Dear Editors and Reviewers,

We thank you very much for taking time to review this manuscript. We really appreciate all your comments and suggestions! The comments and suggestions are valuable and very helpful for revising and improving our manuscript. Based on the instructions provided in your letter, we uploaded the file of the revised manuscript.

Appended to this letter is our point-by-point response to the comments raised by the reviewer. The comments are reproduced and our amended texts are in a different color.

We would like also to thank you for allowing us to submit a revised copy of the manuscript. We hope that the revised manuscript is accepted for publication in The Cryosphere

Sincerely

Weidong Kong, on behalf of all authors

Anonymous Referee #1

Received and published: 14 April 2020

This is a technically correct manuscript on a currently relevant topic in the context of climate change and biogeochemical cycles - the response of microbes to permafrost thawing. The study shows changes in bacterial community structure and richness of drained lake basins with permafrost soil age and permafrost thawing status (active, transition and permanently frozen soil layer). In addition, there is data on soil carbon and nitrogen. The results are presented clearly and the figures are well prepared.

Response:

We sincerely thank reviewer #1 for the careful editing and constructive comments provided. We have carefully amended our manuscript accordingly, please see below.

Major concerns:

Q1. Are the samples in this study from the same soil cores as those in Kao-Kniffin et al. 2015, which is cited in the section on sampling? Kao-Kniffin et al. 2015 also describe bacterial communities with permafrost soil age and thawing status. If the soil cores are the same, please make it clear in the aims why a second analysis of bacterial communities in these samples is needed and explain what new this study adds. In any case, please take the results of Kao-Kniffin et al. 2015 into account in the discussion, especially as their conclusion (communities in active layers converge) seems to be the opposite from this manuscript (no convergence of active layer communities).

Response:

We appreciate the reviewer for this comment. In this manuscript, we focused on the bacterial community only, whereas Kao-Kniffin et al. 2015 investigated the community structure of the entire prokaryotes (both bacteria and archaea). For bacteria, Kao-Kniffin presented the taxonomic composition, community phylogenetic distance, and biomass. Thus, the interactive influence of permafrost age and thawing on bacterial diversity, community structure, and assembly processes still remain unexplored. Therefore, we believe further investigation is necessary and could provide essential knowledge on how permafrost age would influence the bacterial

community interactively with permafrost thawing.

To address the reviewer's comments, we have added the following sentences to clarify our aims and distinguish our work from Kao–Kniffin et al.:

An earlier study at this site has revealed a high abundance of Candidatus *Methanoflorens* archaeon in the community (Kao–Kniffin et al., 2015), but how the bacteria in the permafrost of various ages would respond to thawing remains less understood. Thus, we investigated the interactive influence of permafrost thawing and age on permafrost soil bacterial community.

Following sentences are added to discuss the inconsistency between our work and Kao–Kniffin et al on community convergence:

Our results demonstrated that bacterial community structure did not converge due to permafrost thawing, as reflected by the non-significant difference in sample heterogeneity among the various permafrost layers (Supplementary Fig. 3, Supplementary Table 11). This contradicts previous studies (Deng et al., 2015; Yuan et al., 2018) in the Arctic, but was consistent with Mackelprang (2011). Our results also contradict to Kao–Kniffin et al. (2015), which reported lowered prokaryotic community differences in the active layer than in the transition and permanently frozen permafrost. Several reasons could cause this inconsistency. Firstly, different microbial communities were targeted. Kao–Kniffin et al. (2015) focused on archaeal community, whereas only bacteria were targeted in the present study. Kao–Kniffin et al. (2015) identified a single archaeon OTU accounting for over 30% of the prokaryotic community (Fig. 3 in Kao-Kniffin et al., 2015). An early study revealed that archaea exhibited a lower community variation with increasing soil depths compared with bacteria (Frank-Fahle et al., 2014). Therefore, the community convergence observed by Kao–Kniffin et al. (2015) could be due to the influence of archaea. Furthermore, the inconsistency may be related to the different community dissimilarity metrics used. Kao–Kniffin et al. (2015) used unweighted UniFrac, which only calculates the phylogenetic closeness of OTUs, and the relative abundance is not considered. This is distinctively different from the Bray-Curtis dissimilarity used in

the present study, and it has been reported that unweighted and weighted community metrics examine different features of community strcture (Lozupone et al., 2011).

Q2. I am concerned that the connection of soil layers to thawing status is too simplified and does not take into account variation in the soil profile. Was the soil structure/chemical composition of the profiles homogeneous with depth? The description of organic layer on l. 94-96, Fig. 1 and Kao-Kniffin et al. 2015 and Mueller et al. 2015 cited in the manuscript suggest they were not. In this case, the differences in bacterial communities between soil layers cannot be directly interpreted as a thawing response (l.37., l.318), because the state of the system before thawing is not known and the differences between the layers can be due differences in other soil properties (for example organic vs. mineral layer). It is possible to compare the active, transition and frozen layers with permafrost age but that seems to have already been done by Kao-Kniffin et al. 2015? In any case, the issue of other differences between the soil layers than thawing status should be better taken into account in the manuscript. Do soil carbon and nitrogen explain the community changes?

Response:

We appreciate the reviewer to raise this concern. As the reviewer has pointed out, the physicochemical properties of the soils in different permafrost depths are not homogenous. Hence, the response of microbial community to thawing could be the collective effects of both thawing and the environmental differences. However, microbial transformation (as a result of permafrost thawing) would substantially change the quantity and composition of organic compounds (Mueller et al. 2015). Thus, soil physicochemical properties and bacterial community structure are interactive, and we have to admit that the individual influence would be very difficult to be disentangled. Nevertheless, Mondav et al. (2017) reported that permafrost thawing has a stronger influence on microbial community structure than soil depth. To address the reviewer's concern and emphasize the importance of environmental heterogeneity in the different permafrost layers, we have added the following paragraph to the manuscript:

Bacterial community structure in the active layer is more similar to the transition layer than to the permanently frozen layer (Fig. 3). This is consistent with those observed in other Arctic permafrost (Monteux et al, 2018, Deng et al., 2015), confirming that thawing can homogenize bacterial community structure of different soil depths. However, significant differences in bacterial community were still observed between the active and transition layers (Supplementary Table 8), instead of being identical (Monteux et al, 2018). This could be due to physiochemical heterogeneity between the soils in the different permafrost layers (Fig. 1, Kao-Kniffin, et al., 2015, Mueller et al. 2015). Thus, variations in the measured (such as TN) and unmeasured physicochemical properties (such as pH) among the different permafrost layers also contributed to the bacterial community heterogeneity and led to the significantly different bacterial communities observed.

We have added the following sentence to the conclusion section, which demands further investigation to identify the factors (both environmental and historical) that caused the distinct microbial responses to thawing.

Further studies are required to identify the environmental and historical factors that lead to the distinct responses of bacteria in the permafrost of different ages.

Minor comments:

Q3. 82-91 Please indicate where your replicate samples come from and how many there are. Here it is mentioned that there are four age classes and one soil core per age class, but the figures show a lot more data points (over 40?). Table S6: I am confused how mean relative abundance can be over 100%. Could you clarify? Please also check the definition of SD (should be standard deviation?).

Response:

We thank the reviewer for this comment, the following sentence has been added to clarify the number of replicates used.

In brief, 16 soil cores were collected along a chronosequence of drained lake basins, spanning in age from young (< 50 years old), medium (< 300 years old), old (< 3,000

years old), to ancient (3,000–5,000 years old) in April 2010.

And

For each permafrost age-layer combination, there were four sampling replicates.

For Table S6, we apologize for the mistake. The number presented is the bacterial richness (i.e., number of OTUs observed), but not a percentage number. The spelling of S.D. is corrected throughout the manuscript.

Original table

		Mean±S.D.(%)	Active	Transition	Frozen
Firmicutes	Active	87±15	-	-	-
	Transition	47±15	**0.013**	-	-
	Frozen	49±8	**0.042**	0.986	-
Actinobacteria	Active	128±14	-		
	Transition	106±10	0.062	**0.021**	
	Frozen	71±2	**0.002**	-	
Chloroflexi	Active	36±4	-	-	
	Transition	29±3	0.095	-	
	Frozen	16±6	**0.002**	**0.02**	
Alphaproteobacteria	Active	35±6	-	-	
	Transition	19±7	0.016	-	
	Frozen	16±4	0.016	0.774	
Deltaproteobacteria	Active	25±8	-	-	
	Transition	12±2	**0.028**	-	
	Frozen	11±2	**0.049**	0.976	

Amended table

		Mean±S.D.	Active	Transition	Frozen
Firmicutes	Active	87±15	-	-	-
	Transition	47±15	**0.013**	-	-
	Frozen	49±8	**0.042**	0.986	-
Actinobacteria	Active	128±14	-	-	-
	Transition	106±10	0.062	-	-
	Frozen	71±2	**0.002**	**0.021**	-
Chloroflexi	Active	36±4	-	-	-
	Transition	29±3	0.095	-	-
	Frozen	16±6	**0.002**	**0.02**	-
Alphaproteobacteria	Active	35±6	-	-	-
	Transition	19±7	0.016	-	-
	Frozen	16±4	0.016	0.774	-
Deltaproteobacteria	Active	25±8	-	-	-
	Transition	12±2	**0.028**	-	-
	Frozen	11±2	**0.049**	0.976	-

Minor comments on spelling and grammar:

The spelling and grammar mistakes have been corrected as indicated by the reviewer.

Q4. l. 30 Deltaproteobacterai -> Deltaproteobacteria

Original manuscript:

The bacterial richness was significantly higher in the young and thawed permafrost, and the richness increase was mainly observed in *Firmicutes*, *Actinobacteria*, *Chloroflexi*, ***Deltaproteobacterai***, and *Alphaproteobacteria*.

Amended manuscript:

The bacterial richness was significantly higher in the young and thawed permafrost, and the richness increase was mainly observed in *Firmicutes*, *Actinobacteria*, *Chloroflexi*, ***Deltaproteobacteria***, and *Alphaproteobacteria*.

Q5. l. 95 vary -> varies

Original manuscript:

The surface organic layer thickness **vary** with permafrost age, which was < 5, 10–15, 15–30, and 40–50 cm for the young, medium, old, and ancient–aged permafrost soils (Kao–Kniffin et al., 2015)

Amended manuscript:

The surface organic layer thickness **varies** with permafrost age, which was < 5, 10–15, 15–30, and 40–50 cm for the young, medium, old, and ancient–aged permafrost soils (Kao–Kniffin et al., 2015)

Q6. l. 253 early -> earlier

Original manuscript:

Furthermore, an **early** study on the freshwater ecosystem also confirmed that organic carbon composition determined bacterial richness and community structure

Amended manuscript:

Furthermore, an **earlier** study on the freshwater ecosystem also confirmed that organic carbon composition determined bacterial richness and community structure

Q7. l. 270 Alphaprroteobacterai -> Alphaproteobacteria

Original manuscript:

One possible explanation is that the surface active layer may be the major location for root exudates, which favours ***Alphaprroteobacterai***

Amended manuscript:

One possible explanation is that the surface active layer may be the major location for root exudates, which favors ***Alphaproteobacteria***

Q8. l. 272 Please check language. What enhances their richness?

Original manuscript:

Deltaproteobacteria has been reported to have a strong catabolic potential on the degradation of recalcitrate aromatic and other plant detritus (Jansson and Tas, 2014), thus also enhances their richness in the surface active layer of permafrost soil.

Amended manuscript:

Deltaproteobacteria has been reported to have a strong catabolic potential on the degradation of recalcitrate aromatic and other plant detritus (Jansson and Tas, 2014), which enhances their richness in the surface active layer of permafrost soil.

Q9. l. 292 have -> has

Original manuscript:

Collectively, this suggests that permafrost thawing **have** a stronger influence on the bacterial community structure than permafrost age

Amended manuscript:

Collectively, these suggest that permafrost thawing **has** a stronger influence on bacterial community structure than permafrost age.

Q10. l. 280 results is -> results are

Original manuscript:

Our results **is** consistent with Mondav et al.(2017), who found that permafrost activity better separated the community structure than soil depth in peatland permafrost soil in Sweden.

Amended manuscript:

Our results **are** consistent with Mondav et al. (2017), who found that permafrost activity better separated the community structure than soil depth in peatland permafrost soil in Sweden.

Anonymous Referee #2

Received and published: 19 July 2020

This manuscript reports significant influences of both permafrost thawing and age on bacterial richness and community structure. It also documented that permafrost thawing increased the contribution of determinism to bacterial community assembly, but didn't lead to community convergence. The study then showed that permafrost thawing had a greater influence on bacterial community than permafrost age. They extrapolate their findings to highlight that permafrost thawing in different ages can lead to distinct bacterial community compositions and different soil organic carbon degradation processes. The manuscript is well organized and figures are well prepared. I have several major concerns about this manuscript:

Response:

We sincerely thank reviewer #2 for the careful editing and constructive comments provided. We have carefully amended our manuscript accordingly, please see below.

Q1. I am not an expert in permafrost. But I noticed that the samples used in this study should be the same samples reported in Kao-Kniffinet al. 2015. In this study, the permafrost age of different samples had been measured by Kao-Kniffin et al. 2015. However, basin age is used in that study instead of permafrost age. Does it mean that basin age is equal to permafrost age? If so, Why did this study reported different soil total organic carbon and total nitrogen from Kao-Kniffin et al. 2015?

Response:

The "basin age" used by Kao-Kniffin et al. 2015 was obtained from Hinkel et al., 2003. This age is calculated using radiocarbon dating on sample at the interface of the lacustrine sediment and the *in situ* peat, which represents the point in time of lake drainage and revegetation of the basin surface. The basin drainage is associated with vegetation establishment, organic matter accumulation, and ice-wedge growth below the drained lake basin (Hinkel et al., 2003). Therefore, we used the basin age as a proxy for the formation of permafrost (permafrost age), and explored the influence of permafrost age on the response of bacteria to permafrost thawing. We did not use the

term basin age, as we feel that permafrost age is more meaningful than basin age in this case. We apologize for not stating this clearly, and following sentences are now added to clarify this.

Amended manuscript:

Approximately 20% of the Arctic coastal plains of northern Alaska contain thaw lakes drained at various stages since the mid-Holocene, which were then developed into ice-rich permafrost (Hinkel et al., 2003). These drained lake basins contain soils ranging from freshly developed organic layers on sediments to fully developed ancient permafrost soils (Mueller et al., 2015). By using the drained thaw lake basin age as a proxy for the time of permafrost formation, it provides an opportunity to investigate the influence of permafrost age on microbial community during permafrost degradation. An earlier study at this site has revealed a high abundance of Candidatus *Methanoflorens* archaeon in the community (Kao–Kniffin et al., 2015), but how the bacteria in the permafrost of various ages would respond to thawing remains less understood. Thus, we investigated the interactive influence of permafrost thawing and age on permafrost soil bacterial community.

Regarding the inconsistent environment factors with Kao-Kniffinet al. 2015, the environmental factors (SOC and TN) were independently measured in the present study. They are not substantially different from those in Kao-Kniffinet al. 2015 (Table 1), and the differences could be related to sample storage and the equipment used.

Table 1 Comparison of soil organic carbon (SOC) and total nitrogen (TN) using in the present study and Kao-Kniffin et al.

	SOC (%)		TN (%)	
	The present study	Kao-Kniffin et al. 2015.	The present study	Kao-Kniffin et al. 2015.
Basin age				
Young	8.8 (2.0)	6.9 (1.9)	0.43 (0.09)	0.43 (0.11)
Medium	9.8 (2.5)	8.4 (1.6)	0.45 (0.11)	0.49 (0.09)
Old	12.4 (2.2)	13.4 (1.6)	0.57 (0.10)	0.77 (0.09)
Ancient	13.3 (3.2)	10.7 (2.5)	0.60 (0.13)	0.62 (0.14)
Soil depth layer				
Active	16.9 (2.1)	14.1 (2.1)	0.73 (0.09)	0.78 (0.11)
Transition	10.4 (2.1)	9.6 (1.8)	0.50 (0.09)	0.57 (0.11)
Permafrost	5.6 (1.0)	5.8 (1.4)	0.29 (0.05)	0.36 (0.09)

The values indicate means and standard error (in parentheses)

Q2. It seems that Kao-Kniffin et al. 2015 also used amplicon sequencing of 16S rRNA gene to analyze bacterial communities in different permafrost ages and thawing status. They found that community composition appeared to converge in the active layer, however, the authors in this study didn't observe the community convergence due to permafrost thawing. Can you explain why you reanalyzed bacterial communities of these samples? At least, please compare your study with the results of Kao-Kniffin et al. 2015 and provide more discussion.

Response:

We appreciate the reviewer for this comment. In this manuscript, we focused on the bacterial community only, whereas Kao-Kniffin et al. 2015 investigated the community structure of the entire prokaryotes (both bacteria and archaea). For bacteria, Kao-Kniffin presented the taxonomic composition, community phylogenetic distance, and biomass. Thus, the interactive influence of permafrost age and thawing on bacterial diversity, community structure, and assembly processes still remain unexplored. Therefore, we believe further investigation is necessary and could provide essential knowledge on how permafrost age interact with permafrost thawing. To address the reviewer's comments, we have added the following sentences to clarify

our aims and distinguish our work from Kao–Kniffin et al.:

An earlier study at this site has revealed a high abundance of Candidatus *Methanoflorens* archaeon in the community (Kao–Kniffin et al., 2015), but how the bacteria in the permafrost of various ages would respond to thawing remains less understood. Thus, we investigated the interactive influence of permafrost thawing and age on permafrost soil bacterial community.

Following sentences are added to discuss the inconsistency between our work and Kao–Kniffin et al on community convergence:

Our results demonstrated that bacterial community structure did not converge due to permafrost thawing, as reflected by the non-significant difference in sample heterogeneity among the various permafrost layers (Supplementary Fig. 3, Supplementary Table 11). This contradicts previous studies (Deng et al., 2015; Yuan et al., 2018) in the Arctic, but was consistent with Mackelprang (2011). Our results also contradict to Kao–Kniffin et al. (2015), which reported lowered prokaryotic community differences in the active layer than in the transition and permanently frozen permafrost. Several reasons could cause this inconsistency. Firstly, different microbial communities were targeted. Kao–Kniffin et al. (2015) focused on archaeal community, whereas only bacteria were targeted in the present study. Kao–Kniffin et al. (2015) identified a single archaeon OTU accounting for over 30% of the prokaryotic community (Fig. 3 in Kao-Kniffin et al., 2015). An early study revealed that archaea exhibited a lower community variation with increasing soil depths compared with bacteria (Frank-Fahle et al., 2014). Therefore, the community convergence observed by Kao–Kniffin et al. (2015) could be due to the influence of archaea. Furthermore, the inconsistency may be related to the different community dissimilarity metrics used. Kao–Kniffin et al. (2015) used unweighted UniFrac, which only calculates the phylogenetic closeness of OTUs, and the relative abundance is not considered. This is distinctively different from the Bray-Curtis dissimilarity used in the present study, and it has been reported that unweighted and weighted community metrics examine different features of community strcture (Lozupone et al., 2011).

Q3. The thickness of active, transition and permafrost layers should be different in young, medium, old and ancient permafrost. Please provide more information about the soil profile of different layers in four kinds of permafrost. More variables should be taken into account to undermining the mechanism of bacterial response to permafrost thawing in different permafrost age. I'm not sure that structural equation modelling is a good method to quantify the relative importance of permafrost thawing status and age on bacterial community without any other environmental variables. Please incorporate more variables in structural equation modelling to show how permafrost thawing status and age influenced bacterial community directly or indirectly

Response:

We appreciate the reviewer for this comment. We agree with the reviewer that soil profile characteristics are important in determining the response of bacteria to permafrost thawing. Unfortunately, we have only obtained a small quantity of soil samples, and this does not allow us to measure soil properties in great detail. Therefore, we decided to focus on TOC and TN, which are known to be substantially different in the permafrost with different ages and thawing statuses (Mueller et al., 2015). We have also amended the manuscript to acknowledge the limitation of our work and propose further works that are required to identify the environmental factors that shape bacterial response to permafrost thawing.

Amended manuscript (method section)

Due to sample quantity limitation, two of the most important soil physicochemical properties: total organic carbon (TOC) and total nitrogen (TN) were measured using dry combustion (Vario MAX CNS Analyzer, Elementar, Hanau, Germany). These factors have been reported to substantially vary in samples with different permafrost ages and thawing statuses (Mueller et al., 2015). For other soil properties and soil profile descriptions please see Kao-Kniffin et al (2005).

To address the reviewer's concern and emphasize the importance of environmental heterogeneity in different permafrost layers, we have added the following paragraph

to the manuscript:

Amended manuscript (discussion section)

Bacterial community structure in the active layer is more similar to the transition layer than to the permanently frozen layer (Fig. 3). This is consistent with those observed in other Arctic permafrost (Monteux et al, 2018, Deng et al., 2015), confirming that thawing can homogenize bacterial community structure of different soil depths. However, significant differences in bacterial community were still observed between the active and transition layers (Supplementary Table 8), instead of being identical (Monteux et al, 2018). This could be due to physiochemical heterogeneity between the soils in the different permafrost layers (Fig. 1, Kao-Kniffin, et al., 2015, Mueller et al. 2015). Thus, variations in the measured (such as TN) and unmeasured physicochemical properties (such as pH) among the different permafrost layers also contributed to the bacterial community heterogeneity and led to the significantly different bacterial communities observed.

Amended manuscript (Conclusion section)

Further studies are required to identify the environmental and historical factors that lead to the distinct responses of bacteria in the permafrost of different ages.

Using ranked soil age in SEM to investigate the influence of pedogenic development has been used by Laliberté et al., 2017. Here, we use this idea to investigate the influence of permafrost age on the response of bacteria to permafrost thawing. We repeated the SEM analysis incorporating the TOC and TN as suggested by the reviewer, and a large proportion of the richness and community structure were still explained by the permafrost age and thawing status. Therefore, permafrost age and thawing status can influence the richness and community structure via other unmeasured environmental factors or historical effects. The present study does not attempt to identify the environmental factors that are different in the soils of different permafrost ages and thawing statuses. Instead, we tried to raise the awareness that different aged permafrost may response to climate change induced permafrost

thawing differently. To address this comment, we incorporate TOC and TN into the SEM analysis, and performed Random Forest analysis to identify the importance of permafrost age and thawing status. We also amended the results and conclusion sections to address the needs of identifying environmental/historical factors that directly cause the different responses of bacteria in the permafrost of various ages.

Amended manuscript (Results section)

Structural equation modeling (SEM) revealed that both permafrost thawing status and age significantly contributed to bacterial richness. Permafrost thawing status exhibited a higher contribution than age (standard regression weight of 0.51 and -0.30, respectively, both $P < 0.05$) to bacterial richness (Fig. 4a). However, the influences of TOC and TN on bacterial richness were not detected. This is consistent with the Random Forest analysis results, which only identified permafrost thawing and age as the significant determinants of bacterial richness (Supplementary Fig. 5). For community structure, permafrost thawing exhibited an indirect influence on NMDS1 via TN (standard regression weight of 0.58 and -0.63, both $P < 0.001$, Fig. 4b). In comparison, both permafrost age and thawing status significantly contributed to NMDS2 (standard regression weight of -0.34 and 0.59, respectively, both $P < 0.01$), while TN also exhibited a significant influence on NMDS2 (-0.49, $P = 0.002$). The significant contributions of TN, permafrost thawing, and age were consistently identified using the Random Forest approach (Supplementary Fig. 5).

Amended manuscript (Conclusion section)

Further studies are required to identify the environmental and historical factors that lead to the distinct responses of bacteria in the permafrost of different ages.

Minor comments:

Q4. Fig.2 Can you provide information about bacterial phylogenetic diversity of bacteria in different permafrost age and thawing status?

Response:

We appreciate the reviewer for this comment. We have now included the results of

Phylogenetic diversity and Shannon diversity in the results and discussion sections.

Amended manuscript (Results section)

In comparison, the influence of permafrost age and thawing status on bacteria Shannon diversity was non-significant (Two-way ANOVA, P = 0.058 and 0.53, respectively, Supplementary Fig. 2). This contrastively differed from the phylogenetic diversity, where significant influence was observed for age (P = 0.015) and thawing (P = 0.001).

Amended manuscript (Discussion section)

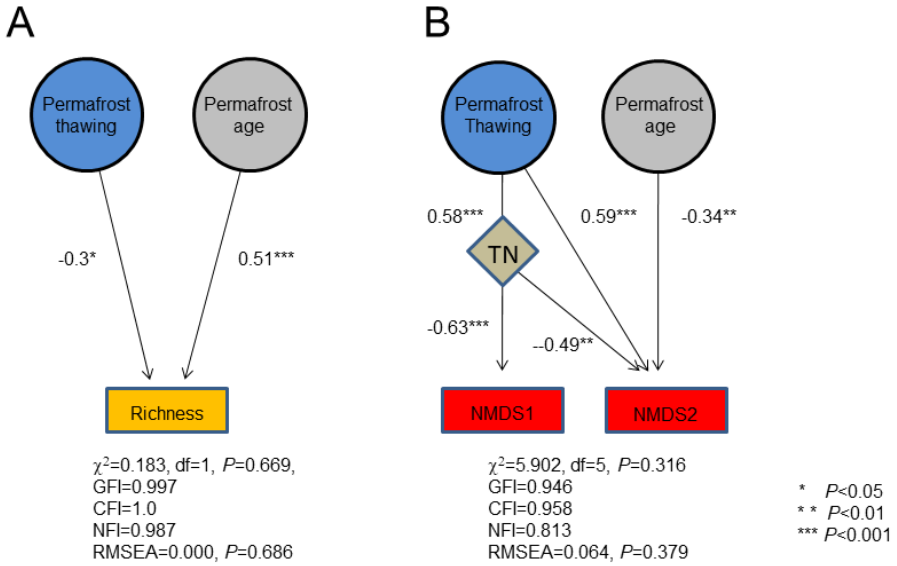
Furthermore, the phylogenetic diversity exhibited a greater sensitivity to permafrost thawing than the Shannon diversity (Supplementary Fig. 2). As phylogenetically close-related microorganisms have similar habitat associations, phylogeny-based community metrics could infer potential community functional change (Stegen et al., 2012). Hence, this suggests that community function could be more sensitive to permafrost thawing than community composition.

Q5. Fig.4 the path coefficients in structural equation modelling can indicate the positive or negative correlations between two variables. Therefore, the raw value should be shown here, instead of the absolute value.

Response:

We appreciate the reviewer for this comment. We have now included the signs of path coefficients in both the figure and manuscript text.

Amended manuscript



Amended manuscript (results section)

Structural equation modeling (SEM) revealed that both permafrost thawing status and age significantly contributed to bacterial richness. Permafrost thawing status exhibited a higher contribution than age (standard regression weight of 0.51 and -0.30, respectively, both $P < 0.05$) to bacterial richness (Fig. 4a). However, the influences of TOC and TN on bacterial richness were not detected. This is consistent with the Random Forest analysis results, which only identified permafrost thawing and age as the significant determinants of bacterial richness (Supplementary Fig. 5). For community structure, permafrost thawing exhibited an indirect influence on NMDS1 via TN (standard regression weight of 0.58 and -0.63, both $P < 0.001$, Fig. 4b). In comparison, both permafrost age and thawing status significantly contributed to NMDS2 (standard regression weight of -0.34 and 0.59, respectively, both $P < 0.01$), while TN also exhibited a significant influence on NMDS2 (-0.49, $P = 0.002$). The significant contributions of TN, permafrost thawing, and age were consistently identified using the Random Forest approach (Supplementary Fig. 5).

Q6. Line 95 vary -> varies

Original manuscript:

The surface organic layer thickness vary with permafrost age, which was < 5, 10–15,

15–30, and 40–50 cm for the young, medium, old, and ancient–aged permafrost soils (Kao–Kniffin et al., 2015)

Amended manuscript:

The surface organic layer thickness varies with permafrost age, which was < 5, 10–15, 15–30, and 40–50 cm for the young, medium, old, and ancient–aged permafrost soils (Kao–Kniffin et al., 2015)

Q7. Line272 Please rewrite this sentence.

Original manuscript:

Deltaproteobacteria has been reported to have a strong catabolic potential on the degradation of recalcitrate aromatic and other plant detritus (Jansson and Tas, 2014), thus also enhances their richness in the surface active layer of permafrost soil.

Amended manuscript:

Deltaproteobacteria has been reported to have strong catabolic potentials on recalcitrate aromatic compounds and plant detritus (Jansson and Tas, 2014). This may explain the enhanced richness of *Deltaproteobacteria* in the active layer of permafrost.

Q8. 280 results is -> results are

Original manuscript:

Our results **is** consistent with Mondav et al.(2017), who found that permafrost activity better separated the community structure than soil depth in peatland permafrost soil in Sweden.

Amended manuscript:

Our results **are** consistent with Mondav et al. (2017), who found that permafrost activity better separated the community structure than soil depth in peatland permafrost soil in Sweden.

Permafrost thawing exhibits a greater influence on bacterial richness and community structure than permafrost age in Arctic permafrost soils

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Running title: Relative influences of permafrost thawing and age on soil bacteria

Abstract

Global warming accelerates permafrost thawing and changes its microbial community structure, but little is known about how microorganisms in permafrost with different ages respond to thawing. Herein, we disentangled the relative importance of permafrost age (young, medium, old, and ancient) spanning from 50 to 5,000 years and thawing status (active, transition, and permanently frozen) in shaping bacterial community structure using Hiseq sequencing of the 16S rRNA gene. Our results revealed significant influences of both permafrost thawing and age on bacterial richness. The bacterial richness was significantly higher in the young and thawed permafrost, and the richness increase was mainly observed in *Firmicutes*, *Actinobacteria*, *Chloroflexi*, *Deltaproteobacteria*, and *Alphaproteobacteria*. Permafrost thawing led to a gradual change in bacterial community structure and increased contribution of determinism. Permutational analysis of variance demonstrated that thawing significantly changed bacterial community structure at all soil ages, but the community convergence due to permafrost thawing was not observed. Structural equation modeling revealed that permafrost thawing exhibited a greater influence on both bacterial richness and community structure than permafrost age. Our results indicate that microorganisms in permafrost with different ages respond differently to thawing, which eventually leads to distinct bacterial community compositions and different organic carbon decomposition processes in Arctic permafrost.

Keywords: Permafrost thawing; permafrost age; bacterial community; richness; Arctic

1 Introduction

Global warming accelerates permafrost thawing, and 200 billion tons of carbon is estimated to be released into the atmosphere from global permafrost over the next 300 years (Turetsky et al., 2019). The degradation of soil organic carbon (SOC) is predominately driven by microorganisms (Frank–Fahle et al., 2014), and the quality and quantity of SOC also control the abundance and community structure of microbial community (Chen et al., 2016). It has been reported that permafrost of different thawing status and ages exhibits distinct labile and recalcitrant carbon quantities, with higher carbohydrates in relation to aliphatic carbon in older than in younger permafrost (Chen et al., 2016; Mueller et al., 2015; Yang et al., 2009). Thus, the distinct SOC composition may subsequently impact the microbial community structure in permafrost soil, and the distinct microbial community structure may respond differently to permafrost thawing. However, the impacts of permafrost age and its interaction with thawing on microbial community remain largely elusive.

In addition to permafrost age, contrasting composition of labile and recalcitrant carbons was also reported in the thawed and frozen permafrost. This was proposed to be due to the distinct microbial transformation process in the different permafrost thawing status (Mueller et al., 2015). The microbes in the frozen permafrost are predominately in a state with reduced metabolism rate (Gilichinskii, 1995), thus labile carbon is protected from microbial degradation (Hobbie et al., 2000). In contrast, permafrost thawing substantially activates a diverse range of oligotrophic and copiotrophic bacteria, and enriches carbohydrate transporter and metabolism–related genes (Schostag et al., 2019). This leads to an increased bacterial richness and converged community metabolic functions, and the soil carbon being dominated by aliphatic carbon resulted from microbial transformation (Deng et al., 2015; Mackelprang et al., 2011; Monteux et al., 2018; Schostag et al., 2019).

Soil development leads to changes in bacterial community structure, predominately due to nutrient accumulation and vegetation colonization (Bardgett and Walker, 2004; Park et al., 2011). Distinct bacterial community structure has been reported in soils of different ages. For example, young soils in the deglaciation chronosequence exhibit significantly lower bacterial richness than aged soils, and autotrophs play a major role in the accumulation of nutrients (Kazemi et al., 2016; Kim et al., 2017; Liu et al., 2016). In contrast, aged soils with vegetations are dominated by heterotrophs, such as *Acidobacteria* and *Actinobacteria* (Kwon et al., 2015). However, little is known about the influence of

permafrost age on soil microbial community.

To explore the effects of permafrost age on the response of bacteria to permafrost thawing, soil bacterial community in Arctic permafrost was characterized using the Illumina sequencing targeting the 16S rRNA gene. Given the continuously changed bacterial community with increasing soil age (Kazemi et al., 2016; Kim et al., 2017), we hypothesized that bacterial richness and community structure would also significantly differ in the permafrost of various ages and response differently to permafrost thawing. Approximately 20% of the Arctic coastal plains of northern Alaska contain thaw lakes drained at various stages since the mid-Holocene, which were then developed into ice-rich permafrost (Hinkel et al., 2003). These drained lake basins contain soils ranging from freshly developed organic layers on sediments to fully developed ancient permafrost soils (Mueller et al., 2015). By using the drained thaw lake basin age as a proxy for the time of permafrost formation, it provides an opportunity to investigate the influence of permafrost age on microbial community during permafrost degradation. An earlier study at this site has revealed a high abundance of Candidatus *Methanoflorens* archaeon in the community (Kao–Kniffin et al., 2015), but how the bacteria in the permafrost of various ages would respond to thawing remains less understood. Thus, we investigated the interactive influence of permafrost thawing and age on permafrost soil bacterial community.

2 Materials and methods

2.1 Site description

The permafrost was sampled in the Barrow Peninsula between 71° 20′ to 71° 27′ N latitude and between 156° 4′ and 156° 7′ W longitude (Kao–Kniffin et al., 2015). Barrow Peninsula is located at the northernmost coast of Alaska and is part of the Arctic Coastal Plain with continuous permafrost. The mean annual temperature is -12°C, and the mean annual precipitation is 104 mm (Mueller et al., 2015). In brief, 16 soil cores were collected along a chronosequence of drained lake basins, spanning in age from young (< 50 years old), medium (< 300 years old), old (< 3,000 years old), to ancient (3,000–5,000 years old) in April 2010. The chronosequence was determined by the degree of plant community succession and ^{14}C carbon dating (Hinkel et al., 2003). At each lake basin, a soil core was collected using a SIPRE corer measuring 80 to 150 cm long and 7.5 cm diameter attached to a Big Beaver earth drill apparatus (Litter Beaver, Inc., Livingstone, TX, USA) mounted on a sled. Each soil core contained three

layers: active, transition, and permanently frozen. The active layer represents the surface soil layer that thaws and refreezes on an annual basis; the transition layer remains frozen, but occasionally thaws during warmer summers; the permanently frozen layer remains annually frozen (Kao–Kniffin et al., 2015). The surface organic layer thickness varies with permafrost age, which is < 5, 10–15, 15–30, and 40–50 cm for the young, medium, old, and ancient–aged permafrost (Kao–Kniffin et al., 2015). For each permafrost age-layer combination, there were four sampling replicates. The frozen soil cores were cut with a chop–saw into sections of soils horizons in a cold room in Barrow, and soils were homogenized, stored, and transported at -20 °C until processed (Mueller et al., 2015). Due to sample quantity limitation, two of the most important soil physicochemical properties: total organic carbon (TOC) and total nitrogen (TN) were measured using dry combustion (Vario MAX CNS Analyzer, Elementar, Hanau, Germany). These factors have been reported to substantially vary in samples with different permafrost ages and thawing statuses (Mueller et al., 2015). For other soil properties and soil profile descriptions please see Kao-Kniffin et al (2005).

2.2 DNA extraction and sequencing

Total DNA was extracted using the MO BIO Power Soil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Universal primer set 515F (5'–GTGCCAGCMGCCGCGGTAA–3') and 806r (5'–GGACTACHVGGGTWTCTAAT–3') with 12–nt unique barcodes was used to amplify the V4 hyper–variable region of the 16S rRNA gene (Caporaso et al., 2012). The PCR mixture (25 µl) contained 1x PCR buffer, 1.5 mM of $MgCl_2$, 0.4 µM each of deoxynucleoside triphosphate bases, 1.0 µM of each primer, 0.5 U of Ex Taq (TaKaRa, Dalian, China) and 20 ng of DNA template. The PCR amplification program included an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR products were pooled in equal molar amounts, and then used for pair–end sequencing (2x250 bp) on the Illumina HiSeq 2500 sequencer at the Magigene (Guangzhou, China).

2.3 Data processing

Three samples generated very low reads, to avoid artifacts from different sequencing batches, these three samples were removed from the downsteam analysis. Raw sequence data were processed using the MOTHUR v. 1.34.3 (Schloss et al., 2009). Paired–end reads were merged and quality screened with the

following settings: as the amplicon size was approximately 300 bp, sequences with length <250 or >350, more than 1 mismatch in the primer region, average quality < 30, ambiguous bases >0 and homopolymer length >9 were removed from the subsequent downstream analysis. The sequences were then aligned against the Silva reference alignment (release 128), which was trimmed to include only the same region amplified, and those sequences that did not align were removed. Chimeric sequences were identified using the UCHIME(Edgar et al., 2011) and removed. The remaining sequences were classified using the Bayesian classifier against the Silva database (release 128), with a minimum confidence score of 80% (Wang et al., 2007), and all Eukaryota, chloroplasts, mitochondria and unknown sequences were removed. Archaeal sequences were also removed to concentrate the study on the bacterial community. Finally, sequences were classified into operational taxonomic units (OTUs) at the 97% identity, and singletons were then removed. The dataset was sub–sampled to an equal depth of 16,144, which was the smallest sample size across the entire dataset. Bacterial richness, evenness, and Shannon diversity indices were calculated using the summary.single command in the Mothur program (Schloss et al., 2009). Bacterial phylogenetic diversity index was calculated using the pd command in the Picante package (Kembel et al., 2010) under the R environment.

2.4 Statistical analysis

Bacterial richness, evenness, Shannon diversity, phylogenetic diversity, total organic carbon, and total nitrogen in the samples with different permafrost ages and thawing statuses were compared using the two–way ANOVA, and pairwise differences were assessed using the Tukey's HSD test. The Levene's test was used to ensure the homogeneity of variances for the dependent variables (bacterial richness, TOC, and TN) for each combination of the independent variables(Brown and Forsythe, 1974). One–way ANOVA was used to examine the significance of the differences among the permafrost soils of different thawing statuses within the same age class. Both Two-way ANOVA and one-way ANOVA were performed using SPSS 23 (SPSS Inc., Armonk, NY, USA).

Non–metric Multidimensional Scaling (NMDS) plot was generated from the Hellinger–transformed bacterial community dataset based on the Bray–Curtis dissimilarity matrix using Primer 6 (Clarke and Warwick, 2006). The contributions of carbon, nitrogen, C:N ratio, permafrost age, and thawing status to the community structure were quantified using the distance–based linear model (DistLM) after normalization. Permutational analysis of variance (PERMANOVA) was used to examine the influence

of permafrost thawing status and age on bacterial community structure (Anderson, 2001) using Primer 6. We compared the multivariate dispersion homogeneity to assess the bacterial community convergence by permafrost thawing status, using permutational analysis of multivariate dispersions (PERMDISP) (Anderson, 2006).

2.5 Quantifying the contribution of stochasticity

Bray–Curtis dissimilarity based normalized stochasticity ratio (NST) index was calculated using the 'NST' package in R (http//www.r-project.org) to represent the contribution of stochasticity to community assembly (Ning et al., 2019). The NST index values range from 0% to 100%, a 0% indicates zero contribution of stochasticity, whereas 100% indicates the community assembly being completely stochasticity–driven.

2.6 Structural equation modeling (SEM) analysis

SEM was conducted using AMOS 21 software (IBM SPSS Inc., Chicago, IL, USA) to assess the relative importance of permafrost thawing status and age in shaping bacterial richness and community structure. The permafrost age was ranked from 1 to 4 for the youngest to the oldest permafrost soils, whereas the thawing status was ranked from 1 to 3 for the active to frozen permafrost. The standardized regression weights were calculated for the bacterial richness and the 1^{st} and 2^{nd} axis coordinates of the NMDS ordination plot. The goodness of fit for the model was judged by the following measures(Guo et al., 2015): (1) comparative fit index (CFI, the model has a good fit when $0.97 \leq CFI \leq 1.00$, and an acceptable fit when $0.95 \leq CFI < 0.97$); (2) goodness–of–fit index (GFI, the model has a good fit when $0.95 \leq GFI \leq 1.00$, and acceptable fit when $0.90 \leq GFI < 0.95$); (3) normed fit index (NFI, the model has a good fit when $0.95 \leq NFI \leq 1.00$ and an acceptable fit when $0.90 \leq NFI < 0.95$); (4) χ^2 test; the model has a good fit when $0 \leq \chi^2/d.f. \leq 2$ and $0.05 < P \leq 1.00$, and an acceptable fit when $2 < \chi^2/d.f. \leq 3$ and $0.01 \leq P \leq 0.05$); and (5) the root mean square error of approximation (RMSEA, the model has a good fit when $0 \leq RMSEA \leq 0.05$ and $0.10 < P \leq 1.00$, and an acceptable fit when $0.05 < RMSEA \leq 0.08$ and $0.05 \leq P \leq 0.10$). The relative contributions of TOC, TN, permafrost age, and thawing status to the richness and community structure were also evaluated by the Random Forest approach using the rfPermute package for R (Archer, 2016).

3 Results

3.1 The influence of permafrost age and thawing status on soil organic carbon and nitrogen

Across all samples, soil total organic carbon (TOC) ranged from 0.5% to 35.6%, and exhibited significant differences by permafrost thawing status (Two–way ANOVA, $P < 0.01$, Fig. 1a), but not by permafrost age ($P = 0.343$, Fig. 1b). The active layer soil exhibited the highest TOC (16.7%), and was significantly higher than the permanently frozen layer soil (5.6%, Tukey's HSD $P < 0.001$). Soil total nitrogen (TN) ranged from 0.1% to 1.5%, and significant differences were only detected by permafrost thawing status ($P = 0.007$, Fig. 1c), but not by permafrost age ($P = 0.446$, Fig.1d). The active layer soil exhibited the highest TN (0.73%), and was significantly higher than the permanently frozen layer soil (0.29%, Tukey's HSD, $P = 0.004$).

3.2 The influence of permafrost age and thawing status on bacteria richness

A total of 1,679,607 bacterial sequences were retained, with an average sequence length of 292 bp. After rarefying to an equal sequencing depth, there were 2,415 bacterial OTUs at the 97% nucleic acid sequence identity retained, and the community was dominated by *Firmicutes* (42%), *Actinobacteria* (28.9%), and *Proteobacteria* (10.6%, Supplementary Fig. 1).

Our results exhibited substantial differences in the bacterial richness among the permafrost soils of different thawing status (Two–way ANOVA, $P < 0.001$; Fig. 2a, Supplementary Table 1) and ages ($P = 0.013$; Fig. 2b). A significantly higher bacterial richness was observed in the active layer soil (358 OTUs) than the transition (287 OTUs; Pairwise Tukey's HSD tests, $P = 0.011$) and the frozen layer soils (248 OTUs, $P < 0.001$, Supplementary Table 2). Young permafrost (380 OTUs) exhibited a significantly higher bacterial richness than the medium (265 OTUs, $P = 0.001$), old (287287, $P = 0.002$), and ancient soils (271 OTUs, $P = 0.009$, Supplementary Table 3). In comparison, the influence of permafrost age and thawing status on bacteria Shannon diversity was non-significant (Two-way ANOVA, $P = 0.058$ and 0.53, respectively, Supplementary Fig. 2). This contrastively differed from the phylogenetic diversity, where significant influence was observed for age ($P = 0.015$) and thawing ($P = 0.001$).

The influence of permafrost thawing on bacterial richness was only significantly observed in the young permafrost (one–way ANOVA, $P < 0.001$, Fig. 2b, Supplementary Table 4), whereas those in the medium,

old, and ancient soils were non–significant (P = 0.445, 0.48, and 0.35, respectively). In the young permafrost, permafrost thawing significantly increased OTU number from 248 in the frozen layer soil to 471 in the active layer soil (Supplementary Table 5). The increased bacterial richness was mainly attributed to the significantly increase detected in *Firmicutes* (ANOVA, P = 0.011), *Actinobacteria* (P = 0.002), *Chloroflexi* (P = 0.002), *Deltaproteobacteria* (P = 0.02), and *Alphaproteobacteria* (P = 0.008; Supplementary Table 6).

3.3 The influence of permafrost thawing status and age on bacterial community structure

Bray–Curtis distance based NMDS ordination plot revealed a clear separation of the bacterial community structure by permafrost thawing status (Fig. 3a), while the separation by permafrost age was less obvious (Fig. 3b). The results of DistLM analyses revealed that the measured soil factors, thawing status, and age explained a total of 10.7% of the bacterial community structure. TN was the most important factor by explaining 7.2% of the community structure (P = 0.001). This was followed by C:N ratio, TOC, soil age and thawing status, which explained additional 3.5% (P = 0.028), 3% (P = 0.083), 2.9% (P = 0.105), and 2.8% (P = 0.111), respectively.

PERMANOVA indicated that significantly different community structure was observed among the various permafrost thawing status and ages (both $P < 0.001$, Supplementary Table 7), and an interactive effect of the two existed ($P < 0.001$). Post–hoc analysis indicated that the community structure differences were significantly different among the soils with distinct thawing statuses (all $P < 0.01$, Supplementary Table 8). In contrast, significant differences were only detected between the young– and older–aged permafrost soils (all $P < 0.05$, Supplementary Table 9), and between the medium–and ancient–aged soils (P = 0.024). PERMDISP analysis indicated that the community homogeneity was not significantly different across the different permafrost thawing status ($F(2, 42) = 0.193$, P = 0.831). A gradual transition of bacterial community structure due to permafrost thawing was observed in each permafrost age category (Figs. 3c–f). Significantly different soil bacterial community structure across the various thawing status was detected in the young, medium, and ancient–aged permafrost (PERMANOVA, P = 0.002, 0.027, and 0.016, respectively, Supplementary Table 10), but not in the old permafrost (P = 0.124). Similarly, significantly different soil bacterial structure was also detected among the permafrost of different ages with the same thawing status (Supplementary Table 11, Supplementary

Fig. 3).

3.4 The influence of permafrost thawing status and age on the community assembly of bacteria

The average contribution of stochasticity to community assembly was 68%, 74%, and 86% in the active, transition, and frozen layers of the permafrost. Significant differences in the contribution of stochasticity were detected between the active and frozen and between the transition and frozen layers (both $P < 0.05$, Supplementary Fig. 4a), but not between the active and transition layers ($P = 0.15$). In contrast, the average contribution of stochasticity was 65%, 76%, 68%, and 76% for the young–, medium–, old–, and ancient–aged permafrost, with no significant contribution differences among the different aged permafrost (all $P > 0.05$, Supplementary Fig. 4b).

3.5 Quantifying the influence of permafrost thawing status and age on bacterial richness and community structure variation

Structural equation modeling (SEM) revealed that both permafrost thawing status and age significantly contributed to bacterial richness. Permafrost thawing status exhibited a higher contribution than age (standard regression weight of 0.51 and -0.30, respectively, both $P < 0.05$) to bacterial richness (Fig. 4a). However, the influences of TOC and TN on bacterial richness were not detected. This is consistent with the Random Forest analysis results, which only identified permafrost thawing and age as the significant determinants of bacterial richness (Supplementary Fig. 5). For community structure, permafrost thawing exhibited an indirect influence on NMDS1 via TN (standard regression weight of 0.58 and -0.63, both $P < 0.001$, Fig. 4b). In comparison, both permafrost age and thawing status significantly contributed to NMDS2 (standard regression weight of -0.34 and 0.59, respectively, both $P < 0.01$), while TN also exhibited a significant influence on NMDS2 (-0.49, $P = 0.002$). The significant contributions of TN, permafrost thawing, and age were consistently identified using the Random Forest approach (Supplementary Fig. 5).

4 Discussion

The bacteria richness was significantly higher in the active layer soil (Fig. 2a), and this is consistent with the previous findings that permafrost thawing significantly increased bacterial richness in soil in the Tibetan Plateau and the high Arctic (Chen et al., 2017; Schostag et al., 2019; Wu et al., 2018). Permafrost

thawing leads to accelerated microbial degradation of soil organic carbon that can generate a wide variety of metabolic products (Mueller et al., 2015). The increased metabolic product diversity would lead an increased nutrient diversity and provide additional ecological niches for bacteria (Hernández and Hobbie, 2010). This would explain the increased bacterial richness observed in our study. Furthermore, the phylogenetic diversity exhibited a greater sensitivity to permafrost thawing than the Shannon diversity (Supplementary Fig. 2). As phylogenetically close-related microorganisms have similar habitat associations, phylogeny-based community metrics could infer potential community functional change (Stegen et al., 2012). Hence, this suggests that community function could be more sensitive to permafrost thawing than community composition.

The soil bacteria in the young permafrost exhibited a stronger response to thawing than those in older permafrost soils (Fig. 2b). The young permafrost soil demonstrated a higher relative abundance of aliphatic carbon but lower carbohydrates than older permafrost soils (Mueller et al., 2015). Thus, bacterial richness could be driven by carbon quality, but not quantity. It has been reported that the degradation of complex carbon molecules requires extensive microbial collaboration, thus leads to a more diverse microbial community in forest soil (Ding et al., 2015). Furthermore, an earlier study on the freshwater ecosystem also confirmed that organic carbon composition determined bacterial richness and community structure (Docherty et al., 2006). This is in agreement with the higher bacterial richness detected in the active layer of the young permafrost soil (Fig. 2b).

The increased bacterial richness due to permafrost thawing was mainly attributed to *Firmicutes*, *Actinobacteria*, *Chloroflexi*, *Deltaproteobacteria*, and *Alphaproteobacteria* in the young permafrost soil (Supplementary Table 6). Increased transcriptional response of *Chloroflexi* has been reported during permafrost thawing (Coolen and Orsi, 2015), and may be attributed to their recalcitrate organic matter degradation capacity (Colatriano et al., 2018). *Firmicutes* and *Actinobacteria* have been reported to be more abundant in the frozen layer than in the active layer of permafrost soil due to their capacities in maintaining metabolic activity and DNA repair mechanisms at low temperature (Johnson et al., 2007; Tuorto et al., 2014). However, our results showed that their diversity may increase during permafrost thawing, despite their reduction in relative abundance. *Alpha–* and *Delta–proteobacteria* were both abundant in the upper permafrost soil in the Tibetan Plateau, and their relative abundance negatively correlated with soil depth (Wu et al., 2017). *Alphaproteobacteria* was identified to be more abundant in

the active layer of the permafrost soil in Norway (Mueller et al., 2018). One possible explanation is that the surface active layer may be the major location for root exudates, which favors *Alphaproteobacteria* (Morgalev et al., 2017). *Deltaproteobacteria* has been reported to have strong catabolic potentials on recalcitrate aromatic compounds and plant detritus (Jansson and Tas, 2014). This may explain the enhanced richness of *Deltaproteobacteria* in the active layer of permafrost.

PERMANOVA, SEM, and Random Forest analyses consistently demonstrated statistically significant contributions of permafrost thawing and age to soil bacterial community structure (Fig. 4b). Bacterial communities were better separated by thawing status than by age on the NMDS plots (Figs. 3a and 3b). Furthermore, a significantly higher contribution of determinism (lower stochasticity) was observed in the thawed permafrost soils (active and transition layers) than in the permanently frozen layer. This contrastive difference from the weak influence of permafrost age on the bacterial community (Supplementary Fig.4). Collectively, these suggest that permafrost thawing has a stronger influence on bacterial community structure than permafrost age. Our results are consistent with Mondav et al. (2017), who found that permafrost activity better separated the community structure than soil depth in peatland permafrost soil in Sweden.

Permafrost thawing significantly increased determinism in bacterial community structure (Supplementary Fig. 4). Increased determinism is frequently attributed to enhanced environmental filtering (Stegen et al., 2012). Our results demonstrated that TN and the C:N ratio explained a greater proportion of the bacterial community structure than TOC. This is consistent with the previous findings that nitrogen availability strongly regulates microbial community structure and function in the permafrost soils of Arctic and Tibetan Plateau (Chen et al., 2018; Chen et al., 2017; Yergeau et al., 2010). Significantly different soil carbon and nitrogen were observed among the various permafrost thawing statuses, but not among the different permafrost ages (Figs. 1a and 1c). Thus the changed nutrients may explain the significant influence of thawing status on the community structure and assembly processes. The community structure change due to permafrost thawing has also been proposed to be due to the colonization of microorganisms in the active layer (Monteux et al., 2018), which coincides with the increased bacterial richness observed here (Fig. 2a).

Bacterial community structure in the active layer is more similar to the transition layer than to the permanently frozen layer (Fig. 3). This is consistent with those observed in other Arctic permafrost (Monteux et al, 2018, Deng et al., 2015), confirming that thawing can homogenize bacterial community structure of different soil depths. However, significant differences in bacterial community were still observed between the active and transition layers (Supplementary Table 8), instead of being identical (Monteux et al, 2018). This could be due to physiochemical heterogeneity between the soils in the different permafrost layers (Fig. 1, Kao-Kniffin, et al., 2015, Mueller et al. 2015). Thus, variations in the measured (such as TN) and unmeasured physicochemical properties (such as pH) among the different permafrost layers also contributed to the bacterial community heterogeneity and led to the significantly different bacterial communities observed.

The influence of permafrost age on bacterial community structure was weaker (Fig. 3b), with only significantly different community structure being observed in the young– and medium–aged permafrost soils (Supplementary Table 8). Substantial influence of permafrost age on community structure has been reported previously (Mackelprang et al., 2017). Investigation on the pedogenesis following deglaciation also revealed distinct microbial community structure along the chronosequence (Freedman and Zak, 2015). However, the community differences between the old and ancient permafrost soils were much weaker than expected (Supplementary Table 9). This is likely due to the strong influence of permafrost thawing, as thawing enhances environmental filtering (Supplementary Fig. 4) and homogenizes community structure in soils of different ages. This is confirmed by the significantly different bacterial community structure in permafrost soils of the same age along the thawing gradient (except the old permafrost soil, Figs 3c–3f, Supplementary Table 10).

Our results demonstrated that bacterial community structure did not converge due to permafrost thawing, as reflected by the non-significant difference in sample heterogeneity among the various permafrost layers (Supplementary Fig. 3, Supplementary Table 11). This contradicts previous studies (Deng et al., 2015; Yuan et al., 2018) in the Arctic, but was consistent with Mackelprang (2011). Our results also contradict to Kao–Kniffin et al. (2015), which reported lowered prokaryotic community differences in the active layer than in the transition and permanently frozen permafrost. Several reasons could cause this inconsistency. Firstly, different microbial communities were targeted. Kao–Kniffin et al. (2015) focused on archaeal community, whereas only bacteria were targeted in the present study. Kao–Kniffin

et al. (2015) identified a single archaeon OTU accounting for over 30% of the prokaryotic community (Fig. 3 in Kao-Kniffin et al., 2015). An early study revealed that archaea exhibited a lower community variation with increasing soil depths compared with bacteria (Frank-Fahle et al., 2014). Therefore, the community convergence observed by Kao–Kniffin et al. (2015) could be due to the influence of archaea. Furthermore, the inconsistency may be related to the different community dissimilarity metrics used. Kao–Kniffin et al. (2015) used unweighted UniFrac, which only calculates the phylogenetic closeness of OTUs, and the relative abundance is not considered. This is distinctively different from the Bray-Curtis dissimilarity used in the present study, and it has been reported that unweighted and weighted community metrics examine different features of community strcture (Lozupone et al., 2011).

The distinct bacterial community structure in the various aged permafrost soils, yet under the same thawing status, confirms the historic effects of permafrost age on the community structure during permafrost thawing. The distinct bacterial community structure is likely to result in different metabolic functions (Brown and Forsythe, 1974), thus the significantly different bacterial structure under the same thawing status may lead to different organic carbon degradation capacities. Furthermore, older permafrosts enriches pathways involved in the degradation of recalcitrant biomass, while decreases pathways associated with starch and sucrose metabolism comparing with younger soils (Mackelprang et al., 2017). Thus, the thawing of permafrost soils of different ages may also lead to distinct soil carbon degradation schemes.

5 Conclusion

Our results demonstrated that permafrost thawing consistently exhibited greater influence on bacterial richness and community structure than permafrost age. However, permafrost age alters the response of permafrost soil bacteria to thawing, with a stronger response to thawing observed in the young than older permafrost soils. The different community structure during permafrost thawing may present distinct metabolic potentials for soil organic carbon cycling, and may ultimately alter the carbon emission scheme. Further studies are required to identify the environmental and historical factors that lead to the distinct responses of bacteria in the permafrost of different ages.

Data availability

Sequence data generated in the present study have been deposited to theNational Center for Biotechnology Information (NCBI) Sequence Read Archive under the ID PRJNA554442.

Author contributions

WK conceived the study and developed the idea with MJ, TZ and HZ performed DNA extraction, MJ conducted the data statistical analysis. MJ and WK wrote the first draft of the manuscript, CL and XD revised the manuscript substantially. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Figure legends

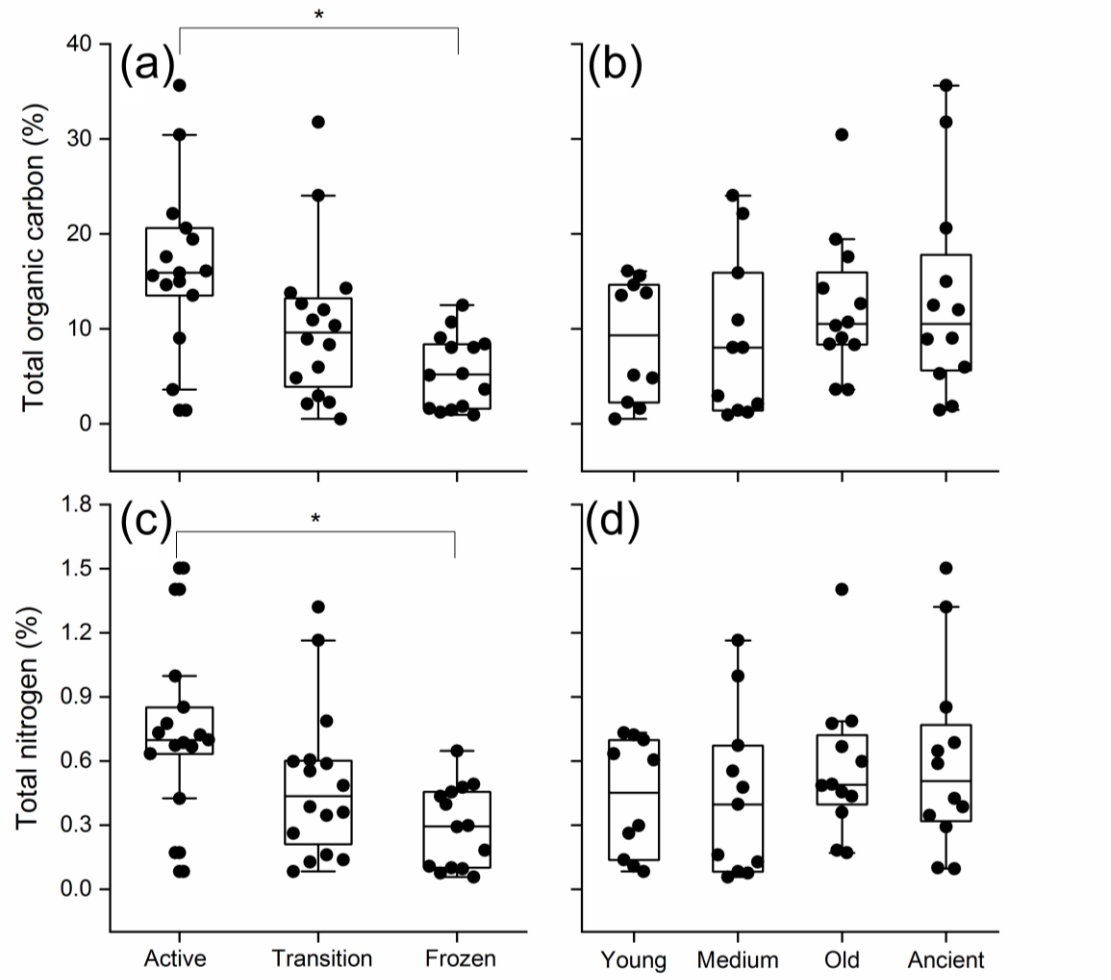
Fig. 1 Total organic carbon (a and b) and total nitrogen (c and d) with the permafrost age (young, medium, old, and ancient) and permafrost thawing status (active, transition and permanently frozen).

Fig. 2 Bacterial richness with the permafrost thawing status(a) and age(b). The richness is indicated by operational taxonomic unit (OTU) number. Different letters indicate significant difference at $P < 0.05$. Young, medium, old, and ancient are permafrost soil ages, active, transition, and permanently frozen are permafrost thawing status.

Fig. 3 NMDS plots showing the bacterial community structure of different thawing status (a) and permafrost age (b). The bacterial community structure of different thawing status in the young, medium, old, and ancient permafrost soils are shown in (c)–(f). Active, transition, and permanently frozen are permafrost thawing status.

Fig. 4 The relative importance of permafrost thawing status and age on bacterial richness (a) and community structure (b) based on structural equation modeling. The community structure variation was assessed by the 1st and 2ndaxis coordinates of the NMDS plot (NMDS1 and NMDS2). Numbers adjacent to arrows are the absolute value of the path coefficients, indicative of the standardized effect size of the relationship. *: $P < 0.05$, **: $P < 0.01$and ***:$P < 0.001$. The arrow thickness represents the strength of the relationship.

548 Fig. 1



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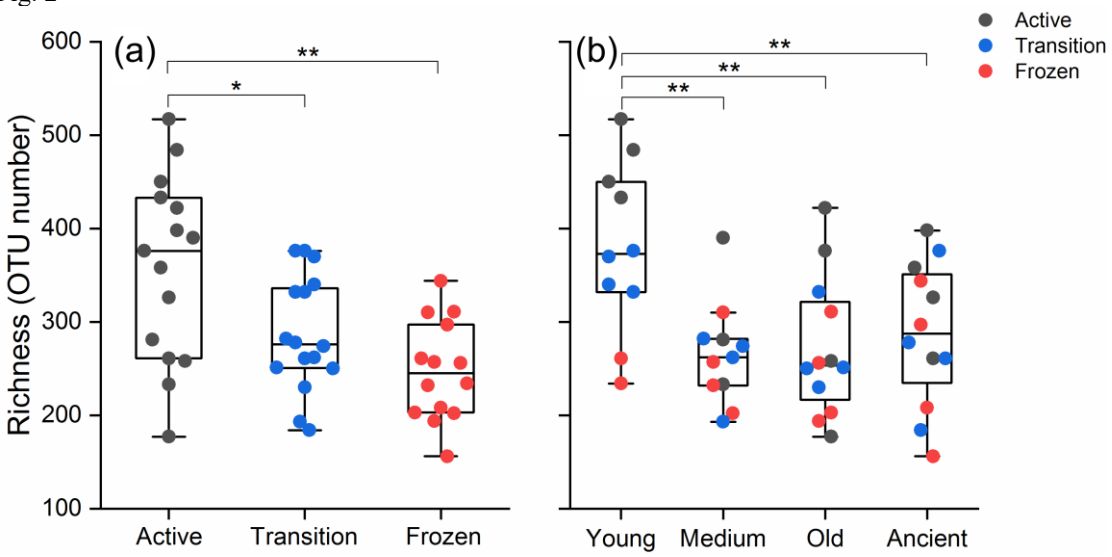
551 Fig. 2



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554 Fig. 3
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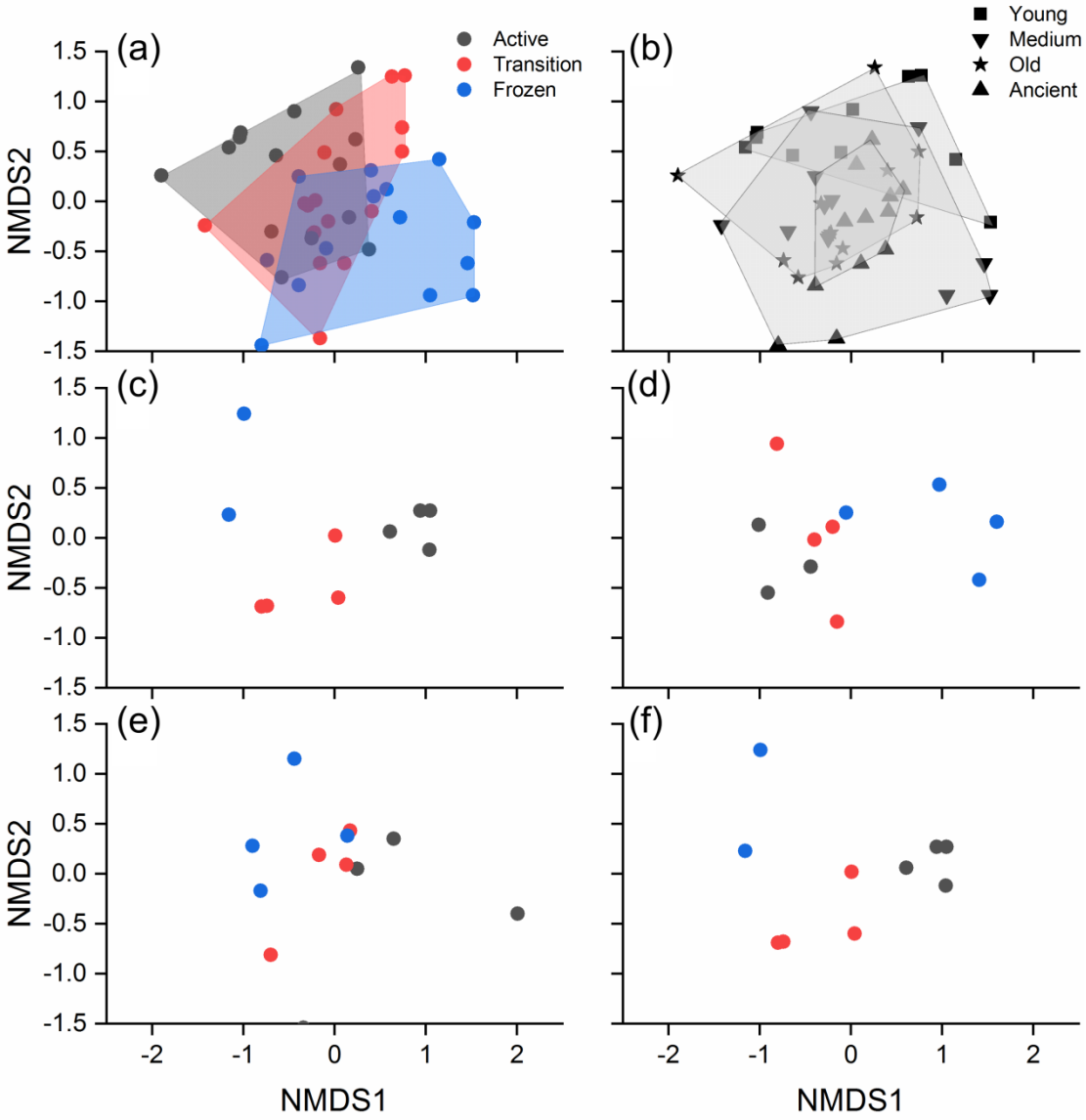

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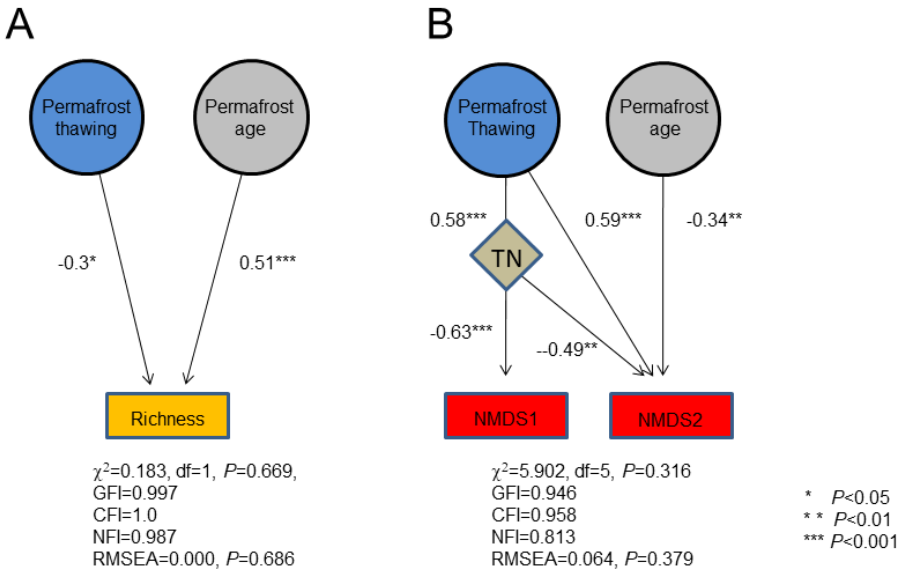
558 Fig. 4



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