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SHIFTS OF SOIL MICROBIAL COMMUNITIES IN ALASKAN TUNDRA IN RESPONSE TO LONG-TERM WARMING

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SHIFTS OF SOIL MICROBIAL COMMUNITIES IN ALASKAN TUNDRA IN RESPONSE TO LONG-TERM WARMING

A THESIS APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

BY

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Dedicated to my parents, Shiyun Li and Yonghong Wang

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Abstract

Permafrost regions store about 33% of the world's soil organic carbon, and are believed to rapidly respond to global warming. The degradation and release of previously stored organic carbon stocks lead to positive feedbacks, potentially further accelerating climate warming. Microorganisms play a crucial role in determining carbon loss in permafrost ecosystems, yet this process is poorly understood. We studied soil microbial community shifts after 5-year experimental warming at the Carbon in Permafrost Experimental Heating Research site (AK, USA) using sequencing of 16S rRNA gene amplicons, and a comprehensive functional gene array (GeoChip 5.0). 16S rRNA analysis showed that the composition and structure of microbial community were significantly changed in response to warming. GeoChip analysis revealed that genes involved in carbon (C) and nitrogen (N) cycling (e.g., C degradation, methane production, N fixation and denitrification) were stimulated under warming. In particular, the significant stimulation of methanogenesis genes suggested that methane release could increase under warming. In addition, both Mantel test and canonical correspondence analysis showed that these changes in diversity and function could be largely explained by thaw depth, plant growth, temperature and moisture. Null model analysis and phylogenetic-based β-nearest taxon index (βNTI) revealed that warming increased the proportion of deterministic processes (i.e., variable selection), indicating that soil microbial communities would be more governed by heterogeneous environmental conditions in a warmer world. Molecular ecological network analysis showed that the network from warmed sites had more complex structure and tighter interactions around environmental factors compared to its counterpart from ambient

sites. Together, this study showed both functional potential and composition of soil microbial communities shifted in response to warming, which was highly related to environmental heterogeneity caused by warming.

Chapter 1: Introduction

High-latitude permafrost-underlain tundra ecosystems have been of great importance for studies on global climate change due to its substantial carbon pool and its vulnerability to climate warming. Permafrost regions represent a unique habitat for microbes which live at subzero temperature (Gilichinsky et al., 2008). In such frozen and saturated conditions, microbial activities are very low, which is critical to protect carbon from microbial decomposition. However, permafrost region is undergoing climate warming; the temperature in high latitude region has increased by 0.6 ℃ per decade in the last 30 years, which is twice as fast as the global average (Stocker et al., 2013). Climate change is threatening to cause large-scale permafrost thaw and thus expose previously protected carbon to microbial break down.

Permafrost Carbon Pool

Permafrost regions store substantial quantities of organic carbon, which is twice as much carbon as there is currently in the atmosphere (Tarnocai et al., 2009; Zimov, Schuur, & Chapin III, 2006). The old carbon from the remnant of plants and animals has been sequestered in permafrost for thousands of years under frozen and saturated soil conditions (Hicks Pries, Schuur, & Crummer, 2012) .

The estimate of carbon pool size in permafrost region contains both surface carbon (0-3m) and deep carbon (>3m) (Anthony et al., 2014; Grosse et al., 2013; Strauss et al., 2013), much deeper than the traditional zone of soil carbon accounting in other terrestrial ecosystems (Ping et al., 2008). Furthermore, carbon that was formed on land during glacial periods but now stored on shallow marine regions is considered to be part of the permafrost carbon pool.

The surface carbon stored in the top 3m depth is estimated to be 1035 ± 150 Pg C (Gustaf Hugelius et al., 2014; G Hugelius et al., 2013), which is approximately 33% of the total surface soil carbon on the earth (Jobbágy & Jackson, 2000). The deep carbon stored within 1.2 million square kilometers of yedoma (i.e., an area of deep sediment deposits that cover unglaciated parts of Siberia and Alaska) is estimated to range from 210 ± 70 (Strauss et al., 2013) to 456 ± 45 Pg C (Anthony et al., 2014). In addition, the deep carbon stock outside yedoma region is estimated to be 350-465 Pg C. Therefore, there could be 1,730-1,980 Pg C in total in the northern permafrost zone with additional subsea permafrost carbon (Schuur et al., 2015).

With such an enormous reservoir, conversion of only a fraction of this frozen carbon pool into the greenhouse gases carbon dioxide (CO_2) and methane (CH_4) and their release into the atmosphere could increase the rate of future climate change (Schuur et al., 2008). The impact of these rising temperatures on the fate of carbon in Arctic ecosystems is thus a serious concern.

Carbon Release Rate and Form

Permafrost thaw induced by warming is expected to make previously protected C stock biologically available, thus accelerating C losses. To have a more accurate prediction for effects of climate change, it's crucial to estimate carbon release rate, which is partly controlled by the overall decomposability of organic carbon. Recent studies showed a portion of organic carbon stored in permafrost is quite vulnerable to breakdown in a short period after thaw (Dutta, Schuur, Neff, & Zimov, 2006; Rachel Mackelprang et al., 2011; Kai Xue et al., 2016). Though the initial carbon release rate is potentially high, it's expected to decline overtime. Soil warming experiments in field

often showed a tendency that the initial increase in CO_2 efflux gradually disappeared and C release rate returned to pre-warming value within a few years (Eliasson et al., 2005; Knorr, Prentice, House, & Holland, 2005; Luo, Wan, Hui, & Wallace, 2001; Melillo et al., 2002; Walter C. Oechel et al., 2000; Rustad et al., 2001). Therefore, experiments in permafrost regions on a long timeframe are needed to have more reliable estimation on carbon release rate.

On the other hand, from the geological perspective, climate warming often leads to changes in soil hydrology in permafrost region. In upland areas, permafrost thaw can result in saturated collapsed areas interspersed with dryer areas (Jorgenson, Racine, Walters, & Osterkamp, 2001; O'Donnell et al., 2012; Vogel, Schuur, Trucco, & Lee, 2009). In this case, both aerobic and anaerobic respiration can be potentially enhanced. Soil moisture is of particular concern because it is the main environmental factor affecting C losses besides temperature (Oberbauer et al., 2007; Walter C Oechel, Vourlitis, Hastings, Ault, & Bryant, 1998; Shaver et al., 2006), especially considering its role in affecting the form of C release. In tundra ecosystems, well-drained areas with low moisture were observed to be more responsive to warming (Oberbauer et al., 2007). However, not only the amount but also the form of C release is of great importance, because CH_4, the major product of anaerobic C decomposition, has 28-34-fold larger global warming potential as compared with CO_2 (Myhre et al., 2013). Multiple *in situ* studies have indicated that soil moisture served as a major driver for determining the form of C release (Kane et al., 2013; Olefeldt, Turetsky, Crill, & McGuire, 2013).

Projecting Change

Various models were come up with to answer the critical question: how fast and severe the resultant carbon release will aggravate the climate warming even further. However, forecasts from models with different scenarios showed great uncertainties. Carbon release in permafrost regions has been estimated to range from 37 to 174 Pg C by 2100 under the Representative Concentration Pathway RCP 8.5, averaged on 92 ± 17 Pg C (Burke, Hartley, & Jones, 2012; Burke, Jones, & Koven, 2013; Koven et al., 2011; MacDougall, Avis, & Weaver, 2012; Schaefer, Zhang, Bruhwiler, & Barrett, 2011; Schaphoff et al., 2013; Schneider von Deimling et al., 2012; Zhuang et al., 2006). More importantly, the momentum behind climate warming would result in a cascading release of greenhouse gases in the future. It has been reported that the carbon release resulted from permafrost thaw would have impacts on the earth for centuries; and 59% of it would occur after 2100.

Multi–omics technologies application

Microbial diversity is highly tremendous (Amann & Fuchs, 2008), though only about 1% of microbes can be studied by lab cultivation (Sogin et al., 2006; Torsvik, Øvreås, & Thingstad, 2002). Over the past several years, novel approaches, such as DNA sequencing technology, provide promising opportunities to study the microbial universe in permafrost region since they bypass the need of lab cultivation. Information of the following perspectives can be obtained through novel multi-omics technologies on an unprecedented scale:

Community composition and diversity

Amplification and sequencing of specifically targeted marker genes, e.g., the widely used 16S rRNA gene amplicons sequencing, can provide the information relevant to community composition and diversity. 16S rRNA gene contains the hypervariable regions which have species-specific signature sequences useful for microbe identification. Also, 16s rRNA gene is highly conserved over billions of years of evolution (Tringe & Hugenholtz, 2008), so it can serve as a phylogenetic marker to reveal evolutionary relationships among microorganisms (Pace, 1997).

In the past few years, high-throughput sequencing technologies are rapidly developing and the resultant effective cost is promising for wide application. The transition from Sanger sequencing to 454 sequencing and then to Illumina sequencing has opened new frontiers in microbial community analysis because it makes it possible to analyze thousands of samples in a single run with unprecedented depth (Caporaso et al., 2011).

16S rRNA-based surveys are extraordinarily valuable for microbial community in permafrost region given that they can be used to document unexplored biodiversity. The microbial diversity present in various permafrost samples was discovered and characterized using this method (Nemergut et al., 2005; Steven et al., 2007; Wallenstein, McMahon, & Schimel, 2007). Soil bacterial and archaeal communities across permafrost thaw gradient at different depths were documented and analyzed through Illumina Miseq sequencing of 16S rRNA gene amplicons (J. Deng et al., 2015). The composition of soil microbial communities was found to strongly correlate the spatial and temporal distribution of snow by sequencing 16S rRNA gene amplicons from

locations with different snow conditions (Zinger, Shahnavaz, Baptist, Geremia, & Choler, 2009). Besides, 16S rRNA-based surveys were also used to study the composition of microbial communities with experimental treatments. The shifts of soil microbial communities composition in response to short-term experimental warming were investigated in Alaska (Kai Xue et al., 2016).

Total community metabolic (functional) potential

Describing the phylogenetic diversity of uncultured microorganisms is only the first step. A greater challenge is to assign ecological roles to them. The uncultured microbiota must play pivotal roles in natural environmental processes and are a large untapped resource for biotechnology applications. "Metagenomics" describes the functional and sequence-based analysis of the collective microbial genomes contained in an environmental sample (Handelsman, Rondon, Brady, Clardy, & Goodman, 1998). The functional potential of permafrost soils was first described in 2010; samples from the active layer soil and 2m permafrost soil exhibited a high similarity on functional genes (Yergeau, Hogues, Whyte, & Greer, 2010). Tas et al.'s study indicated that fire had a wide impact on genes involved in carbohydrate metabolism, methanogenesis and N cycling (Tas et al., 2014).

Functional gene array is an advanced technology to understand and characterize the functional capacity of microbial communities. GeoChip, developed in our lab, is one of the representative comprehensive functional gene arrays, targeting thousands of different gene families involved in many key biogeochemical processes, e.g., C, N, P, and S cycling. This technology has been proved powerful to study the microbial communities residing in various environments, e.g., terrestrial ecosystems (He et al.,

2010; Kai Xue et al., 2016; Zhou et al., 2012), aquatic ecosystems (Lu et al., 2012), and bioreactors (Liu et al., 2012; Zhou et al., 2013).

Total community metabolic (functional) activity

Metatranscriptomics can capture snapshots of metabolic activity at a certain time. There is always a difference between metabolic potential represented by metagenomes and metabolic activity, especially in ecosystems like permafrost. Low temperature serves to keep microbes inactive, so detecting functional genes does not mean the microbes function in the ecosystem. Metabolic activity can be represented by analyzing expressed genes through metatranscriptomic sequencing.

Microbial community assemblage

Microbes occupy a fundamental niche in food webs and ecosystems. Thus, any alteration or disturbance of microbial communities in response to climate warming is of great ecological importance. In the past few years, metagenomic technologies were used to study microbial communities in permafrost regions (Coolen & Orsi, 2015; Rachel Mackelprang et al., 2011; Kai Xue et al., 2016; Yergeau et al., 2012; Yergeau et al., 2010). In these studies, soil microbial communities exhibited large metabolic potential for key biogeochemical pathways, including methanogenesis, fermentation, and N cycling. Also, alteration of both compositional and functional structure of permafrost microbial communities was observed as a result of a rapid response to climate warming (R. Mackelprang et al., 2011; K. Xue et al., 2016). However, very few studies examined the shifts of microbial community assemblage, including interactions among species and mechanisms, in response to warming. Beyond studying the shift of diversity and composition of soil microbial communities in response to warming, network analysis

provides a new perspective on how microbes interact with each other and how the microbial communities develop (Shi et al., 2016). Studies of microbial communities should not only focus on richness and abundance, but also on the complex interactions among species (Olesen, Bascompte, Dupont, & Jordano, 2007). Applications of network analysis on macro-ecology (e.g., food webs, mutualistic networks between plants and animals) have successfully improved our understanding of ecosystem functioning and dynamics (Bascompte & Jordano, 2007; Montoya, Pimm, & Solé, 2006; Proulx, Promislow, & Phillips, 2005; Thompson, 2005). Recently, metagenomic technologies have provided an unprecedented opportunity to detect interactions among microbes and construct networks (Y. Deng et al., 2016; Fuhrman & Steele, 2008; Liang et al., 2016; Raes & Bork, 2008; Steele et al., 2011; Zhou, Deng, Luo, He, & Yang, 2011). The co-occurrence networks derived from operational taxa units (OTUs) have been used to explore microbial assemblages in various environments. For example, several different co-occurrence patterns for different groups of N fixers in soil were identified (Tu et al., 2016); extensive mutualistic interactions were detected in rhizosphere microbiome (Shi et al., 2016).

Furthermore, the mechanism driving microbial assemblage is of great interest. Four major ecological processes (i.e., selection, dispersal, drift, and mutation/speciation) are proposed to govern community assemblage (Hanson, Fuhrman, Horner-Devine, & Martiny, 2012; Vellend, 2010). Among the four processes, selection is a deterministic process and drift as a stochastic process, while speciation and dispersal may contribute to both deterministic and stochastic processes (Chase & Myers, 2011; Zhou et al., 2014). For example, microbial communities in bioreactors were reported to be mainly shaped

by stochastic processes (Zhou et al., 2013), while aquatic bacterial communities were found to be dominated by deterministic processes with strong habitat association (Wang et al., 2013). Determining how deterministic and stochastic processes change in response to climate change is critical for prediction of microbial community assembly in a warmer world and hence how the biodiversity and ecosystem functions will be impacted.

Here, we conducted a in situ study in the Carbon in Permafrost Experimental Heating Research (CiPEHR) project, a whole ecosystem warming experiment, initiated in 2008, Interior Alaska. The successful implementation of this project has greatly improved our knowledge of the impacts of climate warming on plant growth and community shifts, ecosystem C exchange, and soil nitrogen (N) availability, as well as the response of soil microbial communities (Bracho et al., 2016; Deane-Coe et al., 2015; Hicks Pries, Schuur, Natali, & Crummer, 2016; Johnston et al., 2016; Natali et al., 2015; Natali, Schuur, & Rubin, 2012; Natali et al., 2011; Natali, Schuur, Webb, Pries, & Crummer, 2014; Salmon et al., 2016; E. E. Webb et al., 2016; Kai Xue et al., 2016). An integrated metagenomic analysis from the first 1.5 years of warming revealed that short-term warming was sufficient to shift the functional structure of soil microbial communities, with genes involved in both aerobic and anaerobic C decomposition increased (Kai Xue et al., 2016). These findings demonstrate the high sensitivity of microbial communities and vulnerability of soil C to climate warming in permafrost-based tundra ecosystems. Considering that thaw depth difference between warming and control sites continually increased from 2009 to 2013 (Figure 1), it is critical to

understand whether microbial communities continued to respond to a longer-term warming manipulation.

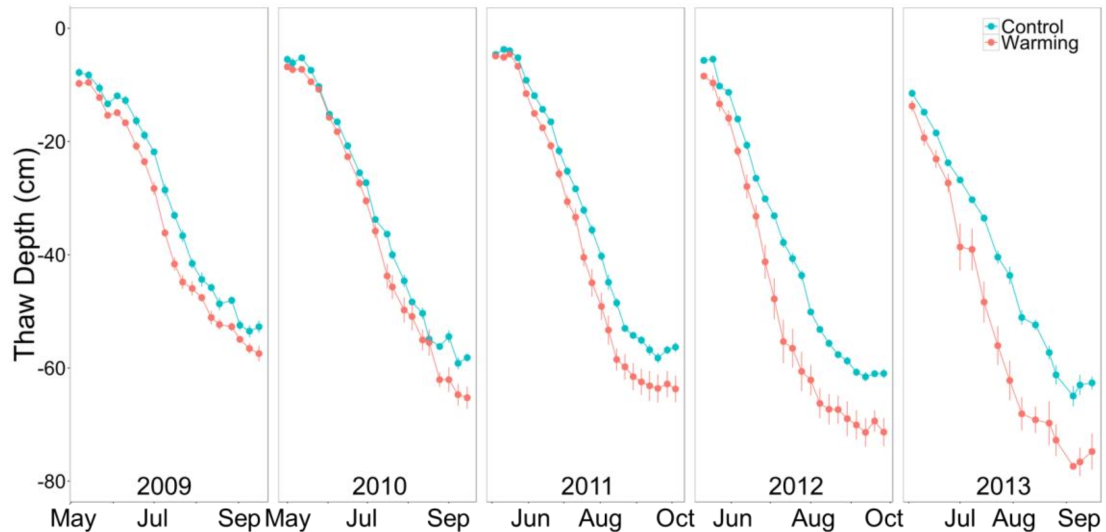

Figure 1. Effect of warming on thaw depth over time (2009-2013).

Warming treatment had marked effects on thaw depth. Each panel represented the dynamics of thaw depth in growing season within a year. Blue dots represented thaw depth in ambient sites in growing season from 2009 to 2013, while red dots represented thaw depth in warmed sites. Error bars represent standard error of the mean.

Therefore, the current study investigated both the functional and taxonomical structure of soil microbial communities after 5 years of warming by using GeoChip 5.0 hybridization and 16S rRNA amplicon sequencing. The major objectives of this study were to address the following questions: (i) how does both the taxonomical and functional structure of the soil microbial communities respond to longer-term warming (5 year), (ii) is the response continual as compared to that observed in our previous 1.5-year study, (3) does the mechanism driving community assembly change, and (4) does 5-year warming manipulation have an impact on the network structure of soil microbial communities. Based on the previous 1.5-year warming study from CiPEHR in our lab, we predicted that warming manipulation would shift the composition and functional

diversity of soil microbial communities. We hypothesized that 1) the trend on how soil microbial communities responded to warming manipulation would continue and amplify because of increasing warming effect resulted from longer warming time; 2) the way on how soil microbial communities assembly would vary because warming manipulation increased environmental heterogeneity. To test these hypotheses, we examined community structure using 16S rRNA gene amplicons sequencing and a functional gene array (GeoChip 5.0) hybridization. Phylogenetic molecular ecological networks (pMEN) and community phylogenetic turnover were also investigated to understand the network structure of microbial communities. Our results indicated that longer-term warming imposed broader impacts on both composition and functional potential of soil microbial communities than 1.5-year warming; the network structure generated from warmed sites showed more complex interactions among microbes; and variable selection dominated the microbial community assemblage, suggesting that higher environmental heterogeneity resulted from warming could be the cause.

Chapter 2: Materials and Methods

Experimental site and sampling

The CiPEHR project , established in 2008, is located within a discontinuous permafrost zone in the northern foothills of the Alaska Range (~670 m elevation) at the Eight Lake Miles study site, AK, USA (63°52'59"N, 149°13'32"W) (Natali et al., 2012; Natali et al., 2011). The soils in the experimental site are gelisols and comprise a 45-65cm thick organic horizon above a cryoturbated mineral mixture of glacial till and loess. The active layer, which thaws annually, is 50-60cm thick in the disturbed areas. The site has a mean annual air temperature of -1.45 ± 0.25 °C during 1977-2013 and a mean growing season precipitation of 216 ± 24 mm during 2004-2013. The dominant vegetation is tussock-forming sedge, *Eriophorum vaginatum*. More detailed information on this site was described previously (Natali et al., 2011).

The soil has been warmed since 2008 at the ecosystem level with soil warming treatment achieved by snow fences (1.5 m tall, 8 m long) in six replicates (Figure 2). The snow fences increased the depth of the snow layer and insulated heat. In addition, snow removal was conducted in the early spring to avoid moisture and meltdown effects of the extra snow. A total of 48 samples were collected in September 2013 after a 5-year period of experimental warming. Soil samples from both warming and control plots (6 replicates each) were collected and separated into four layers: 0-5cm, 5-15cm, 15-25cm, and 45-55cm. The microbial community and environmental monitoring data from soil depths 0-5cm and 5-15cm were combined to investigate the response of microbial communities to experimental warming.

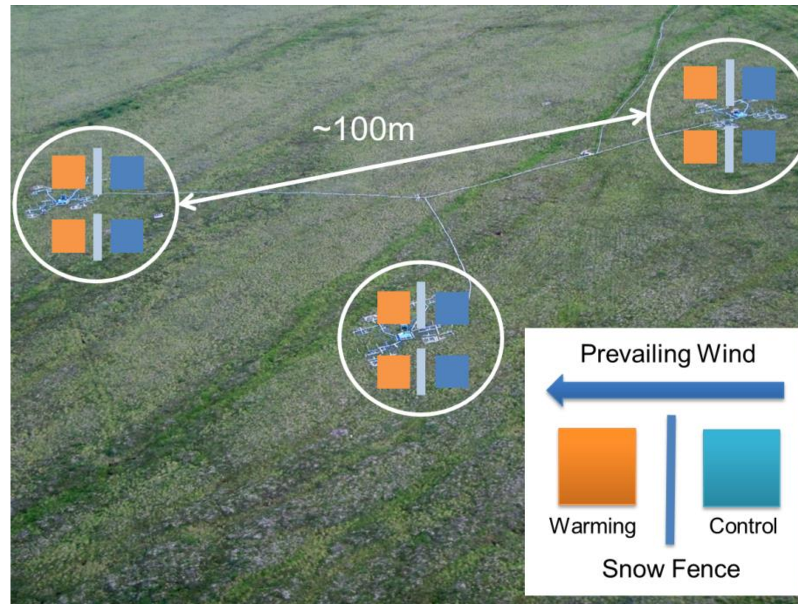

Figure 2. CiPEHR experimental design (photo credit: Edward Schuur)

DNA extraction

Soil DNA was extracted from 3 g of each soil sample by freeze-grinding mechanical cell lysis as described previously (Zhou, Bruns, & Tiedje, 1996) and then further cleaned using a PowerMax Soil DNA Isolation Kit (MO BIO; CA, USA). A NaonoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) was used to assess DNA quality using absorbance ratios of 260/280 and 260/230 nm. Final DNA concentrations were quantified by PicoGreen (Ahn, Costa, & Emanuel, 1996).

16s rRNA gene amplicon sequencing and data preprocessing

The V4 hypervariable regions of 16S rRNA genes were amplified with the primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') using a Miseq (Illumina, San Diego, CA, USA) using 2×150 pair end format (Caporaso et al., 2012; Wu et al., 2015) Sequences were trimmed using BTRIM with a threshold of Quality Scores higher than 20 over a 5 bp

window size and a minimum length of 100bp (Kong, 2011). Forward and reverse reads with at least a 30 bp overlap and 25% mismatches were joined using FLASH (Magoc and Salzberg, 2011). After removing sequences with ambiguous bases, i.e. N, joined sequences with lengths between 245 and 260bp were subjected to chimera removal by U-Chime (Edgar et al., 2011). OTUs were clustered through Uclust at the 97% similarity level (Edgar, 2010). Then taxonomic assignment was conducted through the RDP classifier (Wang et al., 2007) with a confidence cutoff of 0.5, and singletons were removed. The remaining sequences were randomly resampled to a depth of 34,673 reads per sample. The above steps were performed on the Galaxy pipeline (http://zhoulab5.rccc.ou.edu/).

GeoChip 5.0 analysis

To assess the changes of soil microbial communities in response to experimental warming, the functional composition and structure were analyzed using the latest version of GeoChip. GeoChip 5.0 contains 161,961 probes targeting 1,447 gene families involved in 12 major functional categories such as carbon, nitrogen, phosphorus and sulfur cyclings (Tu et al., 2014). For each sample, 1,000 ng of the community DNA was labeled with Cy5 using random primers, dNTP solution and Klenow, purified with the Qiagen QIAquick Kit (Qiagen, Germantown, MD, USA) and dried using a SpeedVac (Thermo Fisher Scientific Inc., Waltham, MA). As shown in Figure 3, labeled samples were hybridized onto GeoChip 5.0M at 67 C in the presence of 10% formamide for 24 hours. After hybridization, gene arrays were washed, dried up and scanned at 100% laser power and photomultiplier tube on an MS200 Nimblegen microarray scanner (Roche, South San Francisco, CA, USA). Scanned images were

then processed and transformed into signal intensities with Agilent's Data Extraction software. Raw signal intensities were uploaded onto the Microarray Data Manager of the Institute of Environmental Genomics at the University of Oklahoma (http://ieg.ou.edu/microarray/) for data clean-up, normalization and analysis. We normalized the signal intensity of each spot by relative abundance, removed spots with <2 signal-to-noise ratio (SNR) (Wu, Liu, Schadt, & Zhou, 2006), and removed outliers based on standard deviation as described in previous study (He et al., 2007).

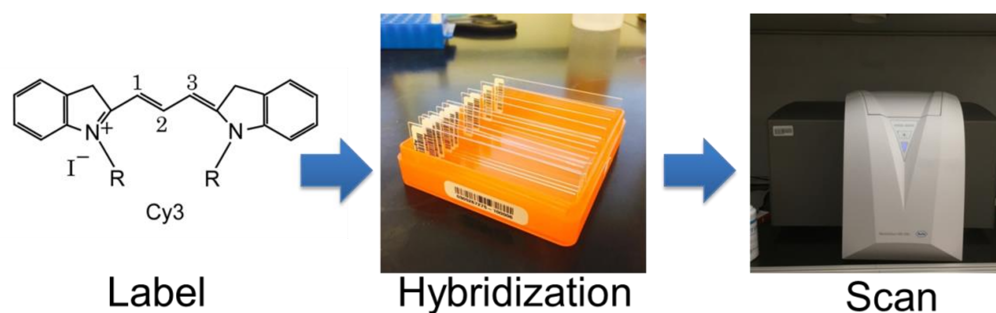

Figure 3. The process of GeoChip 5.0 hybridization

Molecular ecological network analysis

The molecular ecological networks (MENs) were constructed using 16S rRNA sequence data using random matrix theory (RMT)-based network approach (Y. Deng et al., 2012; Zhou et al., 2010; Zhou et al., 2011). OTUs detected in all six replicates remained for network construction to ensure reliability. Then correlation between each pair of OTUs was measured by Spearman's rho to form a correlation matrix. The threshold to remove random noise was determined when the nearest-neighbor spacing distribution of eigenvalues transited from GOE to Poisson distributions. In our analysis, a threshold of 0.98 was identified in both warmed sites and ambient sites networks.

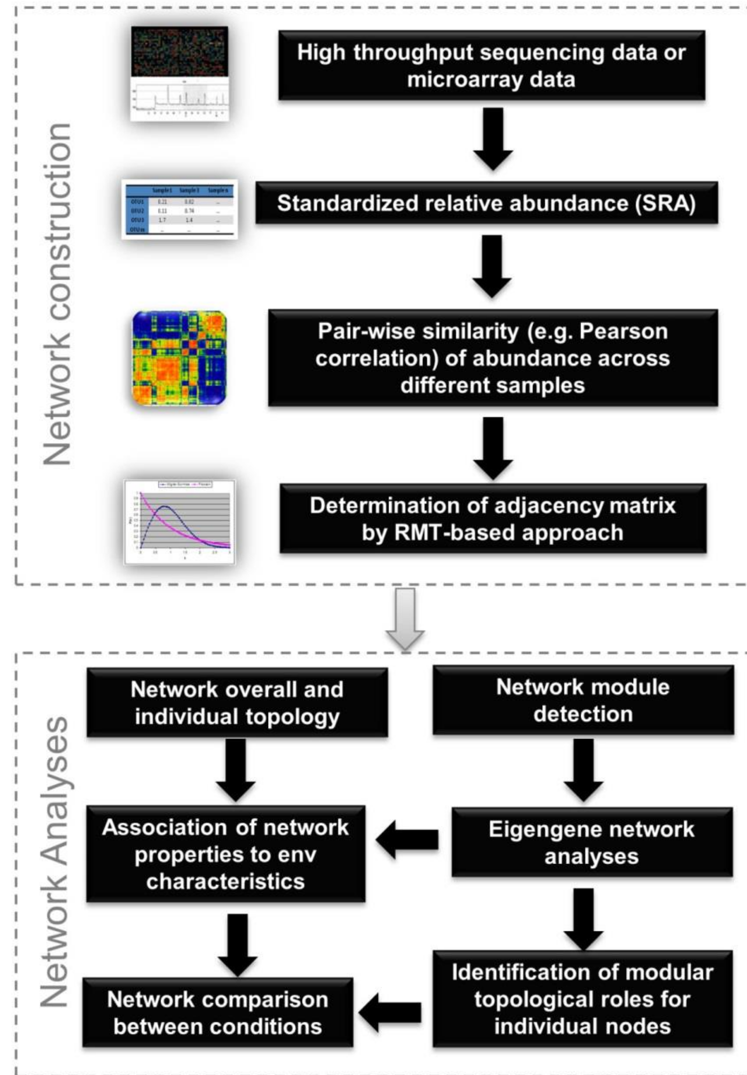

Figure 4. Overview of the Random Matrix Theory (RMT)-based molecular ecological network analysis(Y. Deng et al., 2012)

Quantifying ecological processes shaping microbial community assembly

The relative roles of community assembly processes were quantified using a framework proposed by Stegen *et al.* (Dini-Andreote, Stegen, van Elsas, & Salles, 2015; James C Stegen et al., 2013; James C. Stegen, Lin, Fredrickson, & Konopka, 2015). The influence of selection was estimated based on the nearest taxon index between

communities (βNTI). A turnover between two communities was considered to be governed by "variable" or "homogeneous selection" when the phylogenetic dissimilarity (βMNTD, beta mean nearest taxon distance) was significantly higher (βNTI>2) or lower (βNTI<-2) than null expectation (Fine & Kembel, 2011; James C. Stegen et al., 2015; C. O. Webb, Ackerly, & Kembel, 2008). Then, the turnovers not governed by selection were analyzed using the Raup-Crick metrics (RC) based on Bray-Curtis dissimilarity index to estimate the influence of "dispersal limitation" (RC>0.95) and "homogenizing dispersal" (RC<-0.95) (Chase, Kraft, Smith, Vellend, & Inouye, 2011; James C Stegen et al., 2013), and the remainder was named "undominated" (James C. Stegen et al., 2015). Among pairwise turnovers within the same group, i.e., control or warming group, the percentage of one certain process accounting for all processes, defined as process ratio, is used to determine the relative role of this particular process (James C Stegen et al., 2013).

Statistical analysis

To investigate the difference in overall soil microbial communities between warming and ambient, three complementary dissimilarity tests were used: 1) the multi-response permutation procedure (MRPP) (Van Sickle, 1997), 2) analysis of similarity (ANOSIM) (CLARKE, 1993), and 3) non-parametric multivariate analysis of variance (Adonis) (Zapala & Schork, 2006). Detrended correspondence analysis (DCA) was conducted to visualize the difference in composition of microbial communities (Oksanen & Minchin, 1997). To test whether the abundance of functional genes or species was changed in response to warming, analysis of variance (ANOVA) and paired t-tests were performed. Mantel test and canonical correspondence analysis (CCA) were

conducted to link microbial communities to environmental variables (Ramette & Tiedje, 2007; Zhou, Kang, Schadt, & Garten, 2008). In the CCA model, environmental variables were selected based on variance inflation factor (VIF) and the significance was tested with ANOVA.

Chapter 3: Results

Effects of warming on soil and plant properties

Soil and plant properties were altered by five years of experimental warming. As shown in Table 1, the average soil temperature increased by 0.63℃ ($p < 0.05$) in winter in response to warming. Correspondingly, the maximum thaw depth in 2013 growing season was also increased by 11.37 cm ($p < 0.05$) when soil warming was employed. Also, the maximum thaw depth of each growing season continuously increased from 2009 to 2013, and an increasing difference of thaw depth between control and warming sites was observed (Figure 1). These findings suggest that the soil environment for microbes was progressively altered by warming manipulation over time. However, soil moisture was not altered by warming. Plant biomass also increased by 25.2% ($p < 0.05$) in the warmed sites, suggesting warmer condition stimulated plant growth.

Table 1. Averaged wintertime soil temperature, growing season soil temperature, soil moisture, water table depth, thaw depth, and plant biomass by treatment after 5 years of warming

Environmental Variables	Control	Warming
Wintertime Temperature (°C)	**-1.95 ± 0.25**	**-1.32 ± 0.13**
Growing season Temperature (°C)	6.15 ± 0.46	6.20 ± 0.33
Moisture (%)	37.77 ± 1.39	42.54 ± 2.90
Water Table Depth (cm)	23.82 ± 1.19	21.79 ± 1.90
Thaw Depth (cm)	**40.98 ± 0.70**	**52.35 ± 2.65**
Plant Biomass (g/m^2)	**582.3 ± 24.9**	**728.9 ± 64.6**

Mean and standard error were shown for control and warming samples, separately. The differences between warmed and control plots were tested using paired *t* tests. Bold values indicate $p < 0.05$.

Shifts of taxonomic composition of soil microbial communities

In order to detect the response of soil microbial communities to five years of experimental warming, we examined the phylogenetic composition and structure of microbial communities using 16S rRNA sequencing. Over 1.4 million qualified sequences were obtained from the total 24 samples. OTUs were clustered at 97% sequence identity and singletons were removed. After resampled at a depth of 34,673 reads per sample, 5,117 OTUs remained, and 2,740 of these were mapped to 214 known genera, with Archaea accounting for only 0.12% of the total population. Within the bacterial domain, Proteobacteria were the most abundant phylum (31.00% in relative abundance), followed by Acidobacteria (30.61%), Actinobacteria (12.08%), and Verrucomicrobia (8.34%).

Warming increased the alpha diversity of the microbial communities as increases in both OTU number and Shannon index were marginally significant (p = 0.058 for OTU number, p = 0.089 for Shannon index, Figure 5a) in warmed samples. Also, within-community beta diversity was significantly increased in warmed samples ($p < 0.05$, Figure 5b), indicating that warming resulted in more divergent microbial communities. Although the separation between control and warmed samples were not obvious in DCA analysis (Figure 6a), three dissimilarity tests (MRPP, ANOSIM, and Adonis) consistently showed that the taxonomic structure was differed significantly ($p <$ 0.05) in response to warming (Table 2). These results suggested that warming greatly altered the taxonomic and phylogenetic structure of the microbial communities.

Table 2. Significance tests of warming effect on microbial functional structure revealed by GeoChip, and taxonomical structure revealed by 16S rRNA sequencing.

Dissimilarity Test[a]	MRPP		ANOSIM		Adonis	
	delta	*p*	r	*P*	r^2	*p*
GeoChip	0.001	**0.012**	0.296	**0.012**	0.166	**0.009**
16S	1338.991	**0.04**	0.152	**0.028**	0.162	**0.015**

Three different permutation tests were performed, including the multiple response permutation procedure (MRPP), analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (Adonis), calculated with Bray-cutis distance. Bold values indicate $p < 0.05$.

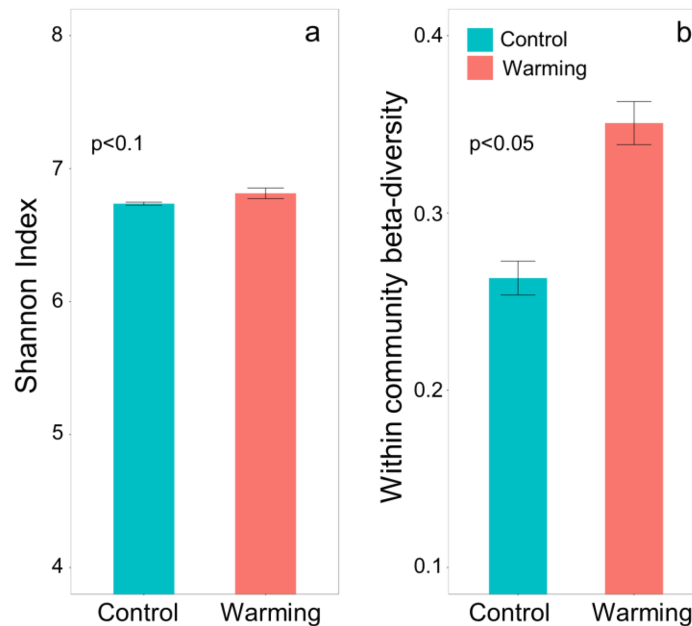

Figure 5. (a) Shannon Index; (b) beta-diversity of 16s rRNA sequencing data

Error bars represent standard error of the mean.

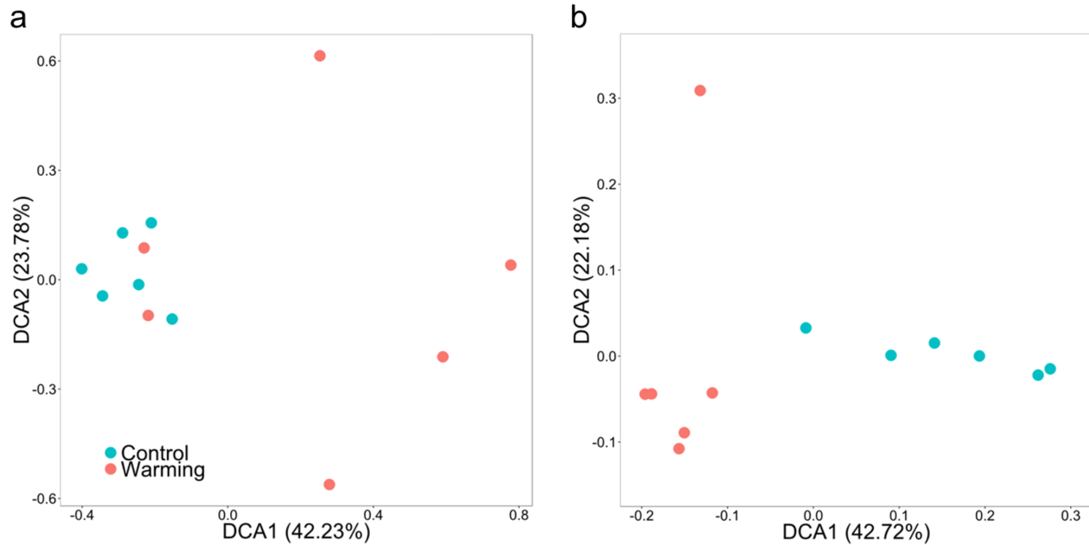

Figure 6. Detrended correspondence analysis (DCA) of (a) 16S rRNA sequencing data; (b) GeoChip data

The relative abundance of 5,117 detected OTUs were used in (a); the normalized signal intensity data for 55,288 detected functional gene sequences were used in (b). Blue circles are for control samples, and red circles for warming samples.

Among the 5,117 OTUs detected in the current study, 3,324 (65.9%) were shared by both control and warming samples. 1,012 (19.8%) OTUs were only detected in warming samples, while 781 (15.3%) OTUs were only observed for control samples (Figure 7a). For example, Methanobacteria, a type of acetoclastic methanogens, and Chitinophaga, a chitin degrader, were only detected in warming samples. For those OTUs shared by both warming and control samples, 228 OTUs were significantly increased ($p < 0.05$) under warming, while 340 OTUs decreased ($p < 0.05$; Figure 7c). For example, 39 OTUs from Actinobacteria significantly decreased, while only 9 OTUs increased in response to warming; warming samples (78 OTUs) contained less unique OTUs from Actinobacteria than control samples (40 OTUs) (Figure 7b). However, warming samples (67 OTUs) contained twice as many unique OTUs from Bacteroidetes

as control samples (30 OTUs) (Figure 7b), suggesting that microbial groups had different responses to experimental warming.

At the phylum level, seven phyla showed significantly ($p < 0.05$) increased relative abundances, including Acidobacteria (30.61%, the second prevalent phylum detected in this study), Bacteroidetes, Betaproteobacteria, Chlorobi, Firmicutes, Gemmatimonadetes, and Verrucomicrobia, and 4 phyla decreased their relative abundances under warming, including Actinobacteria (12.08%), Crenarchaeota, Gammaproteobacteria, and Planctomycetes. At the class level, the relative abundances of 10 classes, accounting for 21.7% of total sequences, significantly ($p < 0.05$) increased in response to warming (e.g., Betaproteobacteria, 2.1% in relative abundance), while the relative abundances of 4 classes, accounting for 12.4% , were significantly ($p < 0.05$) lower in warming sites. On the genus level, we also detected that two genera (Acidiphilium and Granulibacter) from Acetobacteraceae, Rhodospirillales, Alphaproteobacteria were significantly ($p < 0.05$) enriched in warming samples. These findings indicated that warming markedly impacted the taxonomic composition and structure of microbial communities, implying a shift in ecosystem functions performed by these micro-organisms.

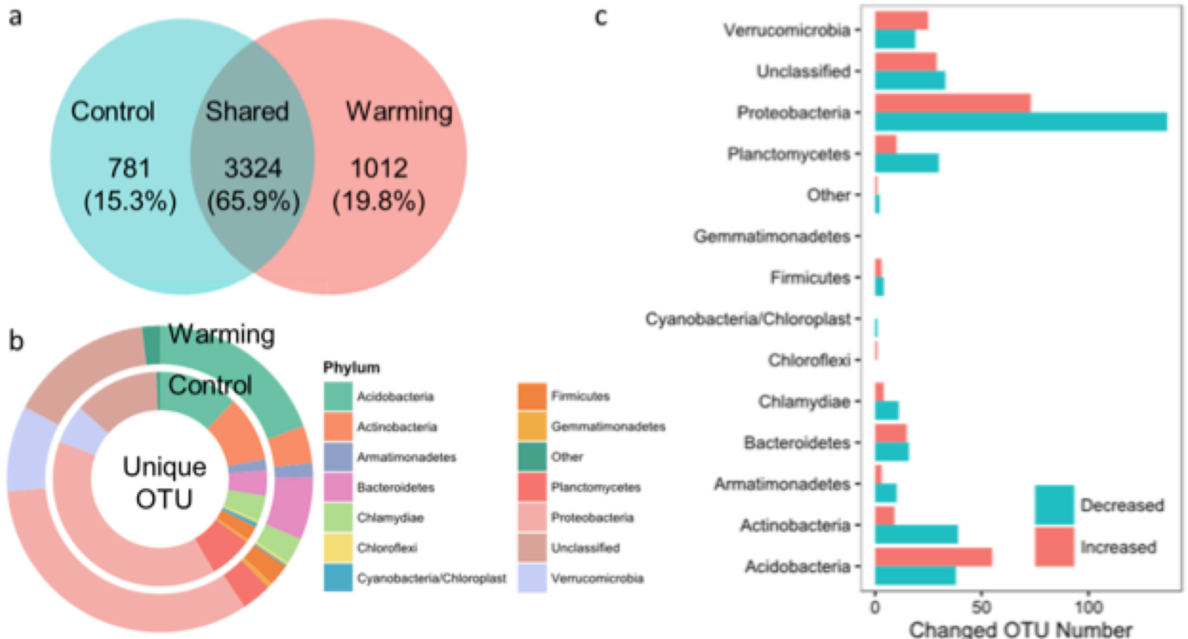

Figure 7. OTUs distribution among phyla

a) shared and unique OTU number between control and warming microbial communities: among the total 5,117 OTUs, 3,324 OTUs were detected in both ambient and warmed sites, accounting for 65.9%; 1,012 OTUs were only detected in warmed sites, accounting for 19.8%; and 781 OTUs were only detected in ambient sites, accounting for 15.3%; b) distribution of unique OTUs in microbial communities residing in ambient (inner circle) and warmed sites (outer circle), respectively; c) numbers of significantly changed OTU among shared OTU in response to warming; blue bars represented the numbers of significantly ($p<0.05$) decreased OTUs while red bars represented the numbers of significantly ($p<0.05$) increased OTUs.

Effects of warming on functional gene abundance

GeoChip 5.0 was employed to investigate the effects of warming on functional potentials of soil microbial communities. Our analysis detected 38,484 probes involved in 1,006 gene families, including genes involved in important biogeochemical processes (e.g., C, N, P, and S cycling). The results showed that the functional composition and structure of soil microbial communities at the CiPEHR project site were significantly ($p < 0.05$) altered by warming, as revealed by three dissimilarity tests (i.e., MRPP, ANOSIM and Adonis, Table 2). Also, DCA showed that samples were well separated between the control and warming treatment groups (Figure 6b), indicating that the functional structures of microbial communities were notably different between these two groups. However, no difference in functional gene diversity based on Shannon-Weiner index was observed.

GeoChip results were further analyzed to understand how warming affects the abundance of key functional genes involved in important biogeochemical cycling, including C, N, P, and S cycling.

C cycling

GeoChip detected 128 genes involved in C cycling, among which 50 genes were associated with degradation of complex carbon compounds, ranging from labile carbon to recalcitrant carbon. Most of the genes (42/50) exhibited significantly higher abundance in warming samples than control samples ($p < 0.05$, Figure 8). Specifically, warming increased the abundance of most functional genes involved in degradation of C, both labile (e.g., starch, hemicellulose, pectin and cellulose), including *amyA*

encoding amylase, xylanase, exoglucanase, pectinesterase and pectate lyase, and recalcitrant (e.g., lignin), including genes encoding phenol oxidase, vanillin dehydrogenase and ligninase. These results suggest that climate warming is most likely to enhance decomposition of both labile and recalcitrant C in the permafrost region, which would lead to an increased release of CO_2

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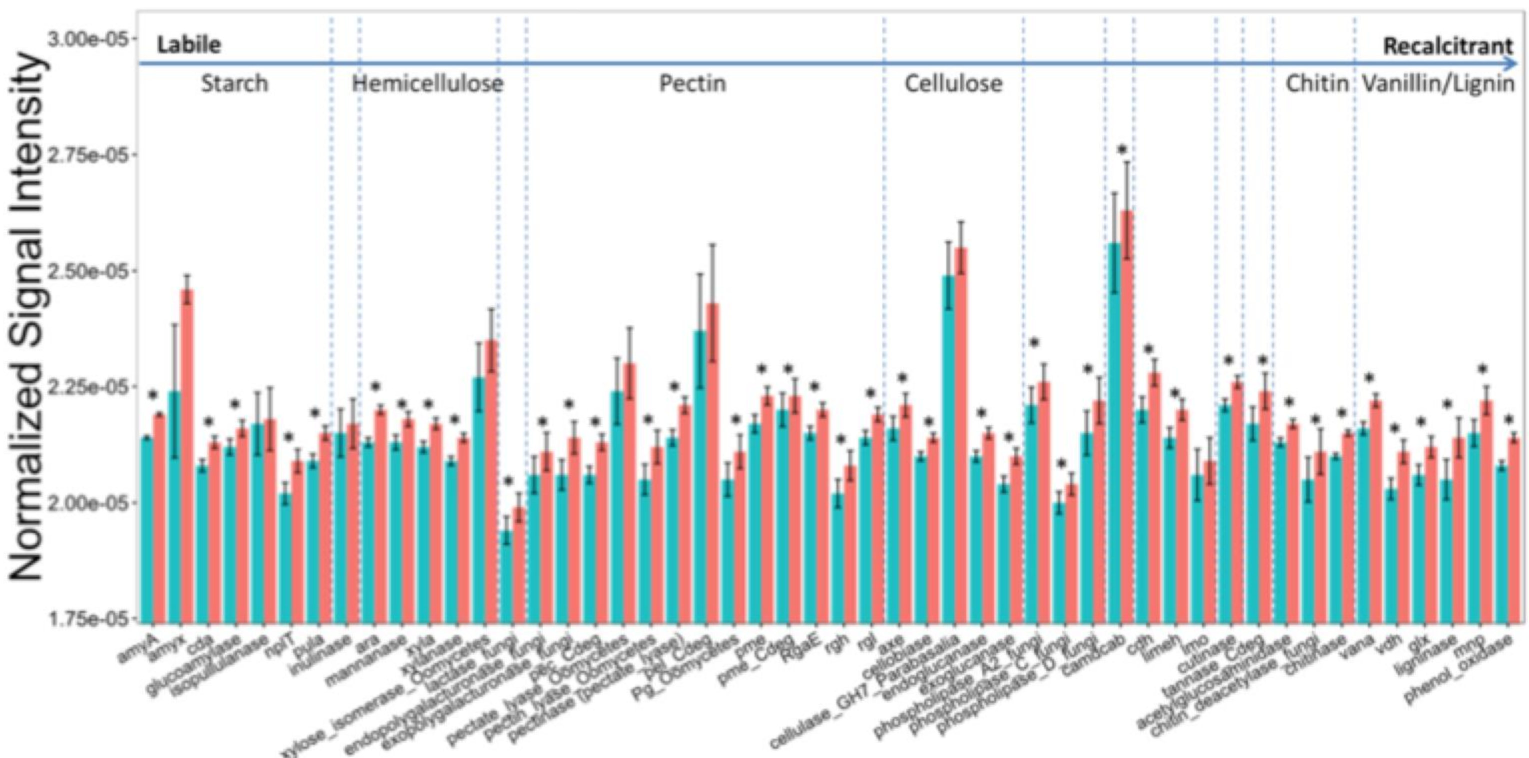

Figure 8. Normalized signal intensities of genes involved in C degradation

Blue bars represented the average normalized signal intensity of probes of each gene in ambient samples, while red bars represented warmed samples. Error bars represent standard error of the mean. The differences between warmed and control plots were tested using ANOVA, indicated by * when $p < 0.05$.

In addition to CO_2 production, methane emission is another important accelerator of climate warming in tundra ecosystems. As shown in Figure 9, 13 genes involved in methanogenesis were detected in our study, including *mcrA* encoding the alpha subunit of methyl coenzyme M reductase. Six genes (i.e., *mcrA*, *mrtH*, *mtaB*, *mtmB*, *mtxX*, and *hdrB*) showed a significantly ($p < 0.05$) higher abundance in warming samples, indicating a potential of higher methane production (Figure 9). However, genes involved in methane oxidation (e.g., *mmoX* and *pmoA* encoding particulate methane monooxygenase) also exhibited significantly ($p < 0.05$) higher signal intensities in warming samples (Figure 9), which could possibly offset potential increases in methane production..

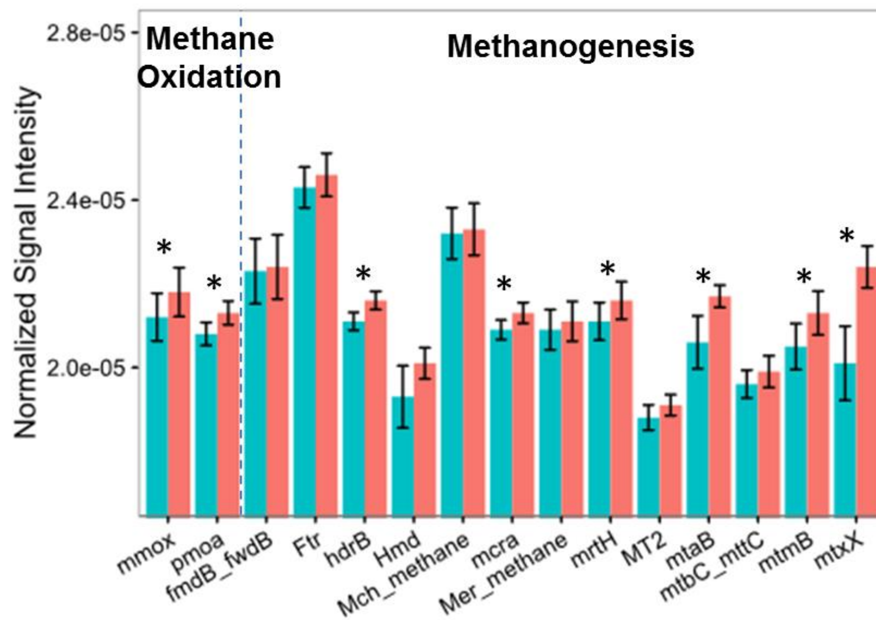

Figure 7. Normalized signal intensities of genes involved in Methane dynamics

Blue bars represented the average normalized signal intensity of probes of each gene in ambient samples, while red bars represented warmed samples. Error bars represent standard error of the mean. The differences between warmed and control plots were tested using ANOVA, indicated by * when $p < 0.05$.

N cycling

N is a limiting nutrient in tundra ecosystems. All of the detected genes involved in N cycling exhibited significantly (p < 0.05) higher signal intensities in warming samples (Figure 10), suggesting that warming potentially enhanced the whole N cycle. Multiple genes functioning in the major pathways of microbially-mediated N cycling were increased, including those involved in N fixation (e.g., *nifH* encoding nitrogenase reductase), nitrification (e.g., *amoA*, *hao*), denitrification (e.g., *narG*, *nirS*, *nirK*, *norB*, *nosZ*), dissimilatory nitrate reduction (e.g., *napA*, *nrfA*), assimilatory nitrate reduction (e.g., *nasA* encoding assimilatory nitrate reductase, *nir* encoding nitrite reductase), N mineralization (e.g., *ureC* encoding urease), and ammonia assimilation (*gdh* encoding glutamate dehydrogenase).

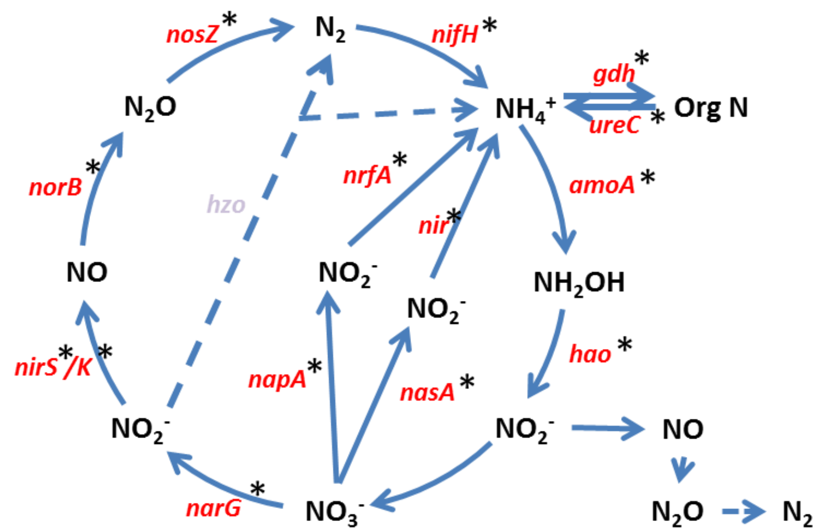

Figure 8. Normalized signal intensities of genes involved in N cycling

The differences between warmed and control plots were tested using ANOVA, indicated by * when $p < 0.05$.

P cycling

Phosphorus deficiency is common in soil ecosystems globally and microbial reactions to an increased P availability are of great interest. Genes involved in *ppx* encoding exopolyphosphatase, and phytase for liberation of inorganic phosphate from phytic acid degradation showed significantly higher signal intensities in warming samples ($p < 0.05$, Figure 11), suggesting that warming could potentially increase the availability of inorganic P in soil.

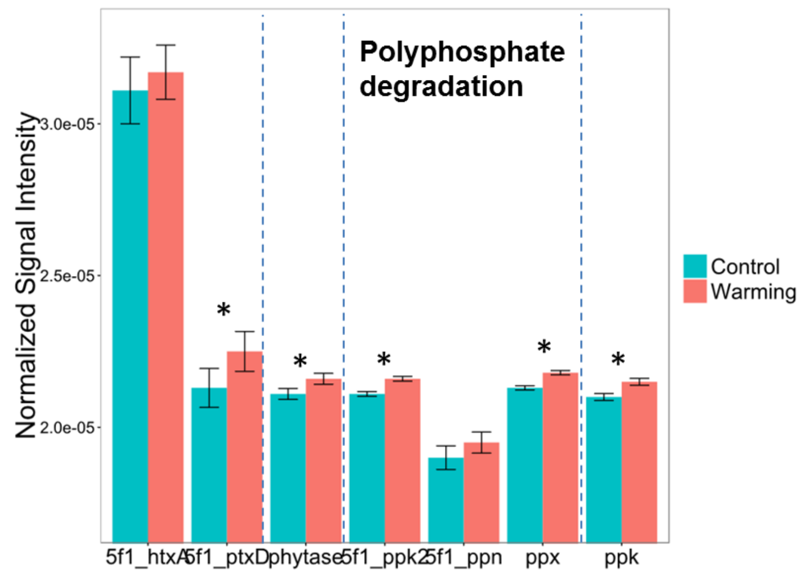

Figure 9. Normalized signal intensities of genes involved in P cycling

S cycling

Twenty-seven genes involved in sulfur metabolism were detected, among which 21 genes showed significantly ($p < 0.05$) higher signal intensities in warming samples, including *dsrA*/*B* for dissimilatory sulfite reductase, *Sir* for assimilatory sulfate reduction, *cysI*/*J* for cysteine biosynthesis required by sulfate reduction, and *soxY* for sulfur oxidation.

Molecular ecological network analysis

We constructed random matrix theory (RMT)-based phylogenetic molecular ecological networks (pMENs) using 16S rRNA sequencing data to explore the possible interactions within microbial communities. Coexistent OTUs in all replicates within the ambient or warmed group, considered as core species, were used for network construction. The network of warmed sites contained 1,568 nodes and 3,375 links, and the network of ambient sites contained 1,644 nodes and 2,898 links (Table 3). Both networks showed general features of biological networks, such as small world, scale free and modularity. In addition, we observed that the two networks differed significantly to their corresponding random network with the same node numbers and average link numbers in terms of average clustering coefficient and modularity (Table 3).

The two networks exhibited very distinct topological structures in terms of average connectivity, average geodesic distance, and modularity (Table 3), although they had similar network sizes. Average connectivity of the warmed network was significantly higher ($p < 0.001$) than that of the ambient network (Table 3), indicating nodes of the warmed network were more connected among each other. Average geodesic distance of the warmed network was significantly lower ($p < 0.001$) than its counterpart in the ambient network (Table 3), indicating that the warmed network had closer interactions. These findings suggested that the network composition and structure were significantly altered by warming.

Table 3. Major topological properties of the empirical pMENs of microbial communities in the ambient and warmed sites and their associated random networks

	Empirical networks							Random networks[c]		
Condition	No. of original OTUs[a]	s_t	Network size[b]	Avg connectivity	Avg geodesic distance	Avg clustering coefficient	Modularity (no. of modules)	Avg geodesic distance ± SD	Avg clustering coefficient ± SD	Avg modularity± SD
Ambient	1877	0.98	1644	3.526[d]	20.294[d]	0.476	0.954(222)	5.366±0.081	0.003±0.001	0.578±0.003
Warmed	1824	0.98	1568	4.305[d]	16.570[d]	0.486	0.926(189)	4.658±0.060	0.005±0.001	0.494±0.003

[a] The number of OTUs was originally used for network construction by the RMT-based network approach.

[b] The number of OTUs (i.e., nodes) in a network.

[c] The random networks were generated by rewiring all of the links of a pMEN with the identical numbers of nodes and links to the corresponding empirical pMEN.

[d] Significant difference ($p < 0.05$) between ambient and warmed networks.

To investigate the topological roles of OTUs identified in the two networks, we drew the *Z-P* plot based on within-module connectivity (Z_i) and among-module connectivity (P_i) (Figure 12). Three module hubs were detected in the warmed site network and the ambient site network, respectively (Table 4). Two connectors were detected in the warmed site network while three connectors were detected in the ambient site network (Table 4). No network hubs were detected in either warmed or ambient site networks.

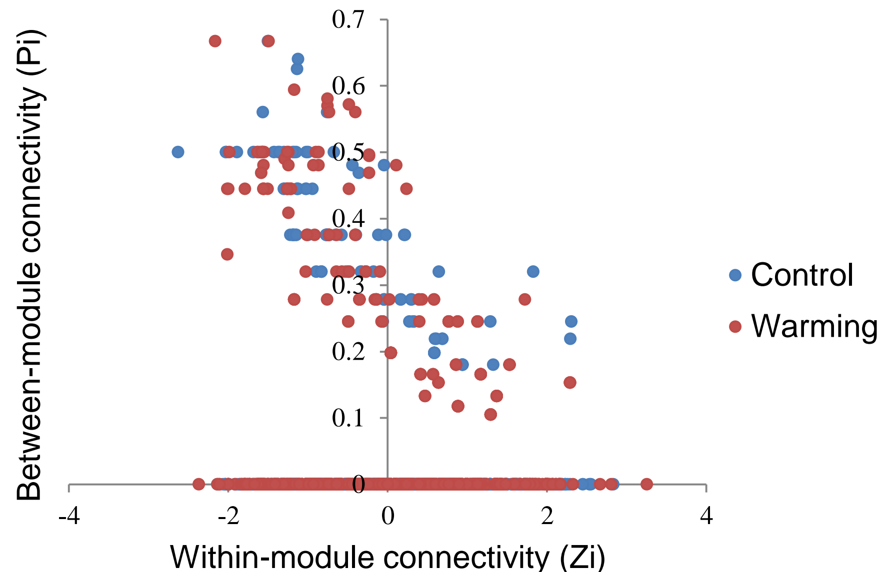

Figure 10. The topological roles of OTUs determined by within-module connectivity (Z_i) and between-module connectivity (P_i).

Table 4. Topological role of OTUs identified in the ambient and warmed networks.

Network	Topological role	OTU	Phylum	Class	Order	Family	Genus
Ambient	Module hubs[a]	OTU_17127	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Unclassified
		OTU_2286	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	Unclassified
		OTU_3347	Proteobacteria	Gammaproteobacteria	Unclassified	Unclassified	Unclassified
	Connectors[b]	OTU_13616	Acidobacteria	Acidobacteria_Gp3	Unclassified	Unclassified	Gp3
		OTU_24635	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Steroidobacter
		OTU_765	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_incertae_sedis	Bauldia
Warmed	Module hubs	OTU_22410	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Chondromyces
		OTU_585	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobineae_incertae_sedis	Aciditerrimonas
		OTU_7016	Acidobacteria	Acidobacteria_Gp3	Unclassified	Unclassified	Gp3
	Connectors	OTU_3505	Bacteroidetes	Unclassified	Unclassified	Unclassified	Unclassified
		OTU_595	Acidobacteria	Acidobacteria_Gp1	Unclassified	Unclassified	Gp1

[a] Module hubs are OTUs with $Z_i > 2.5$.

[b] Connectors are OTUs with $P_i > 0.62$.

To explore the relationships between network topologies with environmental traits, we performed partial Mantel test to calculate the correlation between the connectivity and the OTU significance (GS) of a certain environmental trait when effects of other traits controlled. In warmed network, the GSs of thaw depth and moisture were both significantly ($p < 0.05$) correlated with nodes' connectivity from dominated populations, e.g., Acidobacteria and Actinobacteria (Table 5), suggesting that the OTUs topology in warmed network was significantly associated with thaw depth, and moisture; while in ambient sites, the GSs of wintertime temperature and plant biomass played a more important role in network topology (Table 5). These finding suggested different environmental factors were detected to have high correlation with network topology in the two networks.

Table 5. Mantel test on connectivity vs. the OTU significances of soil geochemical variables

Phylogeny	Ambient				Warmed					
	Wintertime Temperature (°C)		Plant Biomass (g/m^2)		Thaw Depth (cm)		Plant Biomass (g/m2)		Soil Moisture (%)	
	r^a	p^b	r^a	p^b	r^a	p^b	r^a	p^b	r^a	p^b
Acidobacteria	0.079	**0.005**	0.065	**0.008**	0.055	**0.021**	-0.017	0.757	0.065	**0.008**
Actinobacteria	0.125	**0.001**	0.095	**0.019**	0.142	**0.004**	0.011	0.342	0.095	**0.019**
Alphaproteobacteria	0.142	**0.001**	0.068	**0.015**	0.113	**0.001**	0.044	0.084	0.068	**0.015**
Armatimonadetes	0.256	**0.018**	0.056	0.214	0.138	0.086	0.003	0.415	0.056	0.214
Bacteroidetes	0.160	**0.004**	0.044	0.119	-0.007	0.538	-0.051	0.776	0.044	0.119
Betaproteobacteria	0.098	0.118	0.292	**0.012**	0.076	0.195	-0.031	0.489	0.292	**0.012**
Chlamydiae	-0.026	0.538	0.825	0.088	0.184	0.236	-0.167	0.798	0.825	0.088
Chloroflexi	-0.142	0.533	-0.377	0.900	-0.222	0.667	-0.028	0.417	-0.377	0.900
Deltaproteobacteria	0.208	**0.004**	0.216	**0.006**	0.176	**0.010**	0.031	0.248	0.216	**0.006**
Firmicutes	0.060	0.349	0.002	0.458	0.047	0.348	0.825	**0.004**	0.002	0.458
Gammaproteobacteria	0.075	0.061	0.120	**0.017**	0.068	0.100	-0.009	0.497	0.120	**0.017**
Planctomycetes	0.140	**0.004**	0.094	**0.016**	0.124	**0.007**	0.061	0.084	0.094	**0.016**
Unclassified	0.194	**0.001**	0.103	**0.017**	0.037	0.205	0.007	0.384	0.103	**0.017**
Verrucomicrobia	0.134	**0.007**	0.076	**0.040**	0.137	**0.008**	0.060	0.090	0.076	**0.040**

[a] Correlation based on Mantel test.

[b] The significance of Mantel test; Bold values indicate $p < 0.05$.

To explore the roles of environmental factors in microbial community network, we added environmental factors as nodes into microbial community networks. The modules which contained environmental factors were selected from both networks, shown in Figure 13. Modules were much more connected with each other in the warmed site network than its ambient site counterpart, indicative of denser interactions. In the warmed site network, thaw depth had the highest node connectivity with other nodes; while water table depth was the most connected environmental factor in the ambient site network. These results suggested microbes in the warmed sites were more influenced by environment condition and thaw depth was the key environmental variable.

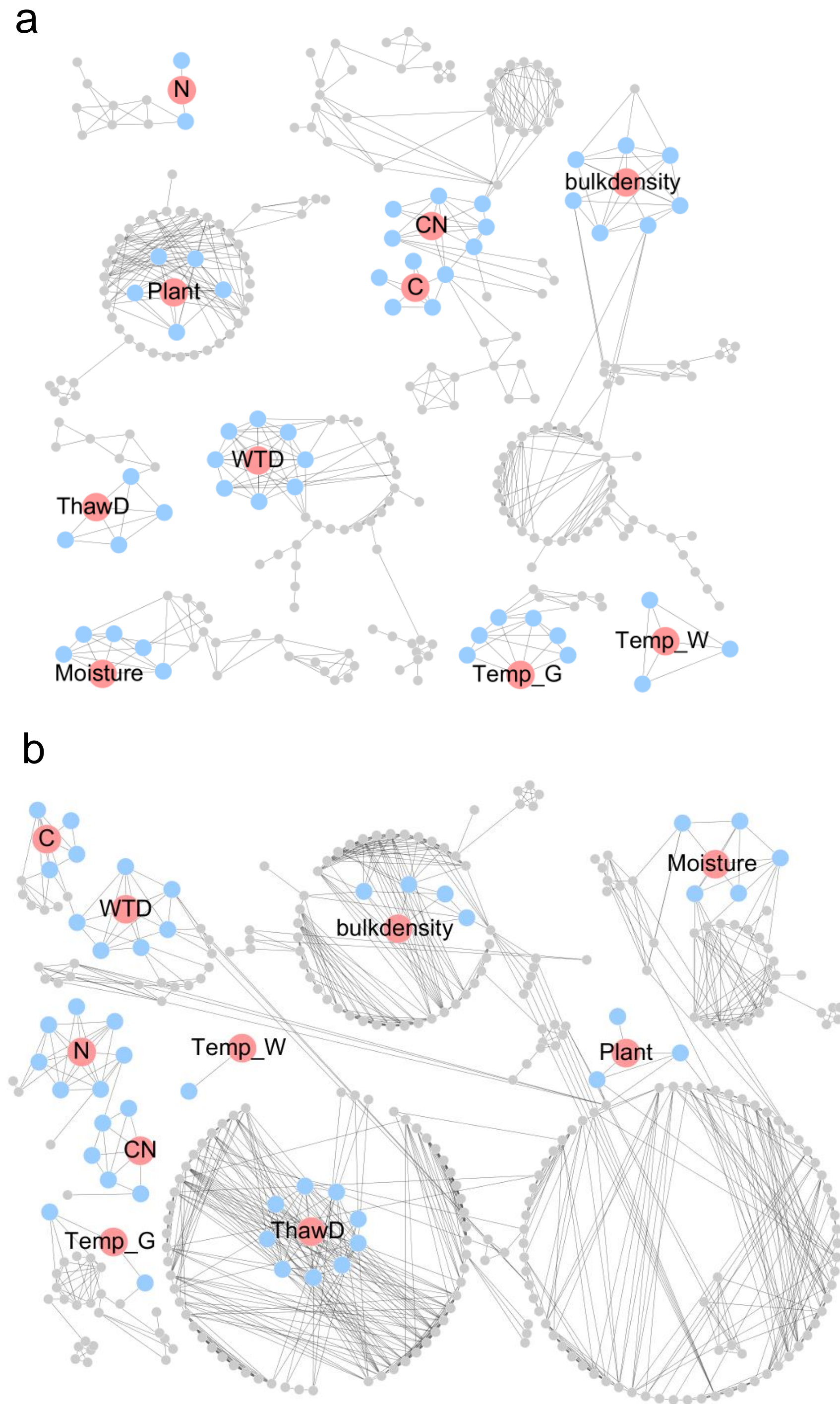

Figure 11. Network interactions between environmental parameters and microbial taxonomic community in the ambient network (a) and the warmed network (b).

The red nodes represent environmental factors. The blue ones represent the nodes directly connected to environmental factors. The grey ones were indirectly connected nodes in the corresponding modules.

Linking microbial community structure to soil properties and plant variables

To identify which environmental factors shape the microbial community, CCA and Mantel test were performed. For taxonomical structure of soil microbial communities, four environmental variables were identified on the basis of VIF (i.e., thaw depth, plant biomass, soil moisture, and wintertime soil temperature), which explained 71.01% of community functional variation in a statistically significant (p = 0.018) CCA model (Figure 14a). For the functional structure of soil microbial communities, five environmental variables were selected: thaw depth, soil moisture, plant biomass, wintertime soil temperature, and soil C/N ratio (Figure 14b), with the CCA model also exhibiting statistical significance ($p = 0.031$). The selected environmental factors in the CCA model were consistent with the Mantel test. In the Mantel test, three factors (e.g., thaw depth, plant biomass, and soil moisture, separately) were indicated to have significant ($p < 0.05$) or marginally significant ($p < 0.1$) influence on the overall taxonomical community (Table 6).

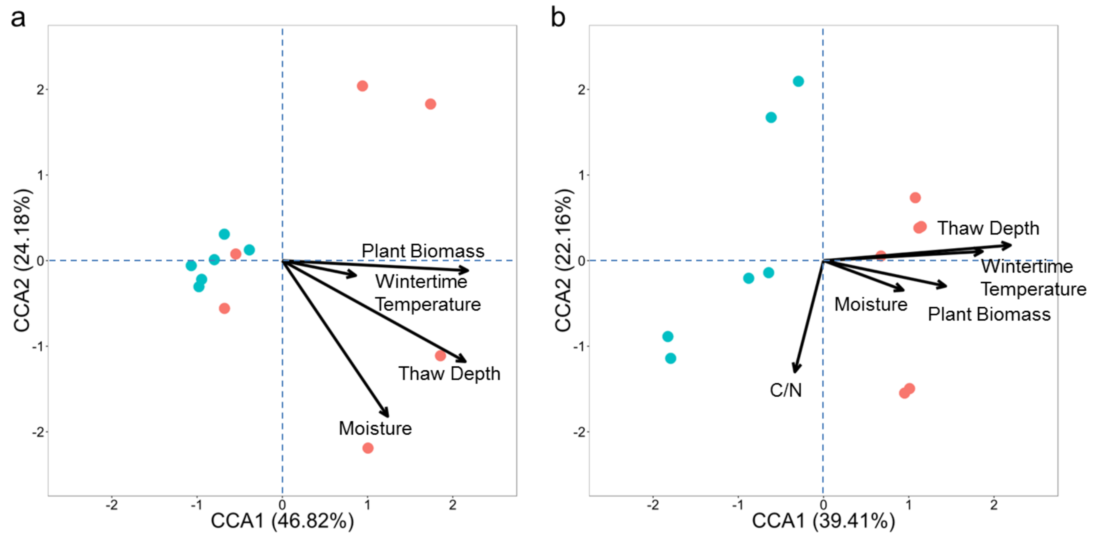

Figure 12. Canonical correspondence analysis (CCA) of a) 16s rRNA sequencing data; b) GeoChip data

Both CCA models were significant with $p < 0.05$. Remained variables were selected by the BIO-ENV procedure.

Table 3. Mantel tests using 16S rRNA gene amplicon sequencing data.

Environmental factors	16S rRNA sequencing		GeoChip 5.0	
	R	***p***	**R**	***p***
Water Table Depth (cm)`	-0.148	0.676	-0.062	0.600
Thaw Depth (cm)	0.489	**0.021**	-0.003	0.402
Soil Moisture (%)	0.231	**0.091**	-0.130	0.870
Growing Season Temperature (℃)	-0.315	0.992	0.232	**0.074**
Wintertime Temperature (℃)	-0.042	0.549	-0.114	0.825
Soil Bulk Density	0.124	0.226	0.125	0.155
Soil Total Nitrogen (%)	0.029	0.374	-0.001	0.466

Soil Total Carbon (%)	0.035	0.397	-0.057	0.666
C/N Ratio	-0.013	0.484	0.028	0.354
Plant Biomass (g/m^2)	0.480	**0.030**	0.020	0.390

Euclidean distance was used to calculate the distance matrices of environmental factors, 16S rRNA sequencing data and GeoChip 5.0 data. Bold values indicate $p<0.1$.

Ecological processes of microbial community assembly

We further determined the relative contribution of deterministic and stochastic processes to microbial community assembly using the null model and β-nearest taxon index (βNTI). βNTI, based on a null model, can factor out influences of stochastic and deterministic ecological processes(James C. Stegen et al., 2015). As shown in Figure 15, ecological processes governing community turnover differed considerably between the ambient and warmed plots. Among the community turnover processes within the ambient group, 46.67% of turnovers were governed by undominated processes. In contrast, 93.33% of community turnovers within the warmed group were determined by variable selection, suggesting that soil microbial community assembly was highly determined by heterogeneous environmental conditions resulting from warming manipulation.

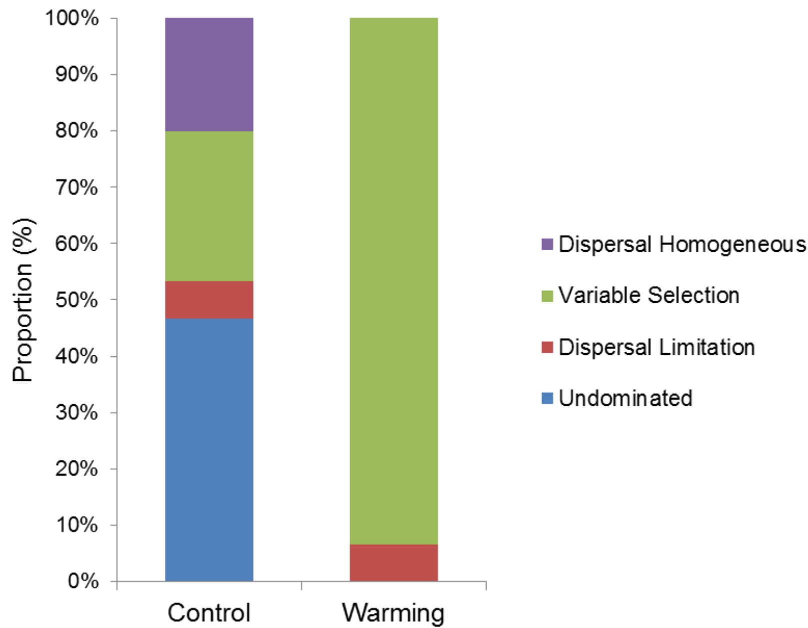

Figure 13. Proportions of microbial assembly processes in ambient or warmed sites

To explore which environmental factors impose the strong selection, we performed the correlation test between βNTI and changes in a given environmental factor. If an environmental factor imposes selection, then higher changes in it are expected to correlate with higher βNTI. Linear regression analysis indicated that βNTI was significantly correlated with changes in thaw depth (R = 0.690, $p < 0.001$, Figure 16a), plant biomass (R = 0.404, $p < 0.001$, Figure 16b), and soil moisture (R = 0.359, $p = 0.003$, Figure 16c). These findings indicated that thaw depth performed as the strongest selective factor, followed by plant biomass and soil moisture. It is in agreement to the results of CCA that thaw depth was identified as the key factor shaping both taxonomical and functional structure of microbial communities.

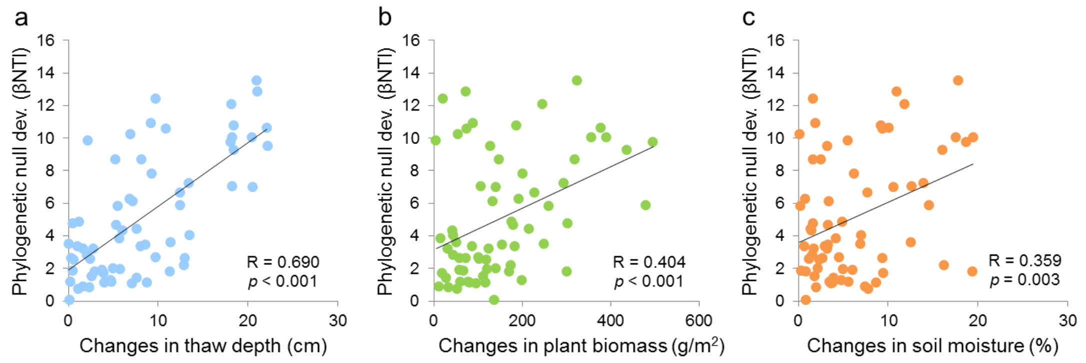

Figure 14. Microbial community turnover in terms of phylogenetic null model deviation. Turnover metrics are related to either changes in thaw depth (a), changes in plant biomass (b), or changes in soil moisture (c).

Chapter 4: Discussion

Microbial functional potential related to the magnitude and form of C losses in response to warming

Microbes serve not only the foundation of food webs but also primary drivers of biogeochemical processes (Madigan, Martinko, Dunlap, & Clark, 2008). Therefore, any alteration or disturbance of microbial communities in response to climate warming is of great ecological importance, influencing the function and activity of entire ecosystem. Considering the huge C stock and the vulnerability to climate warming in permafrost regions, microbial organisms inhabiting there, in particular, have been recognized as the key variable to understand the impacts of climate warming and resultant permafrost thawing on greenhouse gas emission (Graham et al., 2012).

Previous studies have shown a rapid response of permafrost microbes to climate warming, which resulted in the alteration of both compositional and functional structure of microbial communities (R. Mackelprang et al., 2011; K. Xue et al., 2016). Recently, an integrated metagenomic analysis from the first 1.5 years of warming at CiPEHR site demonstrated that short-term warming shifted the functional structure of soil microbial communities, with the abundance of genes involved in both aerobic and anaerobic C decomposition markedly increased (K. Xue et al., 2016). In the current study, more notable changes were observed after 5 years of warming manipulation. For example, the 1.5-year warming samples only showed significant changes in soil microbial functional structure, whereas significant differences in both functional and taxonomical structure were observed after 5 years of warming. Furthermore, 84% of detected genes involved in C degradation were increased after 5 years of warming, as compared to only 54.5%

of these genes increased in the 1.5-year warming samples. Therefore, the rapid microbial response to warming shown at 1.5 years of warming was not a temporary response. Instead, the trend was sustained and amplified after a longer term of warming.

Given the importance of microbial activities in biogeochemical processes, we aimed to obtain a deeper understanding on how microbial response to warming may influence biogeochemical cycles. Permafrost thaw induced by warming is expected to cause previously protected C stock biologically available, and thereby accelerating C losses. In the current study, 5 years of soil warming significantly increased most of the genes involved in degrading substrates ranging from labile (e.g., starch, hemicellulose and cellulose) to recalcitrant (e.g., aromatics and lignin) C (Figure 8). This finding, from the molecular level, is in agreement with recent observation that the annual decomposition rate of a common substrate (cellulose) in soil at 0-10 cm depth was increased by nearly two-fold due to warming (Natali et al., 2015). Also, warming was found to stimulate ecosystem respiration significantly during the non-summer season each year from 2009-2013 (E. E. Webb et al., 2016). These results unambiguously indicate that aerobic C composition accelerated with soil warming. In particular, genes involved in recalcitrant C decomposition (e.g., aromatics and lignin, Figure 8) were significantly increased by soil warming. Consistently, sequencing analysis showed that the abundance of the genus *Chitinophaga*, which was reported to be strongly chitinolytic (SANGKHOBOL & Skerman, 1981), was also increased after warming. Therefore, a potential increase in degradation of old recalcitrant C was expected.

Field warming experiments suggests that the initial increase in CO_2 efflux may gradually disappear, with the rate of C release returning to the pre-warming value

within a few years (Eliasson et al., 2005; Knorr et al., 2005; Luo et al., 2001; Melillo et al., 2002; Walter C. Oechel et al., 2000; Rustad et al., 2001). In contrast, our study demonstrated a continuing potential of increased soil organic carbon (SOC) release (particularly old recalcitrant C) over a 5-year warming manipulation. This phenomenon can be attributed to the following reasons. 1) Continuing warming manipulation led to a larger difference in soil condition between warmed and ambient sites over time, as evidenced by an increased difference of thaw depth (Figure 1). Accordingly, acclimatization of microbial communities is unlikely to occur in our study. 2) The temperature sensitivity of recalcitrant SOC is higher than labile SOC (Bracho et al., 2016; Knorr et al., 2005). The sustained potential of increased C release implies that the tundra ecosystem would continue to contribute toward accelerating climate warming

In addition to accelerated aerobic C decomposition, warming would also facilitate anaerobic C decomposition. GeoChip results revealed that the abundance of most genes involved in methanogenesis was increased as a result of warming (Figure 9). Consistently, 16S rRNA sequencing showed that *Methanobacteria*, a well-known class of methanogens, was only present in the warming samples. Therefore, results from both functional and taxonomical community analysis indicate that warming would lead to higher CH_4 release, which has been recently reported at our studied sites (Natali et al., 2015). It should be noted that warming also increased the abundance of CH_4 oxidizing genes (e.g., *pmoa* encoding particulate methane monooxygenase subunit A and *mmox* encoding methane monooxygenase, Figure 9). This phenomenon was likely resulted from an increase in available CH_4 for oxidation and indicates the potential of altered microbial communities to offset an increased CH_4 release. The increased potential in

methanogenesis could be ascribed to the changed hydrological condition in warmed sites. In the studied sites, although soil moisture was not significantly increased overall, a marginally significant increase in soil moisture was observed during the growing season (e.g., May, June and July). Also, the water table was shallower at warming sites than at control sites (Table 1). These observations indicate that soil warming altered soil moisture and potentially resulted in shallower saturated areas, which favored anaerobic processes. Soil moisture is of particular concern because it is the main environmental factor affecting C losses besides temperature (Oberbauer et al., 2007; Walter C Oechel et al., 1998; Shaver et al., 2006), especially considering its role in affecting the form of C release. In tundra ecosystems, well-drained areas with low moisture were observed to be more responsive to warming (Oberbauer et al., 2007). Furthermore, not only the amount but also the form of C release is of great importance, because CH_4, the major product of anaerobic C decomposition, has 28-34-fold larger global warming potential as compared with CO_2 (Myhre et al., 2013). Multiple *in situ* studies have indicated that soil moisture served as a major driver for determining the form of C release (Kane et al., 2013; Olefeldt et al., 2013). In another study performed at CiPEHR, a higher CH_4 release rate observed in warmed sites co-occurred with higher moisture, and the combined effects of warming and drying was found to increase loss of old permafrost C (Natali et al., 2015). Hence, our results provide additional evidence that methane-related metabolic activities by microbial communities were stimulated by warming, and similar findings were reported in our previously published study (Kai Xue et al., 2016).

In the permafrost region, low temperature and wet soil conditions serve to constrain microbially-mediated biogeochemical processes such as nutrient

mineralization, and plant productivity is limited by a low nutrient supply to roots (F. Chapin III, Miller, Billings, & Coyne, 1980). Also, fertilization studies in various tundra types consistently showed increases in plant growth and net primary production in response to nitrogen and/or phosphorus additions (F. S. Chapin III & Shaver, 1985; Haag, 1974; McKendrick, Batzli, Everett, & Swanson, 1980). As a result, understanding how nutrient cycling responds to warming is a prerequisite for predicting the degree to which the increase in C fixation from higher plant productivity could offset C losses in a warmer climate. Microbial activity and element turnover rates in arctic soils accelerate with increased soil temperature. Consequently, enhanced soil respiration and carbon losses resulting from a rising temperature would likely increase the rates of nutrient release from decomposing organic matter (F. S. Chapin III et al., 2012). In the current study, GeoChip analysis revealed that all of the N cycling-associated genes exhibited higher signal intensities in warming sites (Figure 10). It is in agreement with our previous study on the dynamics of *nifH* harboring microbial communities, in which warming significantly increased both richness and α-diversity of *nifH* genes (Unpublished). Paralleled with a recent study in which 5-year soil warming increased both inorganic N availability and foliar N pools at the CiPEHR site (Salmon 2015), our results provide unambiguous evidence that warming accelerated the whole N cycle.

A larger nutrient pool available to plants facilitated plant growth, which was evidenced by significantly increased plant biomass in our study (Table 1). This phenomenon is consistent with previous field studies on tundra ecosystem, in which warming increased nutrient content in the soil (Deane-Coe et al., 2015; Salmon et al., 2016). Furthermore, positive interactions between warming and fertilization have been

observed (Van Wijk et al., 2004), implying that higher temperature and nutrient availability could have a synergistic effect. Although increased plant growth leads to enhanced C fixation, long-term fertilization experiments in Alaska tundra showed that plant productivity was not able to offset C losses. Instead, a negative net ecosystem exchange was observed (Mack, Schuur, Bret-Harte, Shaver, & Chapin, 2004). Similarly, addition of organic N to the active layer above permafrost has recently been shown to increase SOM degradation by 2~3-fold (Wild et al., 2014). Therefore, an increased soil nutrient availability associated with warming may further amplify C losses and consequently impose a positive feedback to climate warming.

In addition to the significant shift in both taxonomic composition and functional structure of soil microbial communities, phylogenetic network structure was also notably changed in response to warming. The warmed network exhibited more complex network structure and tighter interactions among different phylogenetic groups, suggested by higher average connectivity and shorter average geodesic distance (Table 3). The High interaction in a network is likely to associate with deterministic processes (e.g., environmental filtering) (Cornwell, Schwilk, & Ackerly, 2006), in our case, the higher connectivity of many phylogenetic populations (e.g., Acidobacteria, Actinobacteria, α-proteobacteria, δ- proteobacteria and Verrucomicrobia) in the warmed site was significantly ($p < 0.05$) correlated to thaw depth and moisture (Table 5). Consistently, CCA result using 16S rRNA sequencing data also detected that thaw depth and moisture are the most crucial environmental factors shaping taxonomical composition (Figure 14a). One of the major tasks in ecology is to identify the key populations in a community. We achieved this by measuring the topological role of

individual node based on its within- and among-module connectivity. No overlaps of either module hubs or connectors within the two networks were detected (Table 4), suggesting the network structures of the two networks were influenced by different populations.

To better predict how microbial communities develop when facing an environmental perturbation, we need to understand the mechanisms driving community assembly. Our results showed 5-year warming manipulation led to microbial community assembly dominated by variable selection while the ambient counterpart didn't show such dominance (Figure 15). It should be noted that there is a potential limitation in Stegen et al.'s framework to estimate ecological processes. Both deterministic and stochastic processes contribute to community assembly, however, when determining a pairwise turnover was governed by variable selection, the stochastic part was ignored, thus overestimating the proportion of variable selection in the warmed group. Despite of this limitation, Stegen et al.'s framework is useful to compare the different dominated ecological processes between the ambient and warmed group. Significant increases ($p < 0.05$) in variable selection and decreases ($p < 0.05$) in the undominated fraction in warmed sites were detected compared to the ambient sites (Figure 15). The influence of variable selection in both sites was most strongly related to thaw depth ($R = 0.690$, $p < 0.001$, Figure 16a), consistent with the inference that high interactions in the warmed network could be ascribed to deterministic processes correlated to thaw depth. Permafrost thaw has long been considered to have profound effects local hydrological, thermal, and C dynamics (Hicks Pries, Schuur, & Crummer, 2013; Hicks Pries et al., 2016; O'Donnell et al., 2012; Schuur, Abbott, & Network,

2011). In our study, the warmed sites had higher thaw intensity, indicated by significantly ($p < 0.05$) deeper thaw depth, which could lead to more heterogeneous local environmental conditions, as indicated by the higher standard errors of several environmental attributes, e.g., soil moisture, thaw depth and plant biomass (Table 1). The spatially heterogeneous selective environment resulted from permafrost thaw could explain the dominated role of variable selection in the warmed sites. In addition, soil microbial communities in warmed sites diverged, resulting in higher within β-diversities in warmed samples (Figure 5b). Taken together, high relative contribution of variable selection in the warmed sites suggested microbial community tended to be deterministically assembled rather than randomly developed in a more divergent environment in the warmer world.

Chapter 5: Conclusions

Profiling soil microbial communities in the permafrost underlain tundra regions is crucial for predicting the future dynamics of C release. As compared to our results from 1.5-year warming (K. Xue et al., 2016), the current study demonstrated more notable changes after 5 years of warming manipulation, in terms of both the taxonomical and functional structure of soil microbial communities. Specifically, warming significantly altered the diversity and compositional structure of the microbial communities. The microbial activities in both aerobic and anaerobic C decomposition were increasingly enhanced, leading to a potential increase in greenhouse gas emission. In addition, nutrient cycling, including N, P, and S cycling, could also be facilitated as a result of microbial response. These results suggest that warming markedly influenced the ecological function of microbial communities in permafrost soil, which would in turn change the carbon and nutrient balance of the entire ecosystem. Furthermore, the interactions among different microbial populations were found to be tighter due to warming, indicating an adjustment of microbial communities to the altered environmental condition. Therefore, the rapid response by microbial communities to warming is a sustained and amplified trend. This response will not only alter the structure, function, and interactions of microbial communities themselves, but also provide a positive feedback to warming due to enhanced C decomposition and nutrient cycling.

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