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Temporal variation in glacier snowpack bacterial communities mediated by nitrogen

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Abstract. Global warming accelerates glacier melt, releasing stored carbon and nitrogen, which fertilize downstream ecosystems. Diverse and active microbial communities mediate biogeochemical cycles in snow and are vital to the glacial ecosystem. However, little is known about their temporal changing pattern and the environmental and biotic determinants in snowpacks. Here, we investigated the bacterial community in the surface and subsurface snow (depth at 0-15 and 15-30 cm, respectively) during a nine-day period in the Dunde Glacier of the Tibetan Plateau, based on Illumina MiSeq of 16S rRNA gene sequences. Our results revealed dynamic bacterial communities in both surface and surface snow, and nitrogen is the key determinant of bacterial diversity, composition, community structure, and biotic interactions. Nitrate and ammonium concentration increased and decreased in the surface and subsurface snow over time, therefore indicating accumulation and consumption processes, respectively. This is also evidenced by the dominance of organisms predicted to carry nitrogen fixation and denitrification genes in the surface and subsurface layers, respectively. The nitrogen limitation and the apparent dominance of the denitrification in the subsurface snow suggest stronger environmental and biotic filtering than those in the surface snow. This was associated with lower bacterial diversity, more pronounced community temporal changes, and stronger biotic interactions than in the surface snow. Collectively, these findings significantly advanced our understanding of microbial community variations and bacterial interactions after snow deposition, and revealed the dynamics of nitrogen metabolism in Tibetan snow.

1 Introduction

Global warming accelerates glacier melting across the globe, and the nutrients stored in glaciers are released into downstream ecosystems in melt waters (Hodson et al., 2005; Wadham et al., 2019). The composition and abundance of nutrients in glaciers, which are typically poor in inorganic nutrients relative to aquatic and soil environments (Ren et al., 2019) are regulated by glacier-dwelling microorganisms (Hodson et al., 2008). A range of metabolically active bacteria have

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been reported in snow environments including Bacteroidetes, Actinobacteria, Firmicutes, and Alphaproteobacteria (Miteva, 2008; Maccario et al., 2019; Carey et al., 2016; Lazzaro et al., 2015; Michaud et al., 2014). These microorganisms perform key ecological functions in biogeochemical cycles such as carbon and nitrogen fixation, which are vital to nutrient-limited glacial ecosystems. Changes in their community composition and activities are expected to influence the dynamics of glacial nutrient-storage and release. Thus, it is crucial to understand how the microbial community in glacial snow changes across time and to determine whether those changes are associated with the temporal nutrient differences in snow, to fully estimate the ecological consequences of global glacier melting.

During precipitation events, a new snow layer forms above the previous ones, which creates a stratified structure. Each snow layer has unique physical and chemical characteristics (Lazzaro et al., 2015), which may trigger distinct post-depositional selective processes on microbial communities (Xiang et al., 2009; Møller et al., 2013). For example, Cyanobacteria tend to dominate upper snow layers (0-15 cm) (Carey et al., 2016), while their relative abundance is greatly reduced in the deeper snow layer (Xiang et al., 2009). This is likely due to lower light intensity in the deeper snow, and favors heterotrophic bacteria such as the Actinobacteria and Firmicutes (Carey et al., 2016). Thus, differences in the snow physicochemical conditions shape their distinct bacterial community structures. However, whether microorganisms in the different snowpack layers exhibit similar responses to environmental selection is still largely unknown.

Differences in physicochemical conditions can also indirectly influence microbial community structure through impacts on the types of biotic interactions that dominate an environment (Friedman and Gore, 2017; Khan et al., 2018; Bergk Pinto et al., 2019). On Spitsbergen Island in Svalbard for example, the addition of organic carbon shifted microbial interactions from collaboration to competition (Bergk Pinto et al., 2019). In comparison, intensive collaboration can enhance complex organic carbon degradation and mineralization, which are particularly important for oligotrophic environments such as glaciers (Bergk Pinto et al., 2019; Krug et al., 2020). Collaboration is also known to be essential to biological processes such as ammonia oxidation and denitrification, in which various organisms carry out different steps of these processes (Henry et al., 2005; Madsen, 2011; Yuan et al., 2021). These changes in interactions and network complexity can favor or disadvantage certain microbial groups, thereby changing the microbial community structure (i.e. biofiltering).

Tibetan Plateau is the world's third-largest ice reservoir, after those in Antarctica and Greenland (Qiu, 2012). It is warming at a rate twice of the global average (Chen et al., 2015), causing rapid shrinkage of glaciers and snow (Rauscher et al., 2007; Hall and Fagre, 2003). The glacier melting leads to the enhanced discharge of microorganisms and nutrients into downstream aquatic and terrestrial ecosystems, which makes an impact on their biogeochemical processes. Thus, it is crucial to understand the transformation processes of microbial community and nutrients in the snow.

In the present study, we investigated the bacterial community composition and interaction changes in the surface and subsurface snow layers during a nine-day period at the Dunde Glacier on the northeast of the Tibetan Plateau. Our sampling strategy focused on the ablation zone of the glacier, because it is in this region that mass lost exceeds mass gained. Therefore, ablation zone microorganisms and the biogeochemical processes they are associated with are expected to have a greater impact on downstream processes, relative to that of the glacier accumulation zone. In particular, we aimed to answer the

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following key questions: 1) do the bacterial communities in different snow layers exhibit similar community temporal changes, and 2) are temporal changes within each layer related to environmental filtering, biotic interactions, or both?

2 Materials and methods

2.1 Site description and sample collection

Snow samples were collected from the ablation zone at Dunde glacier (38°06′N, 96°24′E, 5325 m above the sea-level), during the October and November, 2016 (Supplementary Fig S1). Dunde glacier is located in the Qilian mountain region on the northeastern Tibetan Plateau, and it is continuously monitored by the Institute of Tibetan Plateau Research, Chinese Academic of Sciences. Sampling was conducted over a nine-day period (on the 24th, 25th, 26th, 27th, and 29th of October, and the 2nd November, which are referred as day 1, 2, 3, 4, 5, 6, and 9 thereafter). No snow was observed on the glacier surface during the summer, so the snowpack observed during October and November represented fresh snow accumulated during the autumn months. This enabled us to follow the development of microbial communities and the chemical environment through time. On each day, three snow pits were dug within a 5 m × 3 m area and any two snow pits were at least 30 cm apart. Each snow pit was approximately 30 cm deep, and the bottom of the snow pit was a layer of ice, indicating that the snow pits encompassed the depth of the existing snowpack. Each snow pit was further divided into surface and subsurface layers (approximately 15 cm deep for each layer) based on the softness and heterogeneity of the snowpack, after Carey (2016). For each snow pit, the top 1 cm in contact with the air was removed using a sterile spoon to avoid contamination, and then surface and subsurface snow were collected using a sterilized Teflon shovel into 3L separate, sterile sampling bags. An additional 120 mL (meltwater volume) of surface and subsurface snow was sampled for physiochemical analyses. Tyvek bodysuits and latex gloves were worn during the entire sampling process to minimize potential for contamination, and gloves were worn during all subsequent handling of samples. Samples were kept frozen during the transportation to the laboratory and stored at -20 °C until analysis.

2.2 Environmental characterization of snow

For dissolved organic carbon (DOC), total nitrogen (TN), and major ions measurements, 100 mL of snow meltwater was syringe-filtered through a 0.45 μm cellulose membrane (Millipore, Billerica, MA, USA) into 20-mL glass bottles (1% HCl leached, deionized water rinsed, and 450 °C > 3 h combusted). The DOC and TN concentrations were measured with a TOC-VCPH analyzer (Shimadzu Corp., Japan). Major ions (NH_4^+, NO_3^-, Na^+, K^+, and SO_4^{2-}) were analyzed using a Thermo-Fisher ion chromatography system 900 as described previously (Rice et al., 2012). The precision and accuracy of the TOC-VCPH analyzer were both < 3% and the limit of detection was 0.05 mg L^{-1}. The precision and accuracy of the ion chromatography system 900 were < 5% and 0.1 mg L^{-1}, and the limit of detection was 0.01 mg L^{-1}. The concentrations of TN, NH_4^+, and NO_3^- ions were quantified using different methods. The ion chromatography method for quantifying NH_4^+ and NO_3^- ions is susceptible to interference from carbonate and bicarbonate ions in samples (Novic et al., 1997), this may lead to

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an overestimation of nitrate ion concentration, which does not allow direct concentration comparison between TN and NO_3^- ions. Nevertheless, the standard curves were linear with r > 0.99, thus allows comparison among different time points (Supplementary Fig S2).

2.3 DNA extraction

For assessing the bacterial community composition, each of the melted snow samples (3 L) was filtered onto a 0.22 μm polycarbonate membrane (Millipore, USA) with a vacuum pump (Ntengwe 2005). Bacterial community DNA was extracted from the biomass retained in respective filters using a Fast DNA®SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. DNA extraction with no sample added were performed in parallel, and the final elute was used as a negative control.

The raw DNA was checked by electrophoresis in 1% (w/v) agarose gel, and purified from the gel using an Agarose Gel DNA purification kit (TaKaRa, Japan). The concentration and purity of the DNA extracts were measured using a NanoDrop 1000 spectrophotometer (Thermo-Scientific, Wilmington, DE, USA). The extracted DNA was stored at -80°C until amplification.

2.4 Bacterial 16S rRNA amplification and Illumina MiSeq sequencing

In total, 36 DNA samples and one negative control were subjected to amplicon sequencing. Universal primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2012) with 12 nt unique barcodes were used to amplify the V4 hyper-variable regions of the bacterial 16S rRNA gene. Polymerase chain reaction (PCR) was performed under the following conditions: 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds; followed by a final cycle of 10 minutes at 72°C. Each PCR reaction contained 12.5 μL 2x Premix Taq DNA polymerase (Takara Biotechnology, Dalian Co. Ltd., China), 1 μL each primer (0.4 μM final concentration), and 8.5 μL nuclease-free water, 2 μL DNA template (20 ng μL^{-1}) or 2 μL sterile water for the PCR negative controls. PCR products were confirmed using agarose gel electrophoresis, and no PCR band was detected in PCR negative controls. To minimise PCR batch-to-batch variations and maximise the quantity of PCR product, triplicate PCR reactions were performed for each sample, and PCR products were pooled for purification using the OMEGA Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA) following electrophoresis. PCR products from different samples were pooled in equal molar amounts, and then used for 2 × 250 bp paired-ends sequencing on a MiSeq machine (Illumina, San Diego, CA).

2.5 Processing of Illumina sequencing data

MiSeq sequence data were processed using the QIIME 2 pipeline version 2018.8 (Bolyen et al., 2018) following the recommended tutorials (https://docs.qiime2.org/2018.8/tutorials/) and using the plugin demux to visualize interactive quality diagrams and check read quality. Plugin DADA2 (Callahan et al., 2016) was applied to remove primers, truncate poor-quality bases, conduct dereplication, identify chimeras, and merge paired-end reads. Commands included in the feature table

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(McDonald et al., 2012) generated the summary statistics of sequences related to the samples. Further, we trained a Naïve Bayes Classifier with the feature-classifier plugin using the 16S rRNA gene database at 99% similarity of the SILVA 132 QIIME release and based on the 515F/806R primer pair as used for the PCR. Finally, the taxa plugin was used to filter mitochondrial and chloroplast sequences, as well as to generate absolute read count tables of all taxa for each sample. Data were analyzed at the level of amplicon sequence variant (ASV), where ASVs are delineated by 100% sequence identity (Callahan et al., 2017).

After removing singletons, a total of 1,685,186 high-quality reads were obtained, representing 9178 ASVs. Before statistical analysis, the dataset was rarefied to 45,000 reads per sample, which is the lowest read count among samples. Rarefaction curves reached an asymptote before the subsampling, which confirmed that this depth was sufficient to detect the diversity present (Supplementary Fig S3).

2.6 Network analysis

The ASV-ASV associations within the surface and subsurface bacterial communities were explored using Molecular Ecological Network Analyses Pipeline (http://129.15.40.240/mena/) (Deng et al., 2012). The ASVs that occurred in at least 50% of the samples from the surface or subsurface group were selected to construct the network. Spearman's rank correlation coefficient (ρ) was calculated to reflect the strength of association between species. The false discovery rates (Q-values) were calculated from the observed P-value distribution. The resulting correlation matrix was analyzed with the Random Matrix Theory (RMT)-based network approach to determine the correlation threshold for network construction, and the same threshold was used for both the surface and subsurface network, so the topological properties of the surface and subsurface networks are comparable.

2.7 Statistical analysis

Shannon-Wiener and Chao1 indices, which were used to estimate the species richness in the snow community, were calculated using the 'diversity' function in the R package 'vegan' (Oksanen et al., 2010). Functional profiling of bacterial taxa was carried out using the package "Tax4Fun2" in R (Wemheuer et al., 2020). While the application of functional profiles predicted from 16S rRNA gene-based community composition data is limited by the functional information available in databases, we present these data as one possible interpretation of the patterns we detected, and note that the "Tax4Fun2" package performed well compared to older widely used programs (Wemheuer et al., 2020). The pairwise Wilcoxon rank-sum test was used to compare the depth-horizon differences in environmental variables, alpha-diversity, the relative abundance of taxonomic groups at the phylum level, and the relative abundance of putative functional genes. Linear regression models were used to estimate the trend of changes over time. The bacterial community structure was subjected to principal coordinate analysis (PCoA) carried out using the "pcoa" function of the "ape" package in R. Principal component analysis (PCA) was performed in R using the "prcomp" function. The significance of dissimilarity of community composition among samples was tested using permutational multivariate analysis of variance (PERMANOVA) based on

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Bray-Curtis distance metrics with the adonis function in the R package 'vegan' (Oksanen et al., 2010). Test results with $P < 0.05$ were considered statistically significant. The correlations between bacterial diversity and snow variables were calculated using Spearman's rank correlation, as implemented in the 'ppcor' R package. Mantel test based on Spearman's rank correlations was performed using the bacterial dissimilarity and environmental dissimilarity matrix, calculated based on the Bray-Curtis distance metrics and Euclidean distance metrics in the 'vegan' R package, respectively. The normalized stochasticity ratio (NST) based on the Bray–Curtis dissimilarity was calculated using the 'NST' package in R to estimate the determinacy and stochasticity of the bacterial assembly processes with high accuracy and precision (Ning et al., 2019). The NST index used 50% as the boundary point between more deterministic (<50%) and more stochastic (>50%) assembly processes. All environmental variables were normalized before the calculation. All statistical analyses were executed in R version 3.4.3 (R Core Team, 2017).

3 Results

3.1 Environmental characteristics of the snowpack

The concentrations of TN, NO_3^-, and NH_4^+ ranged from 0.18 to 1.26 mg L^{-1}, 0.44 to 5.09 mg L^{-1}, and 0.17 to 0.62 mg L^{-1}, respectively (Supplementary Table S1). The concentrations of TN, NO_3^-, and NH_4^+ were significantly higher in the subsurface layer than in the surface layer (Wilcoxon rank-sum test; all $P < 0.001$, Fig 1a). K^+ and SO_4^{2-} ions in the subsurface layer were also significantly higher (0.29 ± 0.13 and 6.09 ± 3.18 mg L^{-1}, respectively) than those in the surface layer (0.12 ± 0.08 and 3.71 ± 1.64 mg L^{-1}, respectively; Wilcoxon rank-sum test; $P < 0.001$, and $P = 0.015$, respectively). The concentrations of DOC ranged from 0.46 to 5.89 mg L^{-1} and exhibited no significant difference in the surface and subsurface layers (Wilcoxon rank-sum test; $P = 0.310$). The concentrations of Na^+ ion ranged from 0.35 to 7.34 mg L^{-1}, which also exhibited no significant difference in the surface and subsurface layers (Wilcoxon rank-sum test; $P = 0.079$). The concentration of NO_3^-, and NH_4^+ ions in the surface layer significantly increased with time (Fig 1b). In comparison, the concentrations of TN, NO_3^-, and NH_4^+ in the subsurface layer significantly decreased with time. Other environmental factors showed no significant correlation with time in either layer.

3.2 Diversity and composition of bacterial community from the snowpack

The bacterial Shannon and Chao1 indices in the surface layer were 5.61 ± 0.39 and 744 ± 199, respectively, which were not significantly different from those in the subsurface layer (5.52 ± 0.68 and 705 ± 269, respectively) (Fig 2a). In the surface layer, the Shannon and Chao1 indices did not change significantly with time (Pearson correlation; r= 0.15, $P = 0.553$; r = -0.02, $P = 0.939$, respectively, Fig 2b). In contrast, both the Shannon and Chao1 indices both significantly decreased with time in the subsurface layer (Pearson correlation; r = 0.63, $P = 0.003$; r = 0.56, $P = 0.009$, respectively). In the surface layer, the Shannon indices positively correlated with the concentration of DOC (Pearson correlation; r= 0.48, $P = 0.04$, Fig 3a). In

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the subsurface layer, the Shannon and Chao1 indices were positively correlated with the concentration of TN, NO_3^- and NH_4^+ (Pearson correlation; $P < 0.05$, Fig 3b and Supplementary Fig S4).

The surface and subsurface snow layers were both dominated by Alphaproteobacteria (25%), Actinobacteria (20%), Cyanobacteria (15%), Gammaproteobacteria (15%), Bacteroidetes (11%), Firmicutes (4%), Chloroflexi (2%), Gemmatimonadetes (2%), Planctomycetes (1%), Acidobacteria (1%), Deltaproteobacteria (1%), and Deinococcus-Thermus (1%) (Fig 4). The relative abundance of most of these phyla was not significantly differed in the two snow layers, except the Gemmatimonadetes, Planctomycetes, and Acidobacteria, which exhibited significantly higher relative abundance in the surface layer than in the subsurface layer (all $P < 0.05$, Wilcoxon rank-sum test; Supplementary Fig S5).

In the surface layer, the relative abundances of Alphaproteobacteria, Gammaproteobacteria, and Firmicutes significantly decreased with time (Supplementary Fig S6), while those of Cyanobacteria and Deinococcus-Thermus significantly increased (all $P < 0.05$). In the subsurface layer, the relative abundance of Alphaproteobacteria and Firmicutes significantly decreased with time, while Cyanobacteria and Chloroflexi significantly increased (all $P < 0.05$).

3.3 Bacterial community structure and functional genes

The bacterial community structure significantly differed in the surface and subsurface snow (PERMANOVA, $F = 2.78$, $P < 0.001$, Fig 5a), as well as among the different sampling times (PERMANOVA, $F = 3.31$, $P < 0.001$; $F = 2.17$, $P < 0.001$). Additionally, a significant interactive effect was detected between the depth and time (PERMANOVA, $F = 2.68$, $P < 0.001$), indicating that the depth influenced the temporal pattern of bacterial community structure changes. Specifically, only the second principal coordinate (PCoA2) values of the surface layer significantly correlated with time (Pearson correlation; $r = 0.94$, $P < 0.001$, Fig 5b), while the PCoA1 values of the surface layer did not. In comparison, both PCoA1 and PCoA2 values of the subsurface layer correlated with time significantly (Pearson correlation; $r = 0.53$, $P = 0.02$; $r = 0.58$, $P = 0.01$, respectively).

Normalized stochasticity ratio (NST) was used to examine the relative contributions of stochasticity and determinism in shaping bacterial communities. The average NST values were 74% and 46% in the surface and subsurface snow layers, and the contribution of stochasticity was significantly higher in the surface than in the subsurface layers ($P < 0.001$; Supplementary Fig S7).

Mantel tests were performed to evaluate the effects of environmental parameters on bacterial community composition within each layer. No significant correlation was identified between the measured environmental factors and the bacterial community structure in the surface layer. However, the concentrations of TN, NO_3^-, and NH_4^+ were significantly correlated with bacterial community composition in the subsurface layer ($P < 0.05$) (Table 1). The relative abundance of nitrogen-cycling associated functional genes were predicted in the surface and subsurface layers. The relative abundance of nitrogen-fixation marker gene (*nifH*) significantly increased with time in the surface layer, but no clear pattern was observed in the subsurface layer ($P < 0.05$ and $P = 0.46$, respectively, Supplementary Fig S8). The relative abundance of *narG* gene, which is associated with the denitrification process, reduced significantly in the surface layer with time, while increased

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significantly in the subsurface layer ($P < 0.05$ and $P < 0.01$, respectively). The *nirK* gene, which is also associated with the denitrification process, reduced significantly with time in the surface layer, while no significant change was identified in the subsurface layer ($P < 0.01$ and $P = 0.18$, respectively).

3.4 Interspecies interactions at the surface and subsurface layers

Co-occurrence networks were constructed for the surface and subsurface bacterial communities to infer the biotic interactions among species (Fig 6). The surface network comprised a higher number of nodes (each indicating one ASV, n=197) but a lower number of edges (each indicating a significant correlation between two ASVs, n=436) than the subsurface network (n=140 and 523, respectively, Table 2). The network in the subsurface snow demonstrated a higher number of edges per node (3.73 and 2.21, respectively), higher average connectivity (avgK, 7.57 and 4.43, respectively), and lower average path distance (GD, 4.72 and 5.51, respectively), which indicates a substantially more complex network topology. Both networks were dominated by positive (co-presence) relationships, and the subsurface network exhibited a higher positive-to-total interaction ratio (95%) than the surface network (83%).

Modularity, average clustering coefficient (avgCC), and graph density of the surface and subsurface bacterial community networks were all higher than those of random networks (Supplementary Table S2), indicating that snowpack bacterial networks showed non-randomly assemblage and exhibited modular structures. The subsurface networks showed higher values of avgCC (0.39), transitivity (0.49), and connectedness (0.86) than the surface bacterial community network (0.31, 0.45, 0.71, respectively), indicating a greater degree of connectivity (Table 2).

4 Discussion

4.1 Distinct nitrogen-transformation processes in surface and subsurface snow

Nitrogen (including ammonium and nitrate) concentration significantly differed in the surface and subsurface layers and were the only environmental factors that changed significantly with time in the present study (Fig. 1a and 1b). Nitrogen concentration significantly increased in the surface snow, which can be due to atmospheric deposition and/or microbial nitrogen fixation activity. Nitrogen deposition occurs at a rate of 282 kg N km^{-2} yr^{-1} in the region of our investigation (Lü and Tian, 2007), making it a possible source (Björkman et al., 2014). On the other hand, microbial nitrogen fixation can support supraglacial microbial communities when alternative sources of nitrogen become limited (Telling et al., 2011). In the current study, a potential contribution from bacterial nitrogen fixation is supported by the significant increase in the relative abundance of Cyanobacteria and the predicted abundance of *nifH* gene (Supplementary Fig S6 and Fig S8). We did not measure the rates of nitrogen fixation in our system, however, even in a study focused on cryoconite holes, which are expected to have higher rates of biological activity than snowpack, the measured rate of nitrogen fixation (average 0.04 kg N km^{-2} yr^{-1}) were orders of magnitude lower than the nitrogen input from precipitation in the same region (Telling et al., 2011).

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Therefore, we expect atmospheric deposition to be the dominant driver of the elevated nitrogen level observed in the surface snow.

In contrast with the surface layer, nitrogen concentrations (total N, nitrate, and ammonium) significantly decreased in the subsurface with time (Fig. 1). This indicates distinct nitrogen transformation processes, where the surface was associated with nitrogen accumulation, and the subsurface was dominated by nitrogen consumption. We propose that the anaerobic denitrification process could contribute to nitrogen consumption, which is evidenced by the significant increase of predicted genes associated with denitrification processes (*narG*; Supplementary Fig S8), which encodes nitrate reductase (Telling et al., 2011; Zhang et al., 2020). The occurrence of denitrification reactions in subsurface snow has been reported in Arctic snowpacks, where the genes encoding all the required enzymes for denitrification were detected (Larose et al., 2013). The different nitrogen transformation processes in the surface and subsurface layers could be attributed to the substantial differences in geophysical conditions, such as the lower light intensity (Xiang et al., 2009) and anaerobic conditions (Larose et al., 2013) in the subsurface snow layer. Further metagenomic and metatranscriptomic analyses targeting the genes associated with nitrogen cycling are required to further confirm the distinct nitrogen transformation processes between the surface and subsurface layers.

4.2 Nitrogen drives the overall bacterial community structure changes in snow

Bacterial richness and diversity exhibited distinct temporal patterns. Alpha diversity indices remained consistent throughout the nine-day period in the surface snow layer, while they reduced significantly in the subsurface snow layer. This indicates that the microbiome in the subsurface layer may be subjected to greater environmental filtering than in the surface layer (Xiang et al., 2009), and, combined with our chemical data, indicates a relationship with nitrogen. This is consistent with the strong and positive correlative relationships between nitrogen and bacterial diversity in the subsurface layer, which was not observed in the surface layer (Fig. 3). Nitrogen is an essential nutrient for microbial growth and plays important roles in controlling microbial diversity and ecosystem productivity (Vitousek et al., 2002; Xia et al., 2008; Sun et al., 2014). The positive correlations between nitrogen availability and alpha diversity indices in the subsurface layer suggest that nitrogen limitation is an important determinant of bacterial diversity in glacier snowpack as burial removes the influence of surface deposition. Similar conclusions have been reported in subglacial pore waters and sediments (Ren et al., 2019). In comparison, the surface layer is not subjected to nitrogen-limitation, which is consistent with previous studies in supraglacial ecosystems (Cameron et al., 2017; Holland et al., 2020).

The bacterial community also exhibited significant temporal changes in the subsurface layer that were most closely correlated with nitrogen concentration (Table 1). Consistent with the alpha-diversity indices, the bacterial community in the subsurface layer exhibited stronger temporal changes (Fig. 2), again indicating greater environmental filtering. This was also consistent with the higher modelled contribution from deterministic processes relative to stochastic processes in the subsurface layer relative to the surface layer (Supplementary Fig S7). Owing to the accumulation of the surface snow, the external nutrient inputs to the subsurface layer are limited, as is the deposition of microorganisms dispersed via aeolian

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processes. Together, these explain the stronger signal from environment filtering due to nitrogen limitation (Stegen et al., 2012). Despite the microbiome in the surface layer being subjected to less nutrient limitation and environmental filtering, the PCoA2 of the surface layer was strongly correlated with time (Fig. 5b). This suggests that while the richness and diversity did not exhibit any significant temporal changes, the bacterial community structure in the surface layer still changed, potentially due to biotic interactions or unmeasured environmental factors (such as UV radiation) (Maccario et al., 2014).

4.3 Nitrogen transformation processes mediate biotic interactions in snow

Biotic interactions can explain a substantial proportion of the community structure variations (Hacquard et al., 2015; Dang and Lovell, 2016). Our results indicated that the subsurface community network was more complex as evidenced by the higher average connectivity and a shorter path length (GD) than the surface community network (Table 2). This is likely due to the enhanced environmental filtering, as has been observed in other systems subjected to environmental stresses(Ji et al., 2019; Wang et al., 2018). A higher ratio of positive-to-total interactions but lower modularity was identified in the subsurface snow network (Table 2). In general, higher positive interactions indicate increased microbial cooperation (Ju et al., 2014; Scheffer et al., 2012), whereas reduction in modularity indicates microbial niche-homogenization (Ji et al., 2019). The enhanced biotic associations and cooperation in the subsurface layer may be attributed to the dominance of denitrification processes, as denitrification is a multi-step process that involves multiple microbial cohorts to complete (Henry et al., 2005; Madsen, 2011; Yuan et al., 2021). The enhanced collaboration and deterministic succession had been reported in microbial community associated with the anoxic decomposition of microcystis biomass (Wu et al., 2019), and cross-feeding leads to enhanced positive interactions among the different members of the community (Borchert et al., 2021).

The path lengths of the subsurface network were lower than that of the surface layer (Table 2). The shorter path length has been proposed to be associated with a highertransfer efficiencies of information and materials across the microorganisms in the network (Du et al., 2020), which are required for complex biological processes that require extensive microbial collaboration, such as denitrification (Yuan et al., 2021). Thus, the short path length is consistent with the dominance of denitrification processes in the subsurface layer. Previous studies have proposed microbial interactions as biotic drivers that impact microbial diversity (Calcagno et al., 2017; Hunt and Ward, 2015). Thus, those microorganisms who are not adapted to the subsurface environment would be excluded from the environment, which provides an alternative explanation for the reduction in diversity (Scheffer et al., 2012; Ziegler et al., 2018; Bergk Pinto et al., 2019).

5 Conclusion

Our results demonstrated the key roles of nitrogen in shaping the bacterial community in Tibetan glacial snow. The surface and subsurface snow is associated with the accumulation and consumption of nitrogen, respectively. Due to dry deposition and microbial nitrogen fixation activities, nitrogen limitation is unlikely to occur in the surface snow, thus additional nitrogen deposition due to global climate change is unlikely to cause a substantial impact on the bacterial community in

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surface snow. In summary, our results provide a new perspective on the bacterial communities in snowpack of the Tibetan Plateau, and further studies based on metagenome and metatranscriptome can enhance the understanding of microbial functions in snow and predict their future changes.

Data availability. Sequence data generated in the present study have been deposited to the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the ID PRJNA649151.

Author contributions. YL and MJ conceived the study and developed the idea. YC performed DNA extraction. YC and FW performed the environmental characterization measure. YC conducted the data statistical analysis. YC and KS wrote the first draft of the paper, and MJ, TV, and YL revised the paper substantially. All authors read and approved the final paper.

Competing interests. The authors declare that they have no conflict of interest.

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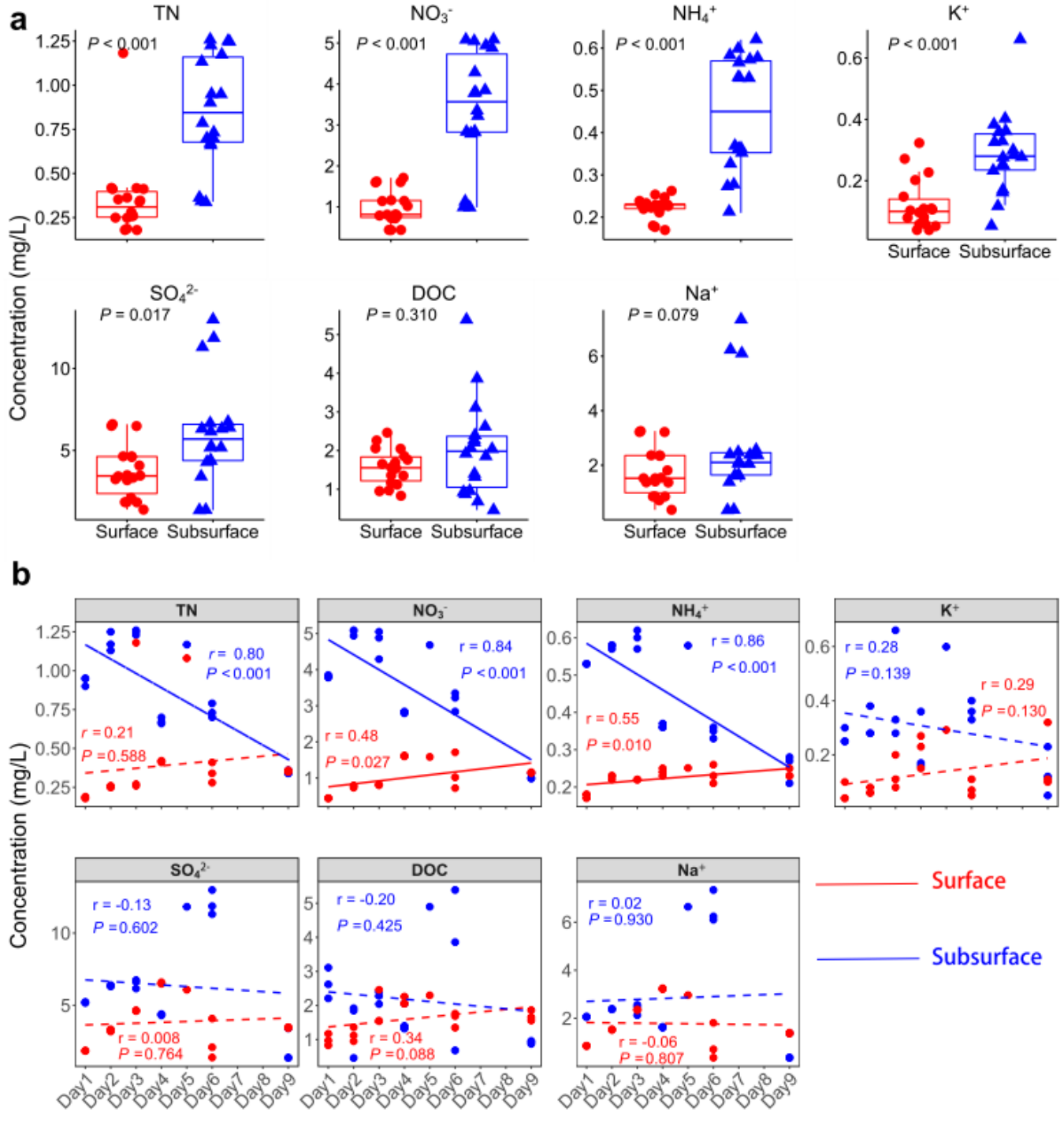

Figure 1: The pattern of environmental factors changes in the surface and subsurface snow layers.
(a) Environmental factor comparisons in the surface and subsurface snow layers. Each dot represents an individual sample. Significantly higher concentrations of TN, NO_3^-, NH_4^+, K^+, and SO_4^{2-} were observed in the subsurface layer based on Wilcoxon rank-sum test.

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(b) Temporal changes of environmental factors in the surface and subsurface layers. The solid and dashed lines indicate significant and non-significant temporal changes, respectively. The concentration of NO_3^- and NH_4^+in the surface layer significantly increased with time while the concentration of TN, NO_3^-, and NH_4^+, in the subsurface layer significantly decreased with time. Regression analysis is based on Pearson correlation.

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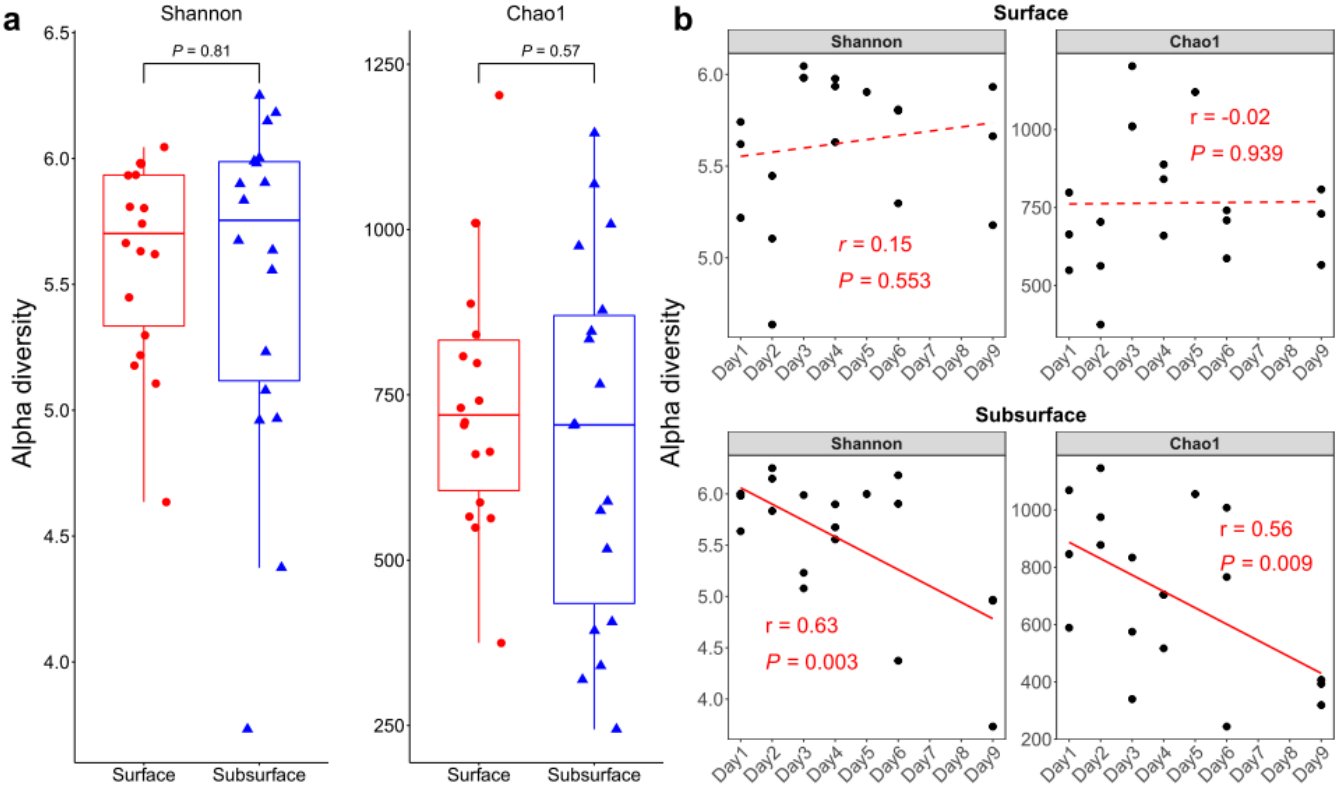

Figure 2: Bacterial alpha diversity in snow layers.

480 **(a) Bacterial alpha diversity comparison between the surface and subsurface layers. Each dot represents an individual sample. For both Shannon and Chao1 indices, no significant difference was observed between the surface and subsurface snow layers. Comparison is based on Wilcoxon rank-sum test.**

(b) Temporal changes of the alpha diversity indices in the surface and subsurface snow layers. For the surface layer, no significant correlation was observed, while both Shannon and Chao1 showed a significantly reduction with time in the subsurface layer.

485 **Regression analysis is based on Pearson correlation.**

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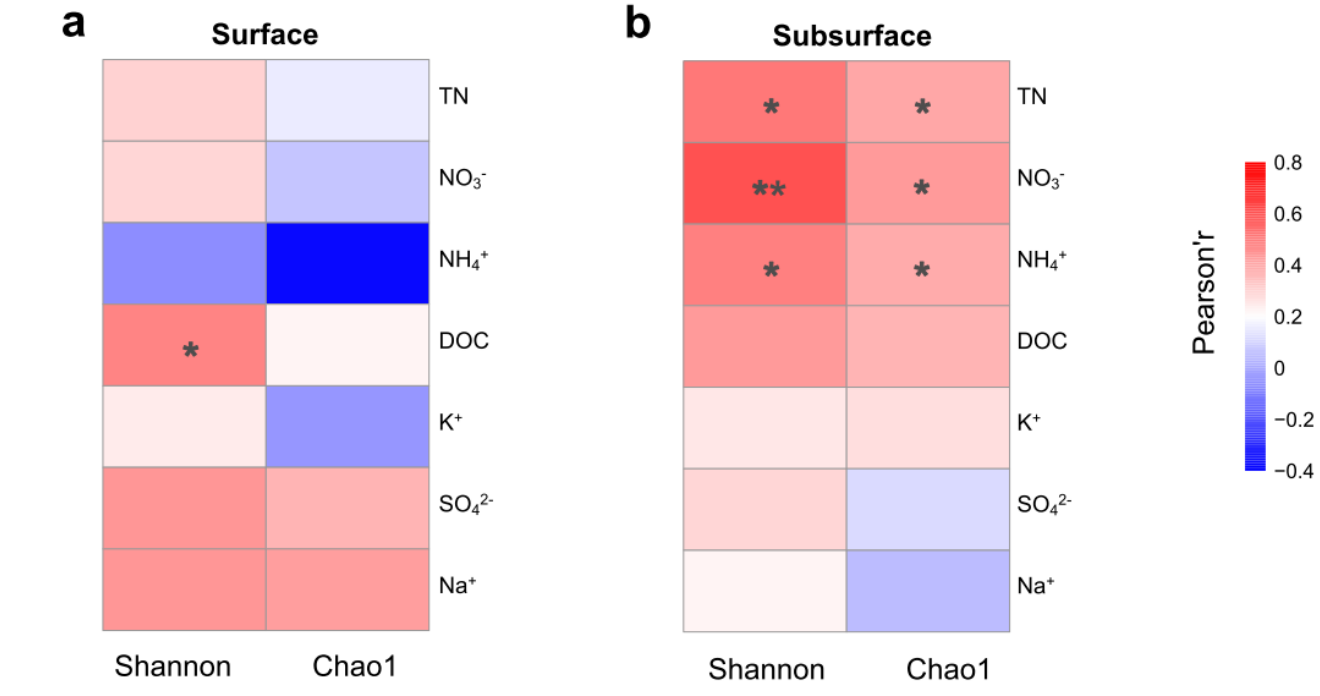

Figure 3: Pearson's correlations (r) between the Shannon and Chao1 indicesand environmental factors in the surface (a) and subsurface (b) snow layers. In the surface layer, Shannon diversity index was significantly correlated with DOC. In the subsurface layer, Shannon diversity index was significantly correlated with TN, NO_3^-, and NH_4^+. Statistical significance is indicated by * *P* < 0.05, ** *P* < 0.01.

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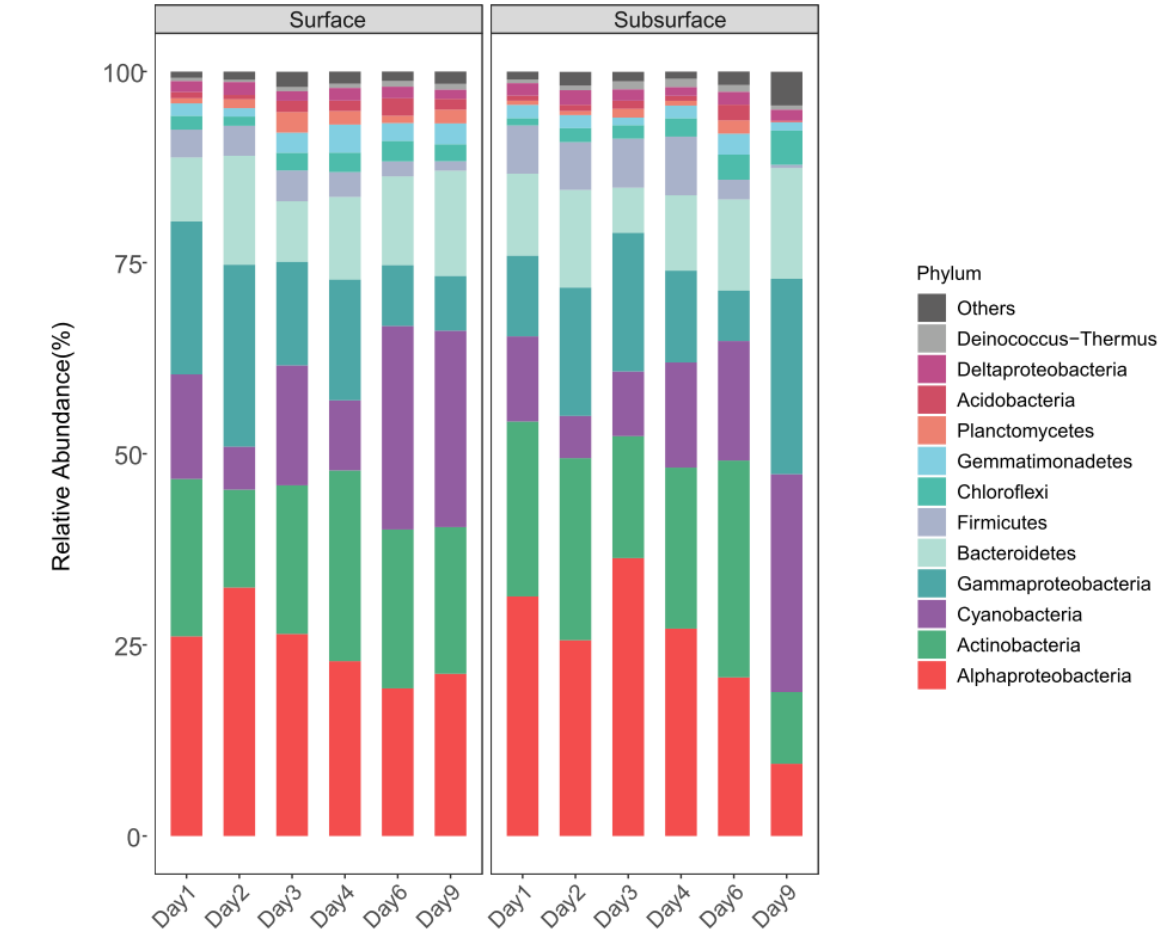

Figure 4: Taxonomic composition of bacterial community in snow. Only dominant phyla are presented (relative abundance > 1%). The snow community are dominated by Alphaproteobacteria, Actinobacteria, Cyanobacteria, Gammaproteobacteria, Bacteroidetes, Firmicutes, Chloroflexi, Gemmatimonadetes, Planctomycetes, Acidobacteria, Deltaproteobacteria, and Deinococcus-Thermus.

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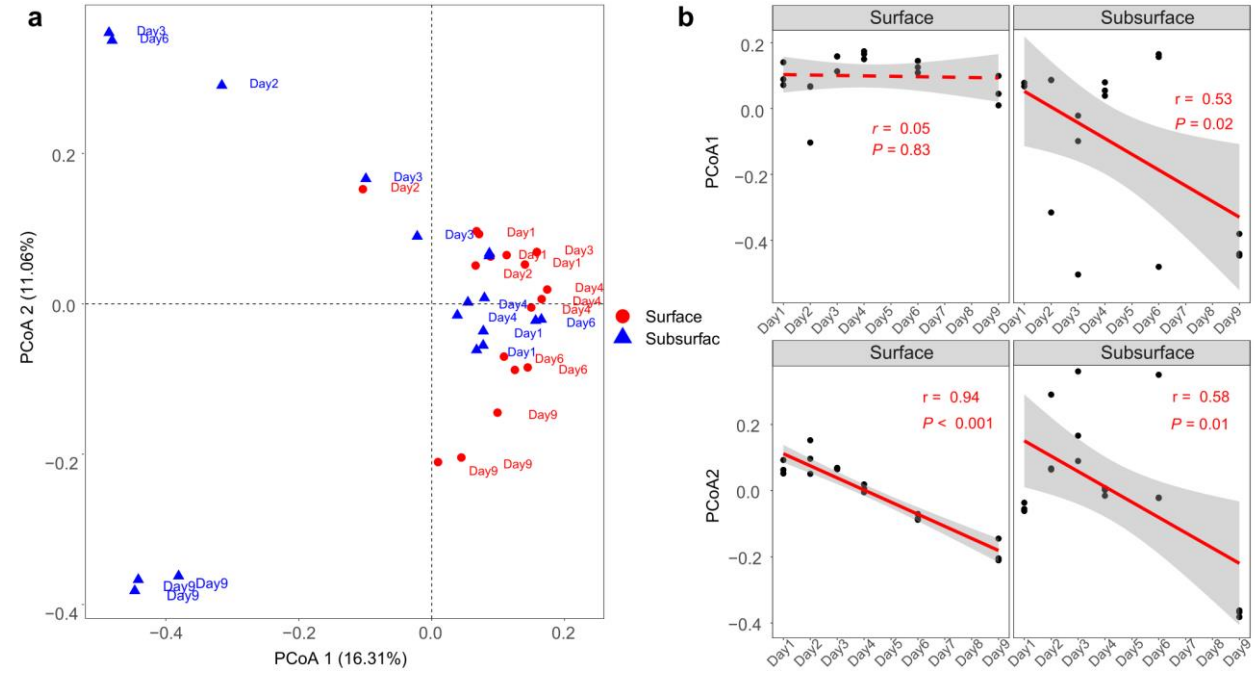

Figure 5: Principal coordinate analysis (PCoA) of microbial communities in the surface and subsurface snow.
(a) Bray-Curtis distance-based PCoA ordination plot. The microbial community structures of the surface and subsurface snows are significantly different (PERMANOVA, $P < 0.001$).
(b) Pairwise regression analysis between PCoA scores and sampling time. The solid and dashed lines indicate significant and insignificant changes (based on Pearson correlations), respectively. The PCoA1 scores for the bacterial community in surface layer exhibit no significant correlation with time, while the PCoA2 scores significantly correlated with time. The PCoA1 and PCoA2 are both significantly correlated with time in the subsurface layer.

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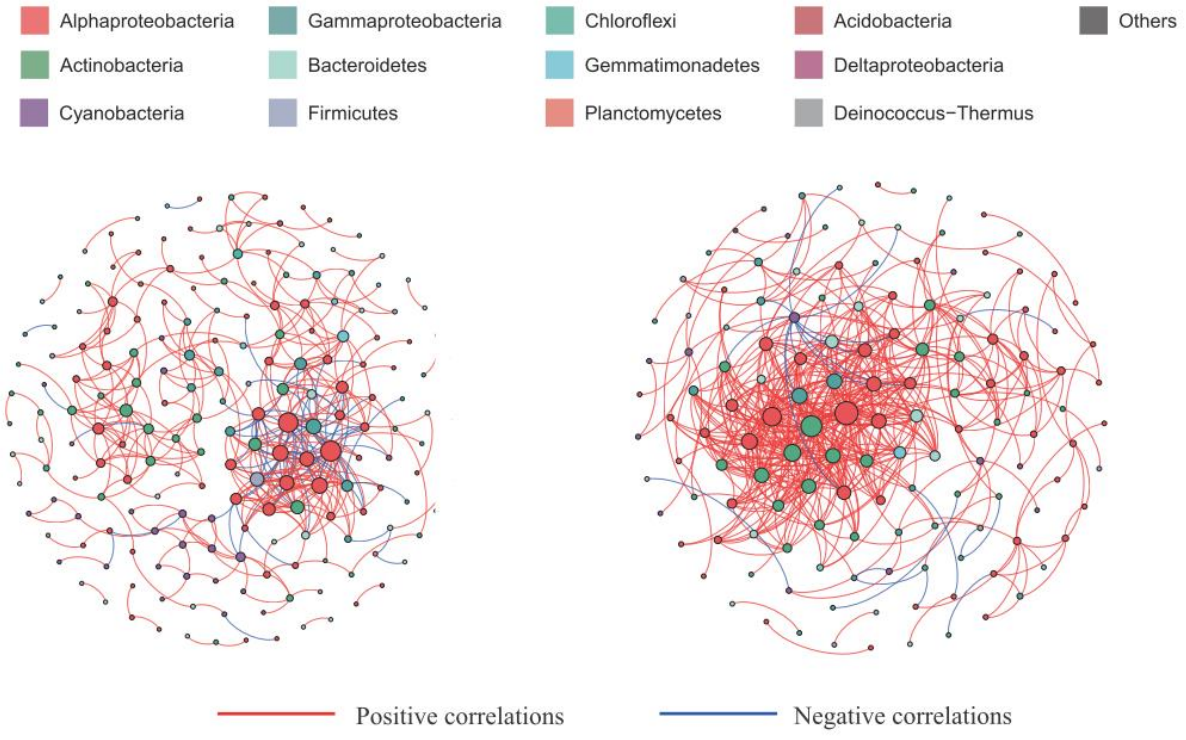

Figure 6: Bacterial Co-occurrence networks for the surface and subsurface layers communities. Each node represents a bacterial amplicon sequence variant (ASV). The red solid lines represent positive correlations, and the blue solid lines represent negative correlations. Nodes are colored by taxonomy at the phylum level. The subsurface community networks are more complex with a higher positive-to-total correlation ratio.

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Table 1. Results of Mantel test showing the relationships between bacterial community composition and environmental factors in the surface and subsurface dnoe layers. Significant correlations are in bold.

Environmental factor	Surface		Subsurface	
	R	*P*	R	*P*
TN	-0.11	0.81	0.36	**0.001**
NO_3^-	0.09	0.21	0.38	**0.005**
NH_4^+	0.01	0.36	0.25	**0.01**
DOC	0.08	0.22	-0.02	0.49
Na^+	0.02	0.40	0.16	0.14
SO_4^{2-}	0.00	0.44	0.25	0.09
K^+	-0.04	0.56	0.11	0.24

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Table 2. Topological properties of the empirical networks and random networks for the surface and subsurface bacterial communities.

	Empirical Network	
	Surface	Subsurface
No. of node	197	140
No. of edges	436	523
Number of edges per node	2.21	3.73
Posotive links	363	500
Negative links	73	22
Ratio of positive-to-total interactions	83%	95%
Modularity	0.65	0.40
No. of modules	23	12
Average connectivity	4.41	7.36
Average clustering coefficient (avgCC)	0.31	0.39
Average path distance (GD)	5.51	4.72
Average degree (avgK)	4.43	7.57
Graph density	0.02	0.06
Transitivity (Trans)	0.45	0.49
Connectedness (Con)	0.71	0.86

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