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

70   permafrost age on soil microbial community.

71   To explore the effects of permafrost age on the response of bacteria to permafrost thawing, soil bacterial

72   community in Arctic permafrost was characterized using the Illumina sequencing targeting the 16S rRNA

73   gene. Given the continuously changed bacterial community with increasing soil age (Kazemi et al., 2016;

74   Kim et al., 2017), we hypothesized that bacterial richness and community structure would also

75   significantly differ in the permafrost of various ages and response differently to permafrost thawing.

76   Approximately 20% of the Arctic coastal plains of northern Alaska contain thaw lakes drained at various

77   stages since the mid-Holocene, which were then developed into ice-rich permafrost (Hinkel et al., 2003).

78   These drained lake basins contain soils ranging from freshly developed organic layers on sediments to

79   fully developed ancient permafrost soils (Mueller et al., 2015). By using the drained thaw lake basin age

80   as a proxy for the time of permafrost formation, it provides an opportunity to investigate the influence of

81   permafrost age on microbial community during permafrost degradation. An earlier study at this site has

82   revealed a high abundance of Candidatus *Methanoflorens* archaeon in the community (Kao–Kniffin et

83   al., 2015), but how the bacteria in the permafrost of various ages would respond to thawing remains less

84   understood. Thus, we investigated the interactive influence of permafrost thawing and age on permafrost

85   soil bacterial community.

86   **2 Materials and methods**

87   *2.1 Site description*

88   The permafrost was sampled in the Barrow Peninsula between 71° 20′ to 71° 27′ N latitude and between

89   156° 4′ and 156° 7′ W longitude (Kao–Kniffin et al., 2015). Barrow Peninsula is located at the

90   northernmost coast of Alaska and is part of the Arctic Coastal Plain with continuous permafrost. The

91   mean annual temperature is -12°C, and the mean annual precipitation is 104 mm (Mueller et al., 2015).

92   In brief, 16 soil cores were collected along a chronosequence of drained lake basins, spanning in age

93   from young (< 50 years old), medium (< 300 years old), old (< 3,000 years old), to ancient (3,000–5,000

94   years old) in April 2010. The chronosequence was determined by the degree of plant community

95   succession and ^{14}C carbon dating (Hinkel et al., 2003). At each lake basin, a soil core was collected using

96   a SIPRE corer measuring 80 to 150 cm long and 7.5 cm diameter attached to a Big Beaver earth drill

97   apparatus (Litter Beaver, Inc., Livingstone, TX, USA) mounted on a sled. Each soil core contained three

98   layers: active, transition, and permanently frozen. The active layer represents the surface soil layer that

99   thaws and refreezes on an annual basis; the transition layer remains frozen, but occasionally thaws during

100 warmer summers; the permanently frozen layer remains annually frozen (Kao–Kniffin et al., 2015). The

101 surface organic layer thickness varies with permafrost age, which is < 5, 10–15, 15–30, and 40–50 cm

102 for the young, medium, old, and ancient–aged permafrost (Kao–Kniffin et al., 2015). For each permafrost

103 age-layer combination, there were four sampling replicates. The frozen soil cores were cut with a chop–

104 saw into sections of soils horizons in a cold room in Barrow, and soils were homogenized, stored, and

105 transported at -20 °C until processed (Mueller et al., 2015). Due to sample quantity limitation, two of the

106 most important soil physicochemical properties: total organic carbon (TOC) and total nitrogen (TN) were

107 measured using dry combustion (Vario MAX CNS Analyzer, Elementar, Hanau, Germany). These factors

108 have been reported to substantially vary in samples with different permafrost ages and thawing statuses

109 (Mueller et al., 2015). For other soil properties and soil profile descriptions please see Kao-Kniffin et al

110 (2005).

111 *2.2 DNA extraction and sequencing*

112 Total DNA was extracted using the MO BIO Power Soil DNA extraction kit (Mo Bio Laboratories,

113 Carlsbad, CA, USA) according to the manufacturer's instructions. Universal primer set 515F (5'–

114 GTGCCAGCMGCCGCGGTAA–3') and 806r (5'–GGACTACHVGGGTWTCTAAT–3') with 12–nt

115 unique barcodes was used to amplify the V4 hyper–variable region of the 16S rRNA gene (Caporaso et

116 al., 2012). The PCR mixture (25 μl) contained 1x PCR buffer, 1.5 mM of $MgCl_2$, 0.4 μM each of

117 deoxynucleoside triphosphate bases, 1.0 μM of each primer, 0.5 U of Ex Taq (TaKaRa, Dalian, China)

118 and 20 ng of DNA template. The PCR amplification program included an initial denaturation at 94 °C

119 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

120 at 72 °C for 10 min. PCR products were pooled in equal molar amounts, and then used for pair–end

121 sequencing (2x250 bp) on the Illumina HiSeq 2500 sequencer at the Magigene (Guangzhou, China).

122 *2.3 Data processing*

123 Three samples generated very low reads, to avoid artifacts from different sequencing batches, these three

124 samples were removed from the downsteam analysis. Raw sequence data were processed using the

125 MOTHUR v. 1.34.3 (Schloss et al., 2009). Paired–end reads were merged and quality screened with the

126 following settings: as the amplicon size was approximately 300 bp, sequences with length <250 or >350,

127 more than 1 mismatch in the primer region, average quality < 30, ambiguous bases >0 and homopolymer

128 length >9 were removed from the subsequent downstream analysis. The sequences were then aligned

129 against the Silva reference alignment (release 128), which was trimmed to include only the same region

130 amplified, and those sequences that did not align were removed. Chimeric sequences were identified

131 using the UCHIME(Edgar et al., 2011) and removed. The remaining sequences were classified using the

132 Bayesian classifier against the Silva database (release 128), with a minimum confidence score of 80%

133 (Wang et al., 2007), and all Eukaryota, chloroplasts, mitochondria and unknown sequences were removed.

134 Archaeal sequences were also removed to concentrate the study on the bacterial community. Finally,

135 sequences were classified into operational taxonomic units (OTUs) at the 97% identity, and singletons

136 were then removed. The dataset was sub–sampled to an equal depth of 16,144, which was the smallest

137 sample size across the entire dataset. Bacterial richness, evenness, and Shannon diversity indices were

138 calculated using the summary.single command in the Mothur program (Schloss et al., 2009). Bacterial

139 phylogenetic diversity index was calculated using the pd command in the Picante package (Kembel et

140 al., 2010) under the R environment.

141 *2.4 Statistical analysis*

142 Bacterial richness, evenness, Shannon diversity, phylogenetic diversity, total organic carbon, and total

143 nitrogen in the samples with different permafrost ages and thawing statuses were compared using the

144 two–way ANOVA, and pairwise differences were assessed using the Tukey's HSD test. The Levene's test

145 was used to ensure the homogeneity of variances for the dependent variables (bacterial richness, TOC,

146 and TN) for each combination of the independent variables(Brown and Forsythe, 1974). One–way

147 ANOVA was used to examine the significance of the differences among the permafrost soils of different

148 thawing statuses within the same age class. Both Two-way ANOVA and one-way ANOVA were

149 performed using SPSS 23 (SPSS Inc., Armonk, NY, USA).

150 Non–metric Multidimensional Scaling (NMDS) plot was generated from the Hellinger–transformed

151 bacterial community dataset based on the Bray–Curtis dissimilarity matrix using Primer 6 (Clarke and

152 Warwick, 2006). The contributions of carbon, nitrogen, C:N ratio, permafrost age, and thawing status to

153 the community structure were quantified using the distance–based linear model (DistLM) after

154 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 1st and 2nd 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 $0.95 \leq GFI$

≤ 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 $0.01 \leq P$

≤ 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

237   Fig. 3).

238   *3.4 The influence of permafrost thawing status and age on the community assembly of bacteria*

239   The average contribution of stochasticity to community assembly was 68%, 74%, and 86% in the active,

240   transition, and frozen layers of the permafrost. Significant differences in the contribution of stochasticity

241   were detected between the active and frozen and between the transition and frozen layers (both $P < 0.05$,

242   Supplementary Fig. 4a), but not between the active and transition layers ($P = 0.15$). In contrast, the

243   average contribution of stochasticity was 65%, 76%, 68%, and 76% for the young–, medium–, old–, and

244   ancient–aged permafrost, with no significant contribution differences among the different aged

245   permafrost (all $P > 0.05$, Supplementary Fig. 4b).

246   *3.5 Quantifying the influence of permafrost thawing status and age on bacterial richness and community*

247   *structure variation*

248   Structural equation modeling (SEM) revealed that both permafrost thawing status and age significantly

249   contributed to bacterial richness. Permafrost thawing status exhibited a higher contribution than age

250   (standard regression weight of 0.51 and -0.30, respectively, both $P < 0.05$) to bacterial richness (Fig. 4a).

251   However, the influences of TOC and TN on bacterial richness were not detected. This is consistent with

252   the Random Forest analysis results, which only identified permafrost thawing and age as the significant

253   determinants of bacterial richness (Supplementary Fig. 5). For community structure, permafrost thawing

254   exhibited an indirect influence on NMDS1 via TN (standard regression weight of 0.58 and -0.63, both P

255   < 0.001, Fig. 4b). In comparison, both permafrost age and thawing status significantly contributed to

256   NMDS2 (standard regression weight of -0.34 and 0.59, respectively, both $P < 0.01$), while TN also

257   exhibited a significant influence on NMDS2 (-0.49, $P = 0.002$). The significant contributions of TN,

258   permafrost thawing, and age were consistently identified using the Random Forest approach

259   (Supplementary Fig. 5).

260   **4 Discussion**

261   The bacteria richness was significantly higher in the active layer soil (Fig. 2a), and this is consistent with

262   the previous findings that permafrost thawing significantly increased bacterial richness in soil in the

263   Tibetan Plateau and the high Arctic (Chen et al., 2017; Schostag et al., 2019; Wu et al., 2018). Permafrost

264   thawing leads to accelerated microbial degradation of soil organic carbon that can generate a wide variety

265   of metabolic products (Mueller et al., 2015). The increased metabolic product diversity would lead an

266   increased nutrient diversity and provide additional ecological niches for bacteria (Hernández and Hobbie,

267   2010). This would explain the increased bacterial richness observed in our study. Furthermore, the

268   phylogenetic diversity exhibited a greater sensitivity to permafrost thawing than the Shannon diversity

269   (Supplementary Fig. 2). As phylogenetically close-related microorganisms have similar habitat

270   associations, phylogeny-based community metrics could infer potential community functional change

271   (Stegen et al., 2012). Hence, this suggests that community function could be more sensitive to permafrost

272   thawing than community composition.

273   The soil bacteria in the young permafrost exhibited a stronger response to thawing than those in older

274   permafrost soils (Fig. 2b). The young permafrost soil demonstrated a higher relative abundance of

275   aliphatic carbon but lower carbohydrates than older permafrost soils (Mueller et al., 2015). Thus,

276   bacterial richness could be driven by carbon quality, but not quantity. It has been reported that the

277   degradation of complex carbon molecules requires extensive microbial collaboration, thus leads to a

278   more diverse microbial community in forest soil (Ding et al., 2015). Furthermore, an earlier study on the

279   freshwater ecosystem also confirmed that organic carbon composition determined bacterial richness and

280   community structure (Docherty et al., 2006). This is in agreement with the higher bacterial richness

281   detected in the active layer of the young permafrost soil (Fig. 2b).

282   The increased bacterial richness due to permafrost thawing was mainly attributed to *Firmicutes*,

283   *Actinobacteria*, *Chloroflexi*, *Deltaproteobacteria*, and *Alphaproteobacteria* in the young permafrost soil

284   (Supplementary Table 6). Increased transcriptional response of *Chloroflexi* has been reported during

285   permafrost thawing (Coolen and Orsi, 2015), and may be attributed to their recalcitrate organic matter

286   degradation capacity (Colatriano et al., 2018). *Firmicutes* and *Actinobacteria* have been reported to be

287   more abundant in the frozen layer than in the active layer of permafrost soil due to their capacities in

288   maintaining metabolic activity and DNA repair mechanisms at low temperature (Johnson et al., 2007;

289   Tuorto et al., 2014). However, our results showed that their diversity may increase during permafrost

290   thawing, despite their reduction in relative abundance. *Alpha*– and *Delta*–*proteobacteria* were both

291   abundant in the upper permafrost soil in the Tibetan Plateau, and their relative abundance negatively

292   correlated with soil depth (Wu et al., 2017). *Alphaproteobacteria* was identified to be more abundant in

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293   the active layer of the permafrost soil in Norway (Mueller et al., 2018). One possible explanation is that

294   the surface active layer may be the major location for root exudates, which favors *Alphaproteobacteria*

295   (Morgalev et al., 2017). *Deltaproteobacteria* has been reported to have strong catabolic potentials on

296   recalcitrate aromatic compounds and plant detritus (Jansson and Tas, 2014). This may explain the

297   enhanced richness of *Deltaproteobacteria* in the active layer of permafrost.

298   PERMANOVA, SEM, and Random Forest analyses consistently demonstrated statistically significant

299   contributions of permafrost thawing and age to soil bacterial community structure (Fig. 4b). Bacterial

300   communities were better separated by thawing status than by age on the NMDS plots (Figs. 3a and 3b).

301   Furthermore, a significantly higher contribution of determinism (lower stochasticity) was observed in

302   the thawed permafrost soils (active and transition layers) than in the permanently frozen layer. This

303   contrastive difference from the weak influence of permafrost age on the bacterial community

304   (Supplementary Fig.4). Collectively, these suggest that permafrost thawing has a stronger influence on

305   bacterial community structure than permafrost age. Our results are consistent with Mondav et al. (2017),

306   who found that permafrost activity better separated the community structure than soil depth in peatland

307   permafrost soil in Sweden.

308   Permafrost thawing significantly increased determinism in bacterial community structure

309   (Supplementary Fig. 4). Increased determinism is frequently attributed to enhanced environmental

310   filtering (Stegen et al., 2012). Our results demonstrated that TN and the C:N ratio explained a greater

311   proportion of the bacterial community structure than TOC. This is consistent with the previous findings

312   that nitrogen availability strongly regulates microbial community structure and function in the permafrost

313   soils of Arctic and Tibetan Plateau (Chen et al., 2018; Chen et al., 2017; Yergeau et al., 2010).

314   Significantly different soil carbon and nitrogen were observed among the various permafrost thawing

315   statuses, but not among the different permafrost ages (Figs. 1a and 1c). Thus the changed nutrients may

316   explain the significant influence of thawing status on the community structure and assembly processes.

317   The community structure change due to permafrost thawing has also been proposed to be due to the

318   colonization of microorganisms in the active layer (Monteux et al., 2018), which coincides with the

319   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

349   et al. (2015) identified a single archaeon OTU accounting for over 30% of the prokaryotic community

350   (Fig. 3 in Kao-Kniffin et al., 2015). An early study revealed that archaea exhibited a lower community

351   variation with increasing soil depths compared with bacteria (Frank-Fahle et al., 2014). Therefore, the

352   community convergence observed by Kao–Kniffin et al. (2015) could be due to the influence of archaea.

353   Furthermore, the inconsistency may be related to the different community dissimilarity metrics used.

354   Kao–Kniffin et al. (2015) used unweighted UniFrac, which only calculates the phylogenetic closeness of

355   OTUs, and the relative abundance is not considered. This is distinctively different from the Bray-Curtis

356   dissimilarity used in the present study, and it has been reported that unweighted and weighted community

357   metrics examine different features of community strcture (Lozupone et al., 2011).

358   The distinct bacterial community structure in the various aged permafrost soils, yet under the same

359   thawing status, confirms the historic effects of permafrost age on the community structure during

360   permafrost thawing. The distinct bacterial community structure is likely to result in different metabolic

361   functions (Brown and Forsythe, 1974), thus the significantly different bacterial structure under the same

362   thawing status may lead to different organic carbon degradation capacities. Furthermore, older

363   permafrosts enriches pathways involved in the degradation of recalcitrant biomass, while decreases

364   pathways associated with starch and sucrose metabolism comparing with younger soils (Mackelprang et

365   al., 2017). Thus, the thawing of permafrost soils of different ages may also lead to distinct soil carbon

366   degradation schemes.

367   **5 Conclusion**

368   Our results demonstrated that permafrost thawing consistently exhibited greater influence on bacterial

369   richness and community structure than permafrost age. However, permafrost age alters the response of

370   permafrost soil bacteria to thawing, with a stronger response to thawing observed in the young than older

371   permafrost soils. The different community structure during permafrost thawing may present distinct

372   metabolic potentials for soil organic carbon cycling, and may ultimately alter the carbon emission scheme.

373   Further studies are required to identify the environmental and historical factors that lead to the distinct

374   responses of bacteria in the permafrost of different ages.

375

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.

Acknowledgements

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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.
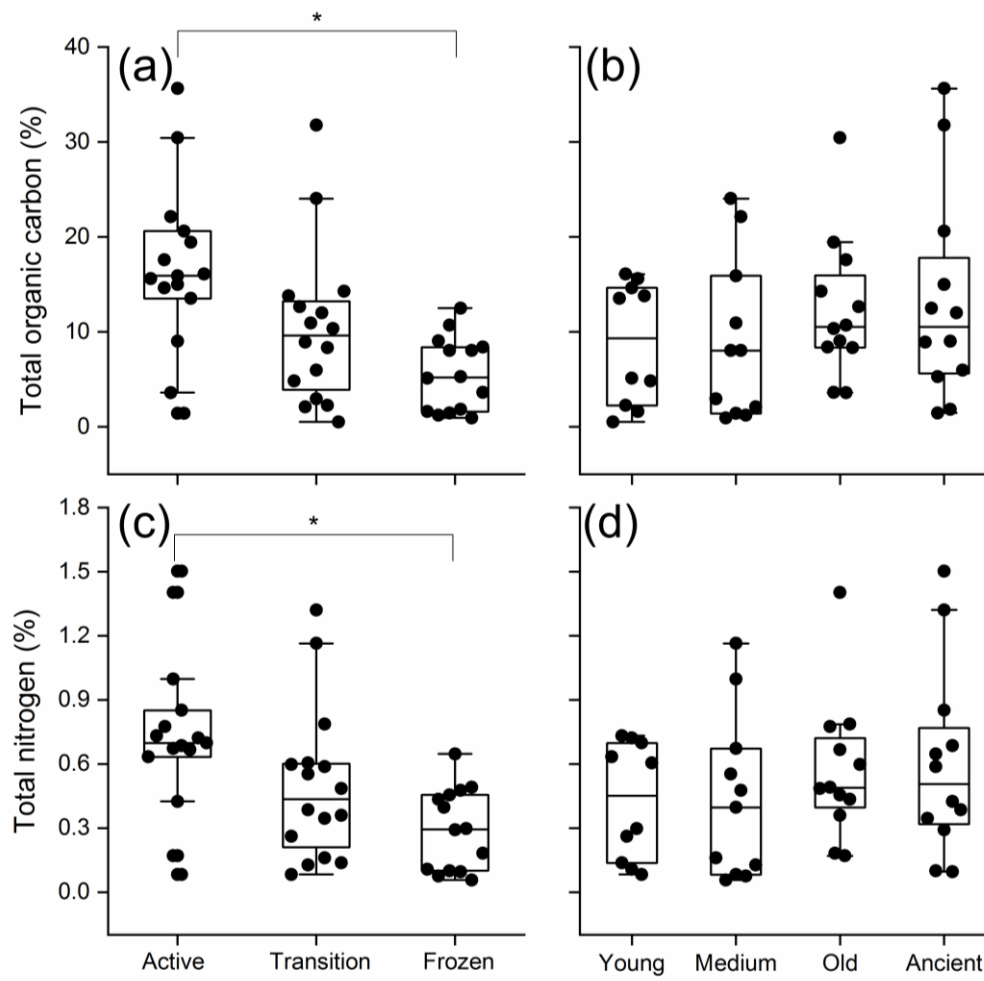
Fig. 3 NMDS plots showing the bacterial community structure of different thawing status (a) and permafrost age (b). The bacterial community structure of different thawing status in the young, medium, old, and ancient permafrost soils are shown in (c)–(f). Active, transition, and permanently frozen are 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 2nd axis 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.

549   Fig. 1
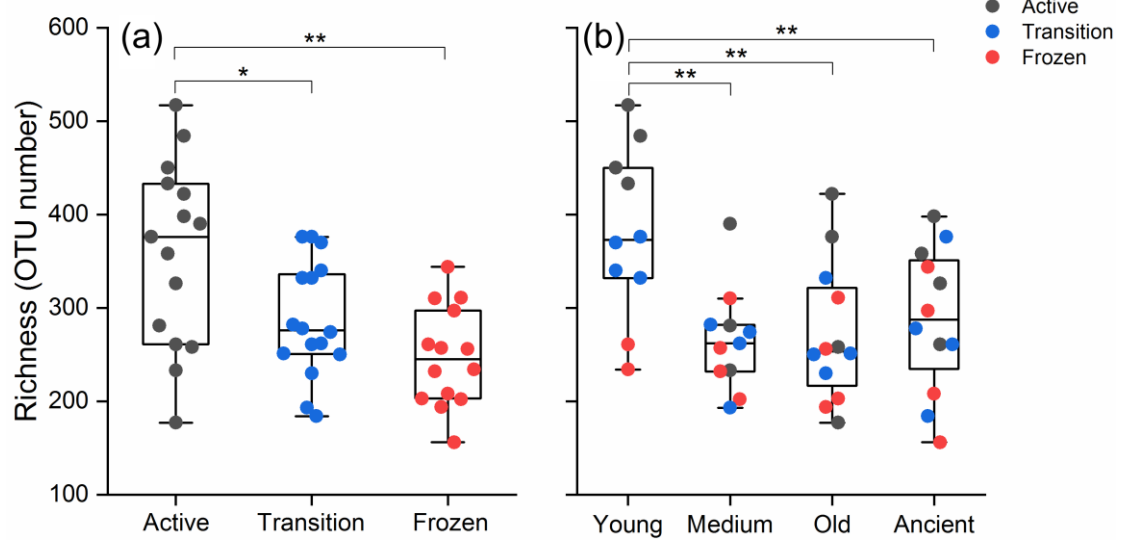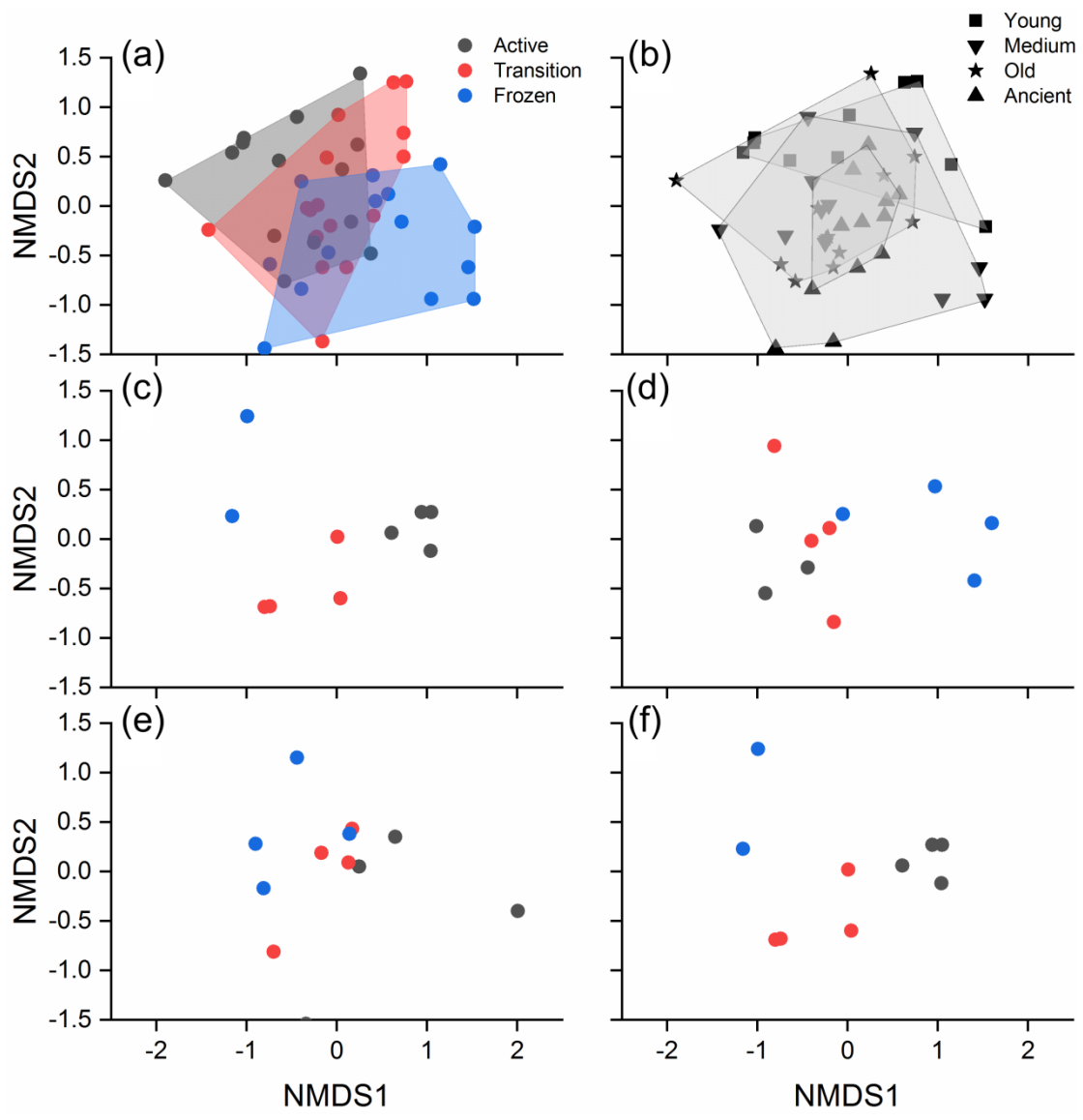
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552   Fig. 2
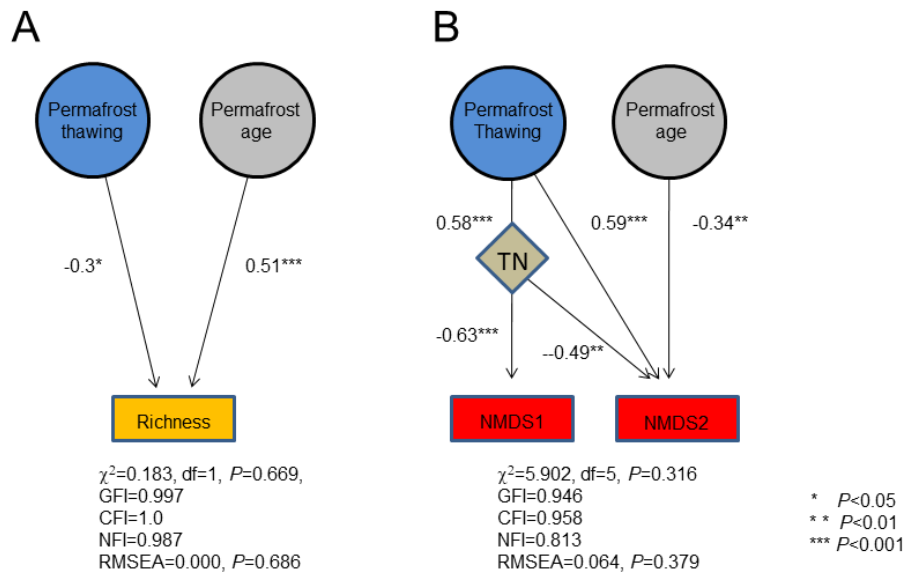
555   Fig. 3
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559   Fig. 4

A

Permafrost thawing — -0.3* → Richness
Permafrost age — 0.51*** → Richness

χ^2=0.183, df=1, P=0.669,
GFI=0.997
CFI=1.0
NFI=0.987
RMSEA=0.000, P=0.686

B

Permafrost Thawing — 0.58*** → TN
Permafrost age — 0.59*** → NMDS2
Permafrost age — -0.34** → NMDS2
TN — -0.63*** → NMDS1
TN — --0.49** → NMDS2

χ^2=5.902, df=5, P=0.316
GFI=0.946
CFI=0.958
NFI=0.813
RMSEA=0.064, P=0.379

* P<0.05
* * P<0.01
*** P<0.001

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