that it references vegetation primarily. I am unsure about that, however, because "ecological" could also be used to describe microbial community composition. Please clarify.

By 'ecological' we mean the shifts associated with autogenic ecological succession seen following thaw in thermokarst bogs, this includes vegetation community, water table position, and temperature. We will be more explicit with this and define what we mean by ecological shifts in the introduction.

(2) Fig 1: I'd recommend explicitly stating how far apart the mature and young bog sites are from one another in panel f (or the caption). You list only one GPS coordinate from the whole site in the figure caption.

Figure 1c includes a scale of 10 m to indicate this distance and we have also roughly defined the distance between these sites in the methods by stating how far they are from the plateau (young bog – L192 and mature bog – L202). We do not have a single exact measurement of the distance between these as cores and dissolved gas depth profiles were taken within specific areas (circles in Figure 1c) rather than a single location.

(3) L158-159: How did you determine that this complex is representative? If the succeeding sentences are meant to serve as evidence for this claim, make that explicit.

This is based on other studies of peatland complexes within the discontinuous zone in the Interior Plains of western Canada. Some of these are added below and we will add some of these references to the text on L159

References

- *Bauer, I. E., Gignac, L. D., & Vitt, D. H. (2003). Development of a peatland complex in boreal western Canada: Lateral site expansion and local variability in vegetation succession and long-term peat accumulation. Canadian Journal of Botany, 81(8), 833–847. https://doi.org/10.1139/b03-076*
- *Beilman, D. W. (2001). Plant community and diversity change due to localized permafrost dynamics in bogs of western Canada. Canadian Journal of Botany, 79(8), 983–993. https://doi.org/10.1139/cjb-79-8-983*
- *Camill, P. (1999). Peat accumulation and succession following permafrost thaw in the Boreal peatlands of Manitoba, Canada. Ecoscience, 6(4), 592–602. https://doi.org/10.1080/11956860.1999.11682561*

- *Pelletier, N., Talbot, J., Olefeldt, D., Turetsky, M., Blodau, C., Sonnentag, O., & Quinton, W. L. (2017). Influence of Holocene permafrost aggradation and thaw on the paleoecology and carbon storage of a peatland complex in northwestern Canada. Holocene, 27(9), 1391–1405. https://doi.org/10.1177/0959683617693899*
- *Vitt, D. H., Halsey, L. A., & Zoltai, S. C. (1994). The Bog Landforms of Continental Western Canada in Relation to Climate and Permafrost Patterns. Arctic and Alpine Research, 26(1), 1. https://doi.org/10.2307/1551870*
- *Vitt, D. H., Halsey, L. A., Bauer, I. E., & Campbell, C. (2000). Spatial and temporal trends in carbon storage of peatlands of continental western Canada through the Holocene. Canadian Journal of Earth Sciences, 37(5), 683–693. https://doi.org/10.1139/e99-097*
- *Zoltai, S. C. (1972). Palsas and Peat Plateaus in Central Manitoba and Saskatchewan. Canadian Journal of Forest Research, 2(3), 291–302. https://doi.org/10.1139/x72-046*
- *Zoltai, S. C. (1993). Cyclic Development of Permafrost in the Peatlands of Northwestern Alberta, Canada. Arctic and Alpine Research, 25(3), 240. https://doi.org/10.2307/1551820*

(4) L551-554: "Overall, the isotopic data indicates a general dominance of hydrogenotrophic methanogenesis in both sites, but a greater contribution of acetoclastic methanogenesis in the young bog relative to the mature bog." Was this difference statistically significant? I suggest adding a p-value after this statement.

This statement is intended to cover the entire section of results discussing isotopic data and includes dissolved gas depth profiles of $\delta^{13}C$-CH_4, $\delta^{13}C$-CO_2, and α_C (L527-552). It summarises these results to close that section, thus we do not perform any statistical analysis or have any p value for it. We can remove the statement if the reviewer deems it unfit for the results section and move it to the discussion. We will make sure to add all relevant p values comparing alpha and delta values in the results section above this to better highlight statistical differences that led to this statement.

(5) L 572-575: *"The δ13C-CH4 signature of CH4 emissions (intercept values from Keeling plots), in the young bog were significantly greater than those observed in the mature bog (Figure 3c; $F_{(1, 4)} = 20.67$, $P < 0.05$)., suggesting a greater influence of acetoclastic CH4 production."*

Why is there no alpha value for the flux measurements? Please provide a source indicating that 13C-CH4 measurements alone (i.e. without concomitant 13C-CO2) are sufficient to discern the relative influence of acetoclastic methanogenesis on total methane production.

Please see above where we have addressed why we do not calculate an alpha value for fluxes

(6) L704-706: *"Evidence of acetoclastic methanogens and CH4 produced via the acetoclastic metabolic pathway was found in the young bog both near the surface and at depths below the thaw transition (i.e., in peat that accumulated prior to permafrost thaw)."*

I have two notes on this:
(1) If the difference in alpha values was not significant in the subsurface (which I am not 100% clear on), this needs to be noted and discussed.

Please see our response above which addresses this. We will add results from statistical analysis comparing both alpha and delta values from below ground dissolved gas samples to show that results from the peat layer that accumulated after thaw are distinct between the young and mature bog.

(2) See my comments regarding L572-575. Make sure your methods for discerning the acetoclastic influence on surface CH4 emissions is sound.

Please see above where we have addressed this.

(7) L765-766: "*The presence of hydrophilic vegetation, particularly graminoids, in the saturated young bog provides the precursors for fermentation..*"

I am confused by this statement. "Precursors" could be interpreted as "reactants", which are primarily sugars. Sugars are ultimately delivered to porewater from other plants too (i.e. Sphagnum spp.). Are you saying that the sugars derived from gramminoids are more labile than those derived from Sphagnum? I would agree with this, but it is necessary to clarify.
Yes, this is exactly what we mean. The references below (Ström et al., 2003; 2012) show how graminoids enhance substrate quality and availability, leading to greater methanogenesis. We will add these citations to the text. We use precursors as a catchall term for all plant derived monomeric compounds formed following extracellular enzyme hydrolysis used in this fermentation step. Precursor is the most suitable term for these, as it is defined by Merriam-Webster dictionary, "a substance, cell, or cellular component from which another substance, cell, or cellular component is formed"

References
Ström et al., Presence of Eriophorum scheuchzeri enhances substrate availability and methane emission in an Arctic wetland, Soil Biology and Biochemistry, Volume 45, 2012, Pages 61-70, ISSN 0038-0717, https://doi.org/10.1016/j.soilbio.2011.09.005.
Ström, L., Ekberg, A., Mastepanov, M. and Røjle Christensen, T. (2003), The effect of vascular plants on carbon turnover and methane emissions from a tundra wetland. Global Change Biology, 9: 1185-1192. https://doi.org/10.1046/j.1365-2486.2003.00655.x

(8) L805-813: I find the thread of this paragraph hard to follow. Please make the connections between sentences clearer.
These sentences will be restructured to better highlight the message we are trying to convey with them. This section attempts to summarise that there are multiple factors influencing the observed differences in the $\delta^{13}C$-CH_4 *signature of* CH_4 *emissions between the young and mature bog. This will be edited to make this clearer and will now read*
"However, increased oxidation above the water table in the mature bog is likely not fully responsible for the observed differences in CH4 surface emissions and depth profiles between the young and mature bog. Lower soil temperatures, a vegetation community associated with reduced substrate availability, the dominance of hydrogenotrophic methanogenesis throughout the peat profile, and a deeper water table position all contribute to the lower CH4 production and higher CH4 oxidation observed in the mature bog.

(9) Fig 2: Please add in a legend.
We have added a legend to this figure in panel Figure 2b