

Biological soil crusts and slope gradient synergistically regulate the community structure and functional differentiation of nitrogen-cycling microorganisms in alpine meadow soils

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Abstract

Background and aims

Biological soil crusts (BSCs) and slope gradient regulate soil nitrogen cycling in alpine meadows, but their combined effects on nitrogen-cycling microbial communities remain unclear. This study investigated how BSCs and slope jointly influence soil nitrogen transformations and the community structure of nitrogen-cycling microorganisms.

Methods

Soils with and without BSC cover were collected from flat, gentle, and steep slopes in an alpine meadow on the Qinghai-Tibetan Plateau. Soil nitrogen nutrients were measured, and high-throughput sequencing was used to characterize the relative abundances, diversity, and co-occurrence networks of five functional genes (archaeal *amoA*, bacterial *amoA*, *nirK*, *nirS*, and *nosZ*).

Results

BSCs significantly promoted nitrification, increasing nitrate nitrogen by 9.1%-56.8% and decreasing ammonium nitrogen by 71.0%-87.5%, with nitrate concentrations further increasing along slope gradient. Microbial groups responded differently to BSCs and slope. BSCs increased archaeal *amoA* and *nirK* abundances but reduced α diversity of AOB, *nirK*, and *nosZ* communities. Slope gradient primarily regulated AOA, *nirK*, and *nosZ* diversity, while their interaction reshaped community structure. Co-occurrence network analysis revealed that AOA communities maintained stable structures through core Thaumarchaeota, whereas AOB and denitrifier communities adapted through network modular differentiation: gentle slopes harbored high-abundance functional modules, while steep slopes enriched stress-tolerant rare modules. Structural equation modeling confirmed that BSCs and slope indirectly regulated microbial diversity by altering soil organic matter and ammonium nitrogen.

Conclusions

This study elucidates the mechanisms by which BSCs and slope gradient jointly regulate nitrogen-cycling microorganisms in alpine meadow soils, providing evidence for nutrient cycling maintenance and ecological conservation.

Introduction

The Qinghai-Tibetan Plateau supports the largest alpine meadow ecosystem in the world (Miehe et al. 2019). This ecosystem plays important roles in soil and water conservation (Immerzeel et al. 2010), water regulation (Guo et al. 2021), nutrient cycling (Fawei et al. 2025), and ecosystem stability (Liu et al.

2018). However, alpine meadows are inherently fragile and highly sensitive to environmental change (Miehe et al. 2019). Soil nitrogen transformations directly constrain vegetation productivity and ecosystem stability (Shun et al. 2024; Ran et al. 2025). Therefore, elucidating the mechanisms driving nitrogen cycling in alpine meadows is essential for understanding regional ecosystem functions and developing sustainable management strategies.

Biological soil crusts (BSCs) are complex biotic assemblages formed on the soil surface through the aggregation and cementation of soil particles by nonvascular plants, including algae, mosses, and lichens, together with microorganisms such as soil bacteria and fungi (Belnap and Lange 2013). On the Qinghai–Tibetan Plateau, BSCs are widespread surface covers and play an important role in the restoration of degraded grasslands (Xie et al. 2024). Through the activities of key microorganisms, including nitrogen-fixing bacteria, rhizobia, and cyanobacteria, BSCs can serve as important sources of carbon and nitrogen inputs to soils (Darby et al. 2007, 2010; Xie et al. 2024). They can also substantially enhance atmospheric carbon and nitrogen fixation, soil organic matter accumulation, and nutrient retention (Xie et al. 2024). Previous studies have shown that BSCs are important nitrogen sources in nitrogen-limited ecosystems (Kurth et al. 2021), with cyanobacterial crusts being particularly important for carbon and nitrogen fixation and the regulation of nutrient cycling (Bao et al. 2019). Studies in arid regions have further demonstrated that BSC development can increase the diversity of nitrogen-cycling genes and functional pathways (Tao et al. 2025). However, in high-altitude ecosystems, especially under different terrain conditions, the mechanisms by which BSCs regulate the nitrogen transformation process and the associated microbial processes remain unclear.

Soil microorganisms are the primary agents driving nitrogen cycling (Deb et al. 2024; Pajares and Ramos 2019). Nitrification is primarily mediated by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) through ammonia monooxygenase encoded by the *amoA* gene. This step is commonly regarded as the rate-limiting stage of nitrification (Schleper and Nicol 2010; Kits et al. 2017). Denitrification involves several functional genes. Among them, *nirK* and *nirS* encode nitrite reductases that catalyze the reduction of NO_2^- to NO , a key step in denitrification (Graf et al. 2014), whereas *nosZ* encodes nitrous oxide reductase, which catalyzes the reduction of N_2O to N_2 and represents the terminal step of denitrification (Walter and Peter 2006). These functional genes have been widely used to investigate the community structure and diversity of nitrogen-cycling microorganisms (Braker et al. 2000; Wasai-Hara et al. 2023).

Slope gradient is an important topographic factor that affects soil moisture (Bao et al. 2024), temperature (Liu et al. 2024), nutrient redistribution (Zhang et al. 2024), and erosion intensity (Zhang et al. 2025). By altering soil microenvironments and the spatial distribution of materials, slope gradient may further regulate microbially mediated nitrogen transformations (Suriyavirun et al. 2019). However, existing studies have generally focused on individual environmental factors, and relatively little is known about changes in soil nitrogen forms and the responses of nitrogen-cycling functional microorganisms to the combined effects of BSCs and topography. In particular, the synergistic mechanisms underlying

these effects have rarely been examined simultaneously in terms of functional gene abundance, community diversity, community structure, and microbial associations.

Accordingly, this study investigated how BSC affected soil nitrogen cycling at different slope gradients in the alpine meadows of the Qinghai–Tibetan Plateau by comparing soil properties sampled from areas with and without BSC cover across three slope positions (flat terrain, gentle slopes, and steep slopes). It further systematically explored the microbial mechanisms through which BSCs and slope gradient jointly regulate the community structure of micro-organisms via high-throughput sequencing and structural equation modeling. The objective of this study was to elucidate the responses of soil nitrogen forms and nitrogen-cycling functional microbial communities to the combined effects of BSCs and slope gradient in alpine meadows on the Qinghai–Tibetan Plateau, and to clarify the potential regulatory pathways mediated by soil environmental factors. This study was underpinned by three hypotheses: (i) BSCs and slope gradient jointly affect soil nitrogen forms and transformation processes in alpine meadows; (ii) BSCs and slope gradient shape the composition, diversity, and association patterns of soil nitrogen-cycling functional microbial communities in unison; and (iii) BSCs and slope gradient are directly or indirectly associated with the diversity of nitrogen-cycling functional microbial communities by altering soil environmental conditions, with changes in soil nutrient availability potentially serving as an important environmental pathway linking BSCs and slope gradient to microbial community responses. The answers to these hypotheses will serve as solid scientific evidence for BSC conservation, proper slope-specific grassland management, and soil nitrogen retention strategy in alpine meadows.

Materials and Methods

Study area description

The study site was located in Henan Mongolian Autonomous County, Huangnan Tibetan Autonomous Prefecture, Qinghai Province, China (101°46'02" E, 34°41'07" N), at an elevation of approximately 3630 m (Fig. 1a). The area has a subhumid cold temperate climate with a distinct continental plateau character: the mean annual temperature ranges from – 1.3 to 1.6°C, mean annual precipitation ranges from 597.3 to 615.5 mm, and the site is characterized by long sunshine duration, intense solar radiation, limited thermal availability, and large diurnal temperature fluctuations. The vegetation is typical alpine meadow, with the dominant species being *Carex alatauensis*, *Carex parvula*, *Carex enervis*, *Stipa purpurea*, *Elymus nutans*, and *Poa pratensis*. The soil is classified as the alpine meadow type. Mixed biological soil crusts composed of cyanobacteria, lichens, and mosses are widely distributed among the herbaceous communities, with moss-dominated crusts accounting for approximately 80% of total BSC cover. Therefore, this study focuses on moss-dominated mixed biological soil crusts.

Soil sampling

At the end of July 2022, representative sites were selected on flat terrain (< 7°), gentle slopes (7°–25°), and steep slopes (> 25°), respectively. Along the contour line at each slope position, three 50 m × 50 m plots were established, with a 25 m buffer between adjacent plots. Each plot was systematically divided

into four 25 m × 25 m subplots, three of which were randomly selected for sampling (Fig. 1b). Within each selected subplot, five points covered by BSCs were randomly chosen, and one surface soil core (0–5 cm) was collected at each point. At the adjacent, paired microsite next to each BSC point, one additional 0–5 cm core was collected from adjacent meadow soil without BSC cover. In this way, five BSC cores and five non-BSC cores were obtained in each subplot. In the same plot, the 15 BSC cores from the three subplots were thoroughly mixed to form one composite BSC soil sample, and the 15 non-BSC cores were similarly combined to form one composite non-BSC meadow soil sample. This design yielded three replicated plots at each slope position, resulting in a total of 12 composite BSC replicates and 12 non-BSC replicates for the four types of terrain. A portion of the mixed soil samples was immediately separated and placed into 1.5 mL cryogenic tubes, and stored in a low-temperature storage box. Subsequently, they were wrapped with dry ice and quickly sent to Shanghai Pansenuo Biotechnology Co., Ltd. for microbial analysis. The remaining soil samples were placed in labelled bags, air-dried in the laboratory, and sieved for the determination of nitrogen-related soil properties. All handling, preservation, and transport of the soil samples followed standard protocols for microbial sampling.

Determination of soil physicochemical properties

Soil organic matter (SOM) was measured using the potassium dichromate–sulfuric acid oxidation method. Total soil nitrogen (TN) was determined with a total soil carbon and nitrogen analyzer. Ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were extracted with 1 mol L⁻¹ KCl and quantified using a continuous flow injection analyzer. Soil total phosphorus (TP) was digested with $\text{H}_2\text{SO}_4-\text{H}_2\text{O}_2$ and determined by molybdenum-antimony colorimetry. Available phosphorus (AP) was extracted with NaHCO_3 and measured by molybdenum-antimony colorimetry. Total potassium (TK) was determined by acid digestion followed by flame photometry, and available potassium (AK) was extracted with ammonium acetate and measured by atomic absorption spectrophotometry (Bao 2000).

Targeted gene sequencing and raw sequence processing

Total soil DNA was extracted from 0.5 g of each soil sample using the OMEGA Soil DNA Kit (OMEGA Bio-Tek, Georgia, USA). DNA purity and concentration were verified using a Nanodrop 2000 spectrophotometer (NanoDrop Technologies, USA). The target functional genes were then amplified on a GeneAmp® 9700 thermal cycler (ABI), with primer sequences provided in Table 1. Amplicons were subjected to paired-end sequencing on the Illumina platform. Raw reads were trimmed to remove primer sequences using cutadapt (v2.3) with the parameter -O 10, and sequences lacking matched primers were discarded. Sequence merging, quality filtering, dereplication, and chimera removal were performed in Vsearch (v2.13.4_linux_x86_64), and high-quality sequences were generated using the Perl scripts documented at <https://github.com/torognes/vsearch/wiki/VSEARCH-pipeline>. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity for each target functional gene, and the sequences of each gene were separately aligned against the corresponding reference databases for

taxonomic annotation, supporting subsequent analysis of nitrogen-cycling microbial community composition.

Table 1
Primers and sequences for functional genes of nitrifying and denitrifying microorganisms

Gene	Primer	Primer sequence	Reference
<i>amoA</i> (AOB)	<i>amoA</i> - 1F	GGGGTTTCTACTGGTGGT	(Weiner and Maizels 1999)
	<i>amoA</i> -2R	CCCCTCKGSAAAGCCTTCTTC	
<i>amoA</i> (AOA)	Arch- <i>amoA</i> F	GACTACATMTTCTAYACWGAYTGGGC	(Park et al. 2008)
	Arch- <i>amoA</i> R	GGKGTCA TRTATGGWGGYAA YGTTGG	
<i>nirK</i>	<i>nirK</i> -1aCuF	ATCATGGT SCTGCCGCG	(Hallin and Lindgren 1999)
	<i>nirK</i> -R3CuR	GCCTCGATCAGRTTGTGGTT	
<i>nirS</i>	cd3aF	GTSAACGTSAAGGARACSGG	(Michotey et al. 2000)
	R3cd	GASTTCGGRTGSGTCTTGA	
<i>nosZ</i>	<i>nosZ</i> -1126qF	GGGCTBGGGCCRTTGCA	(Chen et al. 2012)
	<i>nosZ</i> -1381R	GAAGCGRTCCTTSGARAACTTG	

Statistical analyses

Soil physicochemical properties, including SOM, TN, NO₃⁻-N, NH₄⁺-N, TP, AP, TK, and AK, were initially organized in Excel 2019 and subsequently analyzed using two-way analysis of variance (ANOVA) in IBM SPSS Statistics 26.0. BSC status (BSC-covered meadow and non-BSC meadow) and slope gradient (flat, gentle, and steep slopes) were treated as fixed factors, and their interaction was included in the analysis. Before ANOVA, normality of residuals and homogeneity of variances were assessed to ensure that they conformed to model assumptions. For variables showing significant main or interaction effects, post hoc multiple comparisons were performed using the least significant difference (LSD) test. Statistical significance was defined as *P* < 0.05.

The relative abundance of each nitrogen-cycling functional gene was calculated from amplicon sequencing data as the proportion of valid annotated sequences assigned to that gene relative to the total number of valid annotated sequences across all target functional genes in a sample. This metric reflects relative shifts in the composition of target functional-gene sequences among samples; it does not represent absolute gene copy numbers or directly indicate nitrification or denitrification rates. Therefore, it was interpreted as a relative change in the composition of nitrogen-cycling functional microbial communities rather than a direct measure of nitrogen transformation intensity. To identify the main environmental predictors of functional gene relative abundance, random forest models were constructed using the “rfPermute” package in *R*, in which the relative abundance of each functional gene

was used as the response variable, whereas soil physicochemical properties were treated as the predictor variables. Each model consisted of 500 decision trees. Predictor importance was assessed using percentage increase in mean squared error (%IncMSE) and increase in node purity (IncNodePurity), while variable significance was evaluated using permutation tests.

The α -diversity of nitrogen-cycling functional microbial communities was characterized using the Chao1 richness, Shannon diversity, and Pielou's evenness indices. After quality control and sequencing-depth normalization in QIIME 2, diversity indices were calculated for all samples. The effects of slope gradient, BSC status, and their interaction on α -diversity were assessed using two-way ANOVA. The β -diversity was calculated from Bray-Curtis distance matrices at the operational taxonomic unit (OTU) level and visualized using principal coordinates analysis (PCoA). Permutational multivariate analysis of variance (PERMANOVA) was used to test the effects of slope gradient, BSC status, and their interaction on community composition. Additionally, permutational analysis of multivariate dispersions (PERMDISP) was performed to assess among-group differences in within-group dispersion and to exclude potential confounding effects of heterogeneous dispersion on PERMANOVA results.

Co-occurrence network analysis was performed using OTU-level abundance matrices. To reduce the influence of rare taxa on network construction, OTUs with a relative abundance below 0.01% and absent from more than 80% of samples were excluded. Pairwise associations among OTUs were calculated using Spearman rank correlations, and only significant correlations with $|r| > 0.7$ and $P < 0.01$ were retained. Separate microbial co-occurrence networks were constructed for each combination of slope gradient and BSC status. Network topological properties, including node degree, betweenness centrality, and modularity, were calculated using the "igraph" package in R. Co-occurrence modules were identified using the Louvain community detection algorithm. OTUs within the same module were considered to exhibit similar environmental response patterns and close ecological associations. The relative abundance of each module was calculated across treatment groups to evaluate gradient-related changes in module composition and taxon distribution. Associations between network modules and environmental variables were assessed using Mantel tests and Spearman correlation analyses. Networks were visualized using the "ggnetwork" package in R and the Personalbio cloud platform.

Pearson correlation analysis was used to evaluate relationships between soil physicochemical properties and the relative abundances of nitrogen-cycling functional genes, dominant taxa, and α -diversity indices of functional microbial communities. Data distributions and linear relationships were examined before analysis, and log transformation was applied when necessary. For correlation analyses involving multiple variables, P values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) procedure to reduce the risk of false-positive findings arising from multiple comparisons. Correlation analyses were conducted in SPSS 26.0, and figures were generated using Origin 2019b, GraphPad Prism 7.0, and the Personalbio cloud platform.

A structural equation model (SEM) was further constructed to explore the potential association pathways among slope gradient, biological crusts, soil nutrients and functional microbial diversity, and to

identify statistical associations among observed variables that were consistent with the hypothesized theoretical pathways. Before model construction, continuous variables included in the models were log-transformed or standardized, and correlations and multicollinearity among variables were examined. Variables with an absolute correlation coefficient > 0.8 were not included in the same model. Candidate pathways were specified according to established ecological knowledge and Pearson correlation coefficients. Parsimonious models were obtained by stepwise removal of paths lacking theoretical support or statistical significance. Standardized path coefficients and their confidence intervals were estimated using 5,000 bootstrap resamples. Model fit was evaluated using χ^2/df , the comparative fit index (CFI), normed fit index (NFI), goodness-of-fit index (GFI), root mean square error of approximation (RMSEA), and model P value. The coefficient of determination (R^2) was reported for each endogenous variable. All SEM analyses were performed in IBM SPSS Amos 24.

Results

Effects of BSCs and slope gradient on soil nutrients in alpine meadows

Multiple-comparison results showed that the effects of BSCs on soil nutrient contents varied with slope gradients (Table 2). Compared with non-BSC soils, BSC-covered meadow soils generally had higher SOM, TN, and NO_3^- -N contents, although the magnitude of these responses was inconsistent across slope gradients (Table 2). Specifically, SOM and NO_3^- -N contents in BSC-covered soils on gentle and steep slopes were significantly higher than those in the corresponding non-BSC soils ($P < 0.05$). TN was significantly higher in BSC-covered soil than in non-BSC soil only on gentle slopes ($P < 0.05$), whereas no significant differences were observed on flat terrain or steep slopes.

NO_3^- -N accumulated significantly with increasing slope gradient. Under BSC cover, NO_3^- -N contents on gentle and steep slopes were 63.7% and 73.5% higher, respectively, than those on flat terrain. In contrast, AP content was generally lower in BSC-covered soils than in non-BSC soils, and AP content in BSC-covered soil on flat terrain was significantly lower than that in the corresponding non-BSC soil ($P < 0.05$). NH_4^+ -N content was lower in BSC-covered soils than in non-BSC soils irrespective of slope gradient, although the differences were not statistically significant ($P > 0.05$).

Table 2
Effects of BSCs and slope gradient on soil physicochemical properties in alpine meadows.

Indicators	F		G		S	
	BM	NM	BM	NM	BM	NM
SOM (g/kg)	63.23 ± 3.62ab	55.31 ± 2.92b	73.14 ± 15.69a	53.07 ± 10.69b	74.69 ± 3.75a	58.56 ± 8.37b
TN(g/kg)	4.21 ± 0.74ab	3.77 ± 0.28b	4.94 ± 1.04a	3.76 ± 0.61b	4.51 ± 1.04ab	4.41 ± 0.70ab
NO ₃ ⁻ -N (mg/kg)	6.84 ± 2.91b	6.27 ± 2.00b	11.20 ± 0.35a	7.50 ± 0.42b	11.87 ± 2.17a	7.57 ± 1.56b
NH ₄ ⁺ -N (mg/kg)	4.80 ± 4.80b	16.58 ± 16.80ab	1.06 ± 0.81b	8.51 ± 1.78ab	5.67 ± 7.39ab	22.73 ± 22.60a
TP(g/kg)	0.50 ± 0.04a	0.52 ± 0.09a	0.59 ± 0.14a	0.50 ± 0.06a	0.50 ± 0.04a	0.54 ± 0.07a
AP (mg/kg)	3.88 ± 1.42a	5.65 ± 0.24b	3.57 ± 2.11a	4.52 ± 0.84ab	4.39 ± 0.90ab	4.53 ± 1.18ab
TK(g/kg)	12.26± 4.30a	14.88 ± 1.60a	15.02 ± 2.63a	16.13 ± 1.21a	14.37 ± 2.93a	15.37 ± 1.03a
AK (mg/kg)	208.33± 54.50ab	205.17 ± 30.93ab	209.50 ± 34.58ab	187.33 ± 24.15a	269.50 ± 81.39b	225.50 ± 48.57ab
Note: Different lowercase letters indicate significant differences in soil nutrient contents among treatments ($P < 0.05$). F: flat terrain; G: gentle slope; S: steep slope; BM: BSC-covered meadow; NM: non-BSC meadow.						

Two-way ANOVA showed that BSCs, slope gradient, and their interaction exerted differential effects on soil physicochemical properties (Table 3). BSCs significantly affected SOM, TN, NO₃⁻-N, NH₄⁺-N, and AP ($P < 0.05$), whereas slope gradient significantly affected NO₃⁻-N and AK ($P < 0.05$). In addition, the interaction between BSCs and slope gradient had a significant effect on NO₃⁻-N ($P < 0.05$), indicating that the effect of BSCs on NO₃⁻-N varied with slope gradient.

Table 3
Results of two-way ANOVA for the effects of BSCs, slope gradient, and their interaction on soil physicochemical properties.

Indicators	SOM	TN	NO ₃ ⁻ -N	NH ₄ ⁺ -N	TP	AP	TK	AK
Crust	***	*	***	**	0.64	*	0.07	0.17
Slope	0.141	0.31	***	0.17	0.56	0.38	0.17	*
Crust*Slope	0.24	0.24	*	0.63	0.10	0.29	0.69	0.60

Note: Significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Effects of BSCs and slope gradient on the relative abundance of nitrogen-cycling functional genes

BSCs and slope gradient significantly affected the relative abundances of several nitrogen-cycling functional genes (Fig. 2a). Compared with non-BSC meadows, BSC-covered meadows generally showed higher relative sequence abundances of archaeal *amoA* and *nirK*, with particularly pronounced differences on flat and steep-slope terrains (Fig. 2b). The relative abundance of bacterial *amoA* genes responded to soil texture and slope gradient similarly to those of archaeal *amoA* genes (Fig. 2b). In contrast to the ammonia-oxidation genes, *nirS* had a relative abundance generally significantly higher in non-BSC meadows than in BSC-covered meadows (Fig. 2b). No statistically significant differences in *nosZ* relative abundance were observed between BSC-covered and non-BSC meadows at all slope gradients.

Random forest analysis indicated that the key environmental predictors differed among functional genes. TK and NO₃⁻-N were important predictors of archaeal *amoA* relative abundance, NO₃⁻-N and AP were the principal predictors of bacterial *amoA* relative abundance. SOM and AP showed high explanatory power for *nirK*. SOM and TK were closely associated with variation in *nirS*, and NH₄⁺-N was an important predictor of *nosZ* relative sequence abundance (Fig. 2c).

Effects of BSCs and slope gradient on nitrogen-cycling functional microbial community structure

BSCs and slope gradient jointly influenced the α-diversity and community composition of nitrifying and denitrifying microorganisms in the alpine meadow (Fig. 3). Two-way ANOVA showed that functional groups responded differently to BSCs and slope gradient. α-diversity analysis indicated that BSCs significantly decreased the Shannon diversity of AOA, AOB, *nirK*, and *nosZ* communities. Slope gradient significantly affected the diversity of AOA, *nirK*, and *nosZ* communities, but did not significantly affect the diversity of AOB or *nirS* communities ($P > 0.05$; Fig. 3a).

PCoA based on Bray-Curtis distances and PERMANOVA showed that slope gradient explained significant variation in the composition of all five functional-gene communities. BSCs significantly affected the community composition of all functional groups except AOB (Fig. 3b). In addition, the interaction between BSCs and slope gradient significantly affected the compositions of AOA, *nirK*, and *nirS*

communities, indicating a clear topographic dependency of BSC effects on these functional groups (Fig. 3b).

Spearman correlation analysis was used to examine associations between dominant genera and soil physicochemical properties. The dominant AOA genus was *Nitrososphaera* (mean relative abundance, 77.22%). Its relative abundance was higher on gentle slopes than on steep slopes, where it declined markedly, however, it was not significantly correlated with any measured soil properties (Fig. 4a). The dominant AOB genus was *Nitrosospira* (mean relative abundance, 43.53%), which was more abundant in non-BSC than BSC-covered meadows and was positively correlated with TN and NO_3^- -N (Fig. 4b).

Among dominant *nirK*-harboring taxa, the relative abundance of *Bradyrhizobium* declined significantly on gentle and steep slopes and was negatively correlated with TK. In contrast, the relative abundance of *Mesorhizobium* increased significantly on steep slopes and was negatively correlated with NH_4^+ -N but positively correlated with SOM and NO_3^- -N (Fig. 4c). *Paracoccus* was enriched in non-BSC plots and positively correlated with NO_3^- -N and TK. In the *nirS* community, the dominant taxa were *Azospirillum* (mean relative abundance, 28.38%) and *Cupriavidus* (9.93%). The relative abundance of *Azospirillum* declined markedly on gentle and steep slopes and was negatively correlated with TN, *Bradyrhizobium* was negatively correlated with TK; and *Cupriavidus* increased significantly with SOM (Fig. 4d). The dominant *nosZ*-harboring genus was *Tardiphaga* (mean relative abundance, 62.98%), followed by *Bradyrhizobium* (4.60%). *Tardiphaga* was the least abundant on steep slopes and negatively correlated with NH_4^+ -N, whereas *Paracoccus* was positively correlated with AK (Fig. 4e).

Clear coupling relationships were observed among soil nutrients: TN was positively correlated with TP and NO_3^- -N, whereas NO_3^- -N was negatively correlated with NH_4^+ -N. Mantel tests showed that AOB community composition was significantly regulated by TK and AP, the *nirK*-type denitrifier community was mainly associated with NH_4^+ -N, and the *nirS* community was associated with TN. In contrast, the compositions of AOA and *nosZ*-type denitrifier communities were not significantly associated with the measured soil nutrient properties.

Co-occurrence networks of five nitrogen-cycling functional-gene communities

Co-occurrence networks were constructed at the OTU level for five nitrogen-cycling functional microbial groups: AOA, AOB, *nirK*, *nirS*, and *nosZ*. Ecological co-occurrence modules were identified for each group, comprising three modules for AOA, six for AOB, and seven each for *nirK*, *nirS*, and *nosZ* (Fig. 6a). All AOA modules consisted of Thaumarchaeota, whereas modules of the AOB, *nirK*, *nirS*, and *nosZ* communities were dominated by Proteobacteria (Fig. 6b). Unclassified taxa occurred in AOB Module_6 and *nosZ* Module_7 and were mainly distributed in BSC-covered meadows and non-BSC meadows on steep slopes (Fig. 6b).

Module relative abundances exhibited distinct patterns along the topographic gradient. In the AOA community, Module_1 was the most abundant on gentle slopes, reaching 15.8% and 15.2% in GBM and GNM, respectively, but accounted for < 2% on flat terrain. Module_3 was the most abundant in steep-

sloped non-BSC meadows (51.2%) but decreased significantly in BSC-covered meadows ($P < 0.05$; Fig. 6b). In the AOB community, Module_2 and Module_5 were relatively abundant in flat BSC-covered meadows, accounting for 42.6% and 11.4%, respectively, but were nearly absent from other sites. In the *nirK* community, Module_1 declined from 59.7% in flat BSC-covered meadows to 7.6% in steep BSC-covered meadows, whereas Module_7 was the most abundant in steep-sloped non-BSC meadows (33.4%).

In the *nirS* community, Module_1 was the most abundant on flat terrain, accounting for approximately 30%; Module_3 reached 30%-38% on gentle slopes; and Module_5 occurred only in steep-sloped non-BSC meadows, where it accounted for 18.3%. In the *nosZ* community, Module_1 was the most abundant in flat BSC-covered meadows (59.9%) and declined progressively with increasing slope gradient, whereas Module_3 was the most abundant in steep BSC-covered meadows (34.9%; Fig. 6b).

The effects of BSCs on module abundance were slope dependent. On flat terrain, AOB Module_2, *nirK* Module_1, and *nosZ* Module_1 were significantly more abundant in BSC-covered than non-BSC meadows ($P < 0.05$). On steep slopes, AOB Module_1, *nirK* Module_2, and *nosZ* Module_3 were significantly more abundant in BSC-covered meadows, whereas AOA Module_3, *nirK* Module_7, and *nosZ* Module_7 were significantly less abundant than in non-BSC meadows ($P < 0.05$). On gentle slopes, most modules did not differ significantly between BSC-covered and non-BSC meadows (Fig. 6b).

Comparison of the five co-occurrence networks showed that the AOA community contained only three modules, all composed of Thaumarchaeota, indicating a simple phylum-level composition. Slope gradient and BSCs mainly altered module relative abundance rather than causing clear community-structure differentiation. The AOB community contained six modules and showed strong habitat specialization, including distinct modules in flat BSC-covered and steep BSC-covered meadows. The *nirK*, *nirS*, and *nosZ* denitrifier communities each contained seven modules, and module abundances changed continuously along the slope gradient. Dominant *nirS* modules were concentrated on flat terrain and gentle slopes, the *nirK* community developed numerous specialized rare modules on steep slopes, and the *nosZ* community contained both BSC-associated dominant modules and modules specific to non-BSC meadows. Functional nitrogen-cycling modules displayed differentiated distribution patterns along slope gradient: gentle slopes maintained high-abundance modules for most genes, flat BSC-covered meadows enhanced core nitrification and N₂O-reduction modules, and steep-sloped non-BSC meadows were enriched with diverse rare modules that might serve as a reservoir of nitrogen-cycling microorganisms (Table S1).

Environmental drivers of nitrogen-cycling functional microbial diversity

Pearson correlation analysis and SEM were jointly used to explore relationships between the α -diversity of key nitrogen-cycling functional microbial communities and environmental factors. All SEMs showed good model fit (CMIN/DF < 2 , CFI ≥ 0.99 , GFI ≥ 0.95 , and RMSEA ≤ 0.05). The α -diversity of the AOA, AOB, *nirK*, *nirS*, and *nosZ* communities was characterized using the Chao1 richness, Shannon diversity, and Pielou_e evenness indices.

The results showed that NO_3^- -N was significantly negatively correlated with NH_4^+ -N and TK, whereas BSCs were significantly positively correlated with SOM, TN, and NO_3^- -N ($P < 0.05$; Fig. 7). SEM showed that slope gradient was positively associated with NH_4^+ -N ($r = 0.32$), while BSCs were positively associated with SOM ($r = 0.64$) and negatively associated with NH_4^+ -N ($r = -0.45$; Fig. 7). These findings suggest that BSCs and slope gradient may be associated with changes in functional microbial diversity through changes in soil carbon and nitrogen forms.

For nitrifying microbial communities, the Chao1 richness and Shannon diversity of archaeal *amoA*-harboring AOA communities were significantly negatively correlated with NH_4^+ -N ($P < 0.05$; Fig. 7). SEM further showed significant negative associations of AOA Chao1 richness and Shannon diversity with BSCs (Chao1: $r = -0.65$; Shannon: $r = -0.54$) and NH_4^+ -N (Chao1: $r = -0.51$; Shannon: $r = -0.45$; Fig. 8a). AOB Chao1 richness and Shannon diversity were significantly negatively correlated with BSCs, whereas TK was significantly positively correlated with AOB Shannon diversity ($P < 0.05$; Fig. 8). SEM indicated direct negative associations between BSCs and AOB diversity (Chao1: $r = -0.79$; Shannon: $r = -0.58$; Fig. 8b).

For denitrifying microbial communities, SOM was significantly negatively correlated with *nosZ* Shannon diversity, and TP was significantly negatively correlated with *nosZ* Chao1 richness. BSCs were significantly negatively correlated with the Chao1, Shannon, and Pielou_e indices of the *nosZ* community and with *nirK* Shannon diversity ($P < 0.05$; Fig. 8). AK was significantly positively correlated with *nirS* Shannon diversity ($P < 0.05$). SEM showed that BSCs were negatively associated with *nirK* Shannon diversity ($r = -0.56$), whereas *nirK* Chao1 richness was negatively associated with NH_4^+ -N ($r = -0.46$; Fig. 8c). *nosZ* diversity was negatively associated with BSCs, with a path coefficient of -0.73 for Shannon diversity; slope gradient was positively associated with *nosZ* Shannon diversity ($r = 0.35$; Fig. 8e). The diversity of the *nirS* community showed no consistent significant association with environmental factors in either correlation analysis or SEM (Fig. 8d).

Discussion

Effects of BSCs and slope gradient on soil nitrogen pools

This study showed that BSCs and slope gradient jointly influenced soil nitrogen forms and their spatial distribution in alpine meadows. BSCs significantly affected TN, NO_3^- -N, and NH_4^+ -N contents ($P < 0.05$). Relative to non-BSC meadows, BSC-covered soils had significantly higher NO_3^- -N and lower NH_4^+ -N contents (Table 2), supporting our first hypothesis and suggesting that BSCs may promote the conversion of NH_4^+ -N to NO_3^- -N and the accumulation of NO_3^- -N (Hu et al. 2024). This pattern may be attributable to improved surface-soil aeration and greater thermal and moisture stability under BSCs (Bo et al. 2016), which create favorable microsites for ammonia-oxidizing microorganisms (Nevins et al. 2022). In addition, nitrogen fixed by BSCs may be released partly as NH_4^+ , providing substrate for

subsequent nitrogen transformations (Belnap 2002). By enhancing aggregate stability, BSCs may also increase NO_3^- -N retention and reduce leaching losses (Li et al. 2026).

Slope gradient had a highly significant effect on NO_3^- -N ($P < 0.001$) and interacted significantly with BSC status ($P < 0.05$; Table 3). Across slope gradient, BSCs increased NO_3^- -N contents by 49.3%-56.8%. Although slope gradient had no significant main effect on TN or NH_4^+ -N (Table 3), under BSC cover, TN increased by 31.4% and NH_4^+ -N decreased by 87.5% on gentle slopes (Table 2). This finding suggests that gentle slopes may provide relatively favorable conditions for nitrogen accumulation and transformation in BSC-covered meadows. Slope-induced water redistribution (Bao et al. 2024; Xiao et al. 2024), erosion (Perakis et al. 2015), and hydrothermal conditions (Barry and Blanken 2016) may jointly regulate nitrogen retention, transport, and loss. Stronger runoff and drainage on steep slopes may reduce soil moisture, weaken the anaerobic conditions required for denitrification, and thereby decrease gaseous nitrogen loss (Yu et al. 2021). These processes may also promote the transport and redistribution of nitrification products along slopes (Ayoubi et al. 2025), helping explain the observed increase in NO_3^- -N with slope gradient. Furthermore, slope gradient may regulate BSC development, photosynthesis, respiration, and nutrient-retention capacity by altering hydrothermal and erosional conditions (Zhou et al. 2020; Richardson et al. 2022; Miralles et al. 2013; Raggio et al. 2017). Different slope positions may favor BSC assemblages with distinct compositions and functions, whose nitrogen-fixation efficiencies and nutrient-release patterns could further influence soil nitrogen cycling (Rodríguez-Caballero et al. 2019). Thus, BSCs and slope gradient jointly shape nitrogen transformation and distribution in alpine meadow soils through hydrological, erosional, and microclimatic processes.

Differential responses of nitrogen-cycling genes and microbial communities to BSCs and slope gradient

Functional gene relative abundances and community characteristics showed distinct responses to BSCs and slope gradient, supporting hypothesis (ii). At the same slope gradient, archaeal *amoA* and *nirK* had higher relative abundances in BSC-covered meadows, particularly on flat terrain and steep slopes, whereas *nirS* was generally more abundant in non-BSC meadows (Fig. 2b). The contrasting responses of *nirK* and *nirS* suggest that denitrifying functional groups have different niche preferences in BSC-covered meadows, owing potentially to differences in oxygen availability and carbon-source accessibility (Florio et al. 2019; Jones et al. 2013; Graf et al. 2014).

Random forest analysis further indicated that the environmental drivers of functional-gene relative abundance differed among genes (Fig. 2c). Archaeal *amoA* was mainly associated with TK and NO_3^- -N, bacterial *amoA* with AP and NO_3^- -N, *nirK* with SOM and AP, *nirS* with SOM and TK, and *nosZ* with NH_4^+ -N. This differentiation reflects the distinct requirements of functional groups for nutrient substrates and environmental conditions (Nshimiyimana et al. 2024; Pan et al. 2022).

BSCs significantly reduced the α -diversity of the AOB, *nirK*, and *nosZ* communities, with the strongest effects observed for AOB and *nirK* ($P < 0.001$), whereas slope gradient significantly affected the diversity

of the AOA, *nirK*, and *nosZ* communities (Fig. 3a). The lack of significant variation in *nirS* diversity among the treatments may indicate a relatively broad environmental tolerance of this community (Byun et al. 2024). β -diversity analysis showed that slope gradient significantly drove compositional differentiation of all five functional communities along slope gradient. BSCs significantly altered the communities except AOB, and the BSC $\times$ slope interaction significantly influenced the compositions of the AOA, *nirK*, and *nirS* communities (Fig. 3b).

The low NH_4^+ -N environment associated with BSCs may constrain AOB communities that preferentially occupy habitats with higher ammonium availability, while selecting AOA taxa adapted to oligotrophic conditions (Delgado-Baquerizo et al. 2016). The higher NO_3^- -N content and increased relative abundance of archaeal *amoA* in BSC-covered soils further suggest that AOA may make a greater relative contribution to ammonia oxidation under these conditions. *Nitrososphaera* was the dominant AOA genus, and its relative abundance was the highest on gentle slopes but was unrelated to the measured soil properties, suggesting that its distribution may be primarily regulated by slope-related hydrothermal conditions (Yuan et al. 2015). The dominant AOB genus, *Nitrosospira*, was more abundant in non-BSC meadows and positively correlated with TN and NO_3^- -N, consistent with its sensitivity to nitrogen availability (Nshimiyimana et al. 2024; Daims et al. 2015).

Among denitrifying communities, significant changes in *nirK* and *nosZ* diversity indicate sensitivity to environmental shifts caused by BSCs and slope gradient (Huang et al. 2018; Florio et al. 2019). Because the N_2O reductase encoded by *nosZ* is oxygen sensitive (Jones et al. 2013), improved soil porosity and aeration under BSCs may be unfavorable for strictly anaerobic denitrifiers (Sun et al. 2022). BSCs may also influence particular denitrifying taxa by altering organic-matter composition or releasing secondary metabolites (Baran et al. 2015). At the genus level, the contrasting slope distributions of *Bradyrhizobium* and *Mesorhizobium* indicate niche differentiation in the *nirK* community (Wang et al. 2022). Mantel-test results further showed that AOB composition was mainly associated with TK and AP, *nirK* composition with NH_4^+ -N, and *nirS* composition with TN (Fig. 5). These results suggest fundamental differences in the environmental preferences of nitrogen-cycling functional groups, which may underlie their divergent patterns of relative abundance and diversity.

Differentiation and topographic distribution of co-occurrence network modules

Co-occurrence network modules represent OTU groups with similar environmental responses and strong statistical associations (Gui et al. 2023). The AOA network contained only three modules, all affiliated with Thaumarchaeota. BSCs and slope gradient mainly changed their relative abundances without markedly altering overall network structure, suggesting that AOA communities may maintain a relatively stable ammonia-oxidation potential through a limited number of core taxa (Yuan et al. 2015; Zhao et al. 2024).

The AOB and three denitrifier networks were dominated by Proteobacteria, a phylum containing numerous microorganisms involved in nitrification and denitrification (Nshimiyimana et al. 2024). AOB

Modules_5 and 2 were dominant in flat BSC-covered meadows. AOA Module_1, *nirS* Module_3, and *nosZ* Module 5 maintained relatively high abundance on gentle slopes, whereas distinct modules, including AOA Module_3, *nirK* Module_7, *nirS* Module_5, and *nosZ* Module_7, were enriched in steep-sloped non-BSC meadows (Fig. 6b). These patterns suggest that gentle slopes may be important sites for the aggregation of high-abundance functional modules, whereas steep non-BSC habitats may retain more disturbance-tolerant or rare modules.

BSCs and slope gradient may continuously filter microbial communities by altering moisture, temperature, and nutrient conditions (Zhou et al. 2020). Slope gradient can also affect root growth and soil carbon inputs, thereby influencing the growth and module distribution of nitrogen-cycling microorganisms (Lyu et al. 2025). The strong environmental adaptability of Proteobacteria may enable them to respond to habitat fluctuations through module differentiation and niche expansion (Li et al. 2025). Similar adaptations of functionally related taxa to environmental variation may also contribute to stable co-occurrence patterns (Crocker et al. 2025).

Drivers of nitrogen-cycling microbial diversity

SEM results showed that BSCs, slope gradient, and soil properties were associated with nitrogen-cycling microbial diversity through multiple pathways, supporting hypothesis (iii). BSCs had a significant positive direct association with SOM ($r = 0.64$) and a significant negative direct association with $\text{NH}_4^+\text{-N}$ ($r = -0.45$), while also being negatively associated with the diversity of the AOB, *nirK*, and *nosZ* communities (Fig. 8). These relationships may be related to BSC-induced changes in soil porosity and aggregate structure (Huang et al. 2025). Secondary metabolites released by BSCs may also exert selective or allelopathic effects on particular microbial taxa (Baran et al. 2015).

$\text{NH}_4^+\text{-N}$ was an important intermediate variable linking slope gradient and BSCs with AOA diversity. Slope gradient was positively associated with $\text{NH}_4^+\text{-N}$ ($r = 0.32$), whereas $\text{NH}_4^+\text{-N}$ was negatively correlated with AOA Chao1 richness and Shannon diversity (Fig. 7), indicating that AOA communities may be environmentally filtered along the ammonium gradient (Delgado-Baquerizo et al. 2016). In addition, slope gradient had a direct positive association with *nosZ* Shannon diversity ($r = 0.35$), potentially reflecting slope-related changes in drainage and aeration (Ayoubi et al. 2025; Jones et al. 2013).

Overall, BSCs and slope gradient differentially regulated the structure, diversity, and network-module distribution of nitrogen-cycling functional microbial communities in alpine meadows by altering SOM, $\text{NH}_4^+\text{-N}$, and related soil microenvironmental conditions.

Conclusions

This study demonstrates that BSCs and slope gradient jointly influence soil nutrient characteristics and nitrogen-cycling functional microbial communities in alpine meadows. BSCs increased soil $\text{NO}_3^-\text{-N}$ while

decreasing $\text{NH}_4^+\text{-N}$, and $\text{NO}_3^-\text{-N}$ accumulated with increasing slope gradient. Responses to BSCs and slope gradient differed among functional genes: archaeal *amoA* and *nirK* relative abundances were higher in BSC-covered meadows, particularly on flat terrain and steep slopes, whereas *nirS* relative abundance was higher in non-BSC meadows. BSCs significantly reduced the Shannon diversity of AOB, *nirK*, and *nosZ* communities, while slope gradient significantly affected AOA, *nirK*, and *nosZ* diversity. Network modules also exhibited clear topographic differentiation. Gentle slopes maintained more high-abundance modules, flat BSC-covered meadows supported relatively abundant AOB and N_2O -reduction-associated modules, and steep non-BSC meadows contained several low-abundance modules. Collectively, BSCs and slope gradient influenced the diversity, composition, and module distribution of nitrogen-cycling functional microbial communities by modifying soil properties, particularly SOM, $\text{NO}_3^-\text{-N}$, and $\text{NH}_4^+\text{-N}$. These findings provide a basis for understanding the coupling between soil nitrogen forms and nitrogen-cycling functional microbial communities in alpine meadows.

Declarations

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Figures

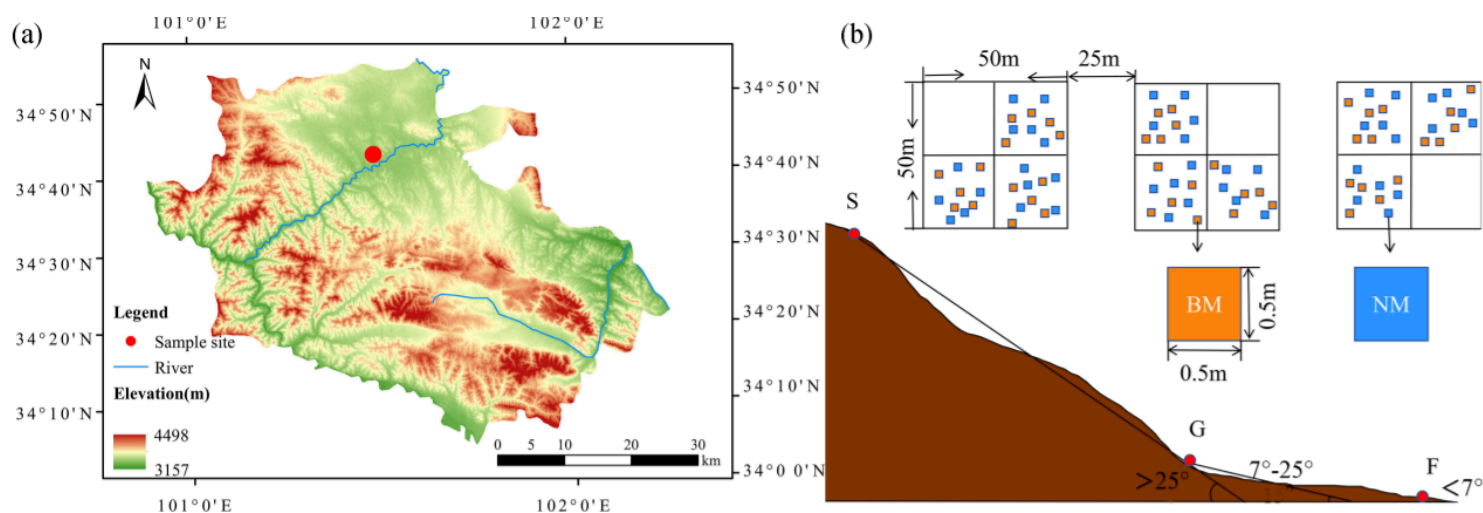


Figure 1

Geographical location of the study area (a) and sampling plot layout (b). F: flat; G: gentle slope; S: steep slope; BM: BSC-covered meadow; NM: non-BSC meadow. Quadrat color: three replicates.

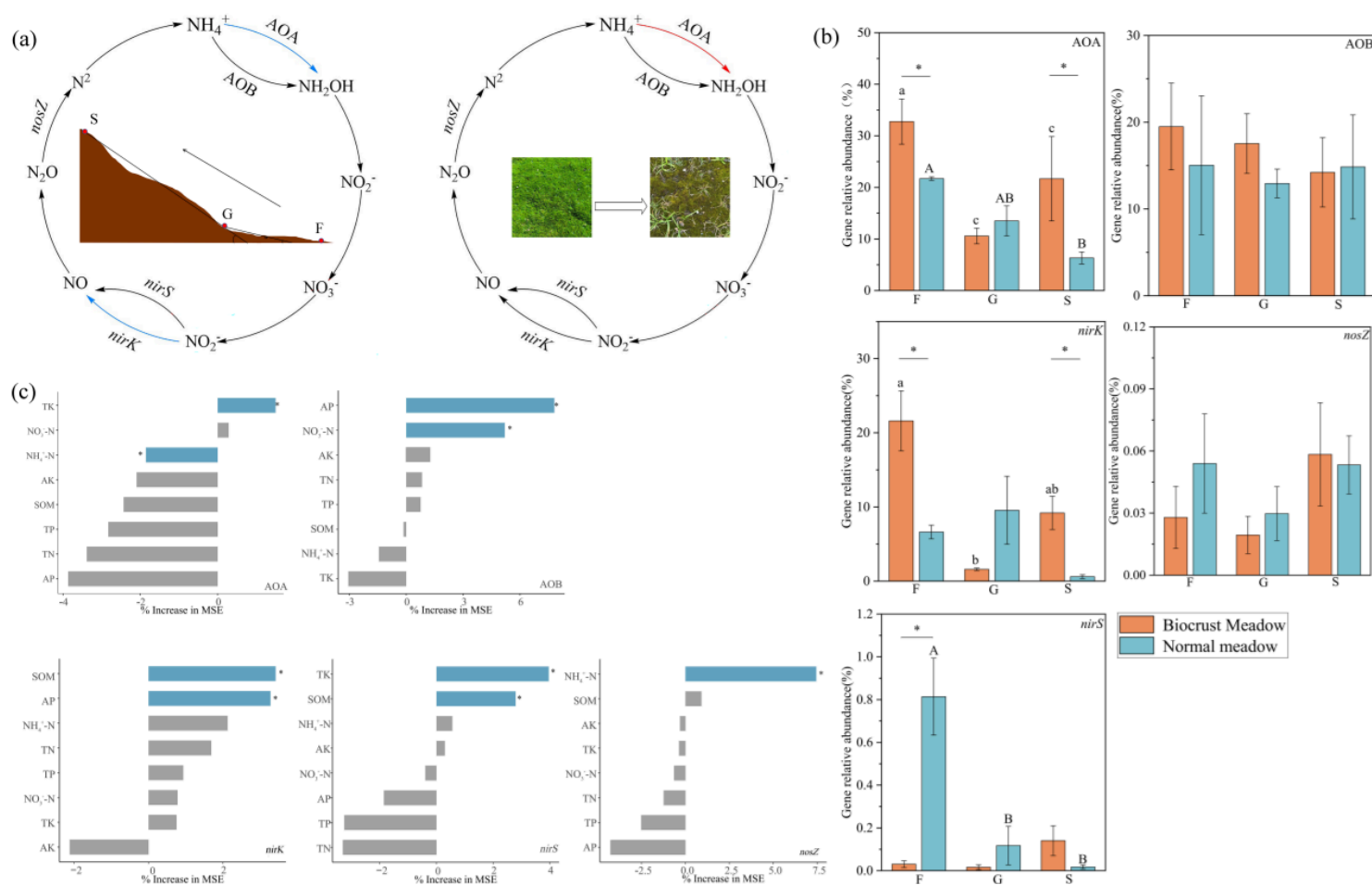


Figure 2

Effects of BSCs and slope gradient on soil nitrogen transformation processes, the relative abundances of nitrogen-cycling functional genes, and their environmental drivers in alpine meadows. (a) Conceptual

diagram illustrating soil nitrogen transformation processes and changes in the relative abundances of associated functional genes. Red arrows indicate significantly increased relative abundance of the corresponding functional gene, whereas blue arrows indicate significantly decreased relative abundance ($P < 0.05$). **(b)** The relative abundance of soil nitrogen cycling functional genes in BSC-covered and non-BSC meadows under different slope conditions. Different lowercase letters indicate significant differences among slope-gradient treatments in BSC-covered meadows, whereas different uppercase letters indicate significant differences among slope-gradient treatments in non-BSC meadows ($P < 0.05$). **(c)** Random forest analysis quantifying the contributions of soil physicochemical properties to functional-gene relative abundance using %IncMSE and IncNodePurity. Differences were tested by one-way ANOVA. F: flat terrain; G: gentle slope; S: steep slope; BM: BSC-covered meadow; NM: non-BSC meadow.

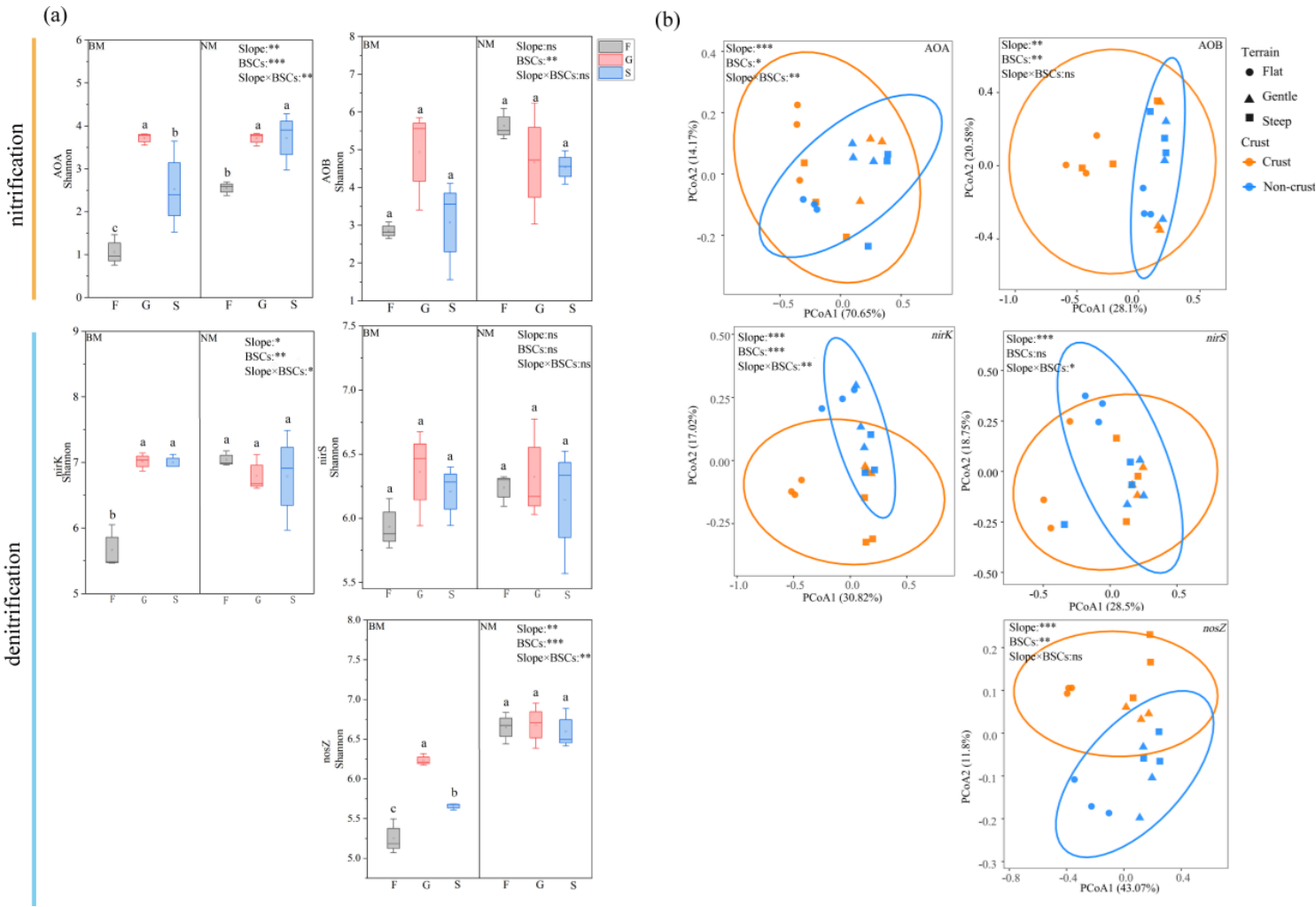


Figure 3

Effects of BSCs and slope gradient on α diversity and community structure of ammonia-oxidizing and denitrifying microorganisms in the alpine meadows. **(a)** Shannon diversity indices of nitrogen-cycling functional gene communities. Two-way ANOVA results show the significance of BSC, slope-gradient, and interaction effects. **(b)** PCoA based on Bray-Curtis distances. The significance of BSC, slope-gradient,

and interaction effects from PERMANOVA is indicated on the right, ellipses represent 95% confidence intervals. F: flat terrain; G: gentle slope; S: steep slope; BM: BSC-covered meadow; NM: non-BSC meadow.

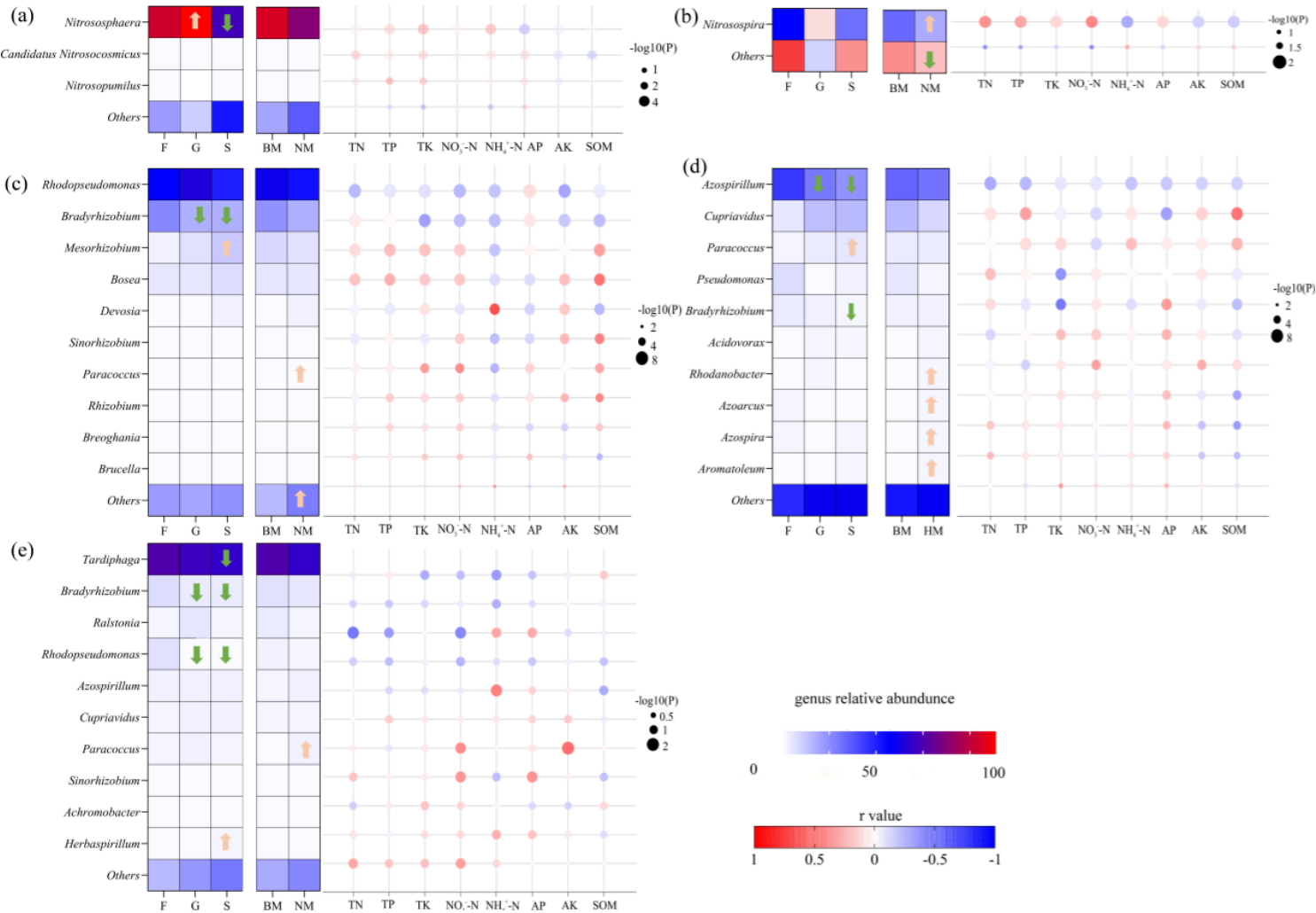


Figure 4

Differences in dominant genera associated with nitrogen-cycling genes and their correlations with environmental factors in BSC and slope-gradient treatments. Heatmaps show the distribution of relative abundance of dominant genera. The color blocks on the left indicate differences in taxon abundance among slope-gradient and BSC treatments. Red upward arrows indicate a significant increase in relative abundance relative to flat terrain and BSC-covered meadows, whereas green downward arrows indicate a significant decrease. Bubble plots show Pearson correlation coefficients between the relative abundances of dominant genera and soil physicochemical properties. Panels (a-e) correspond to AOA, AOB, *nirK*, *nirS*, and *nosZ*, respectively.

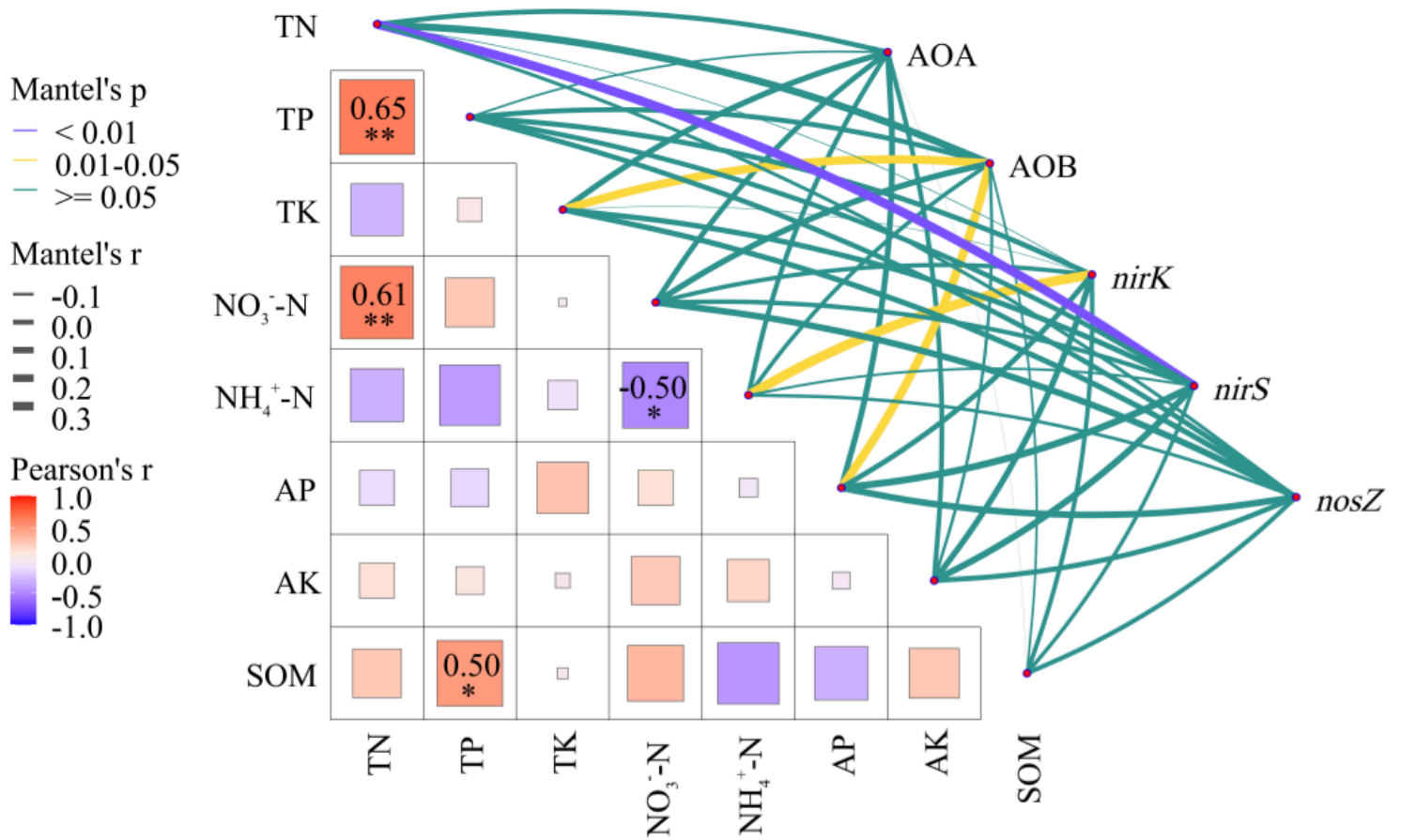


Figure 5

Partial Mantel-test heatmap showing relationships between soil fertility indicators and soil nitrogen-cycling functional microbial communities. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

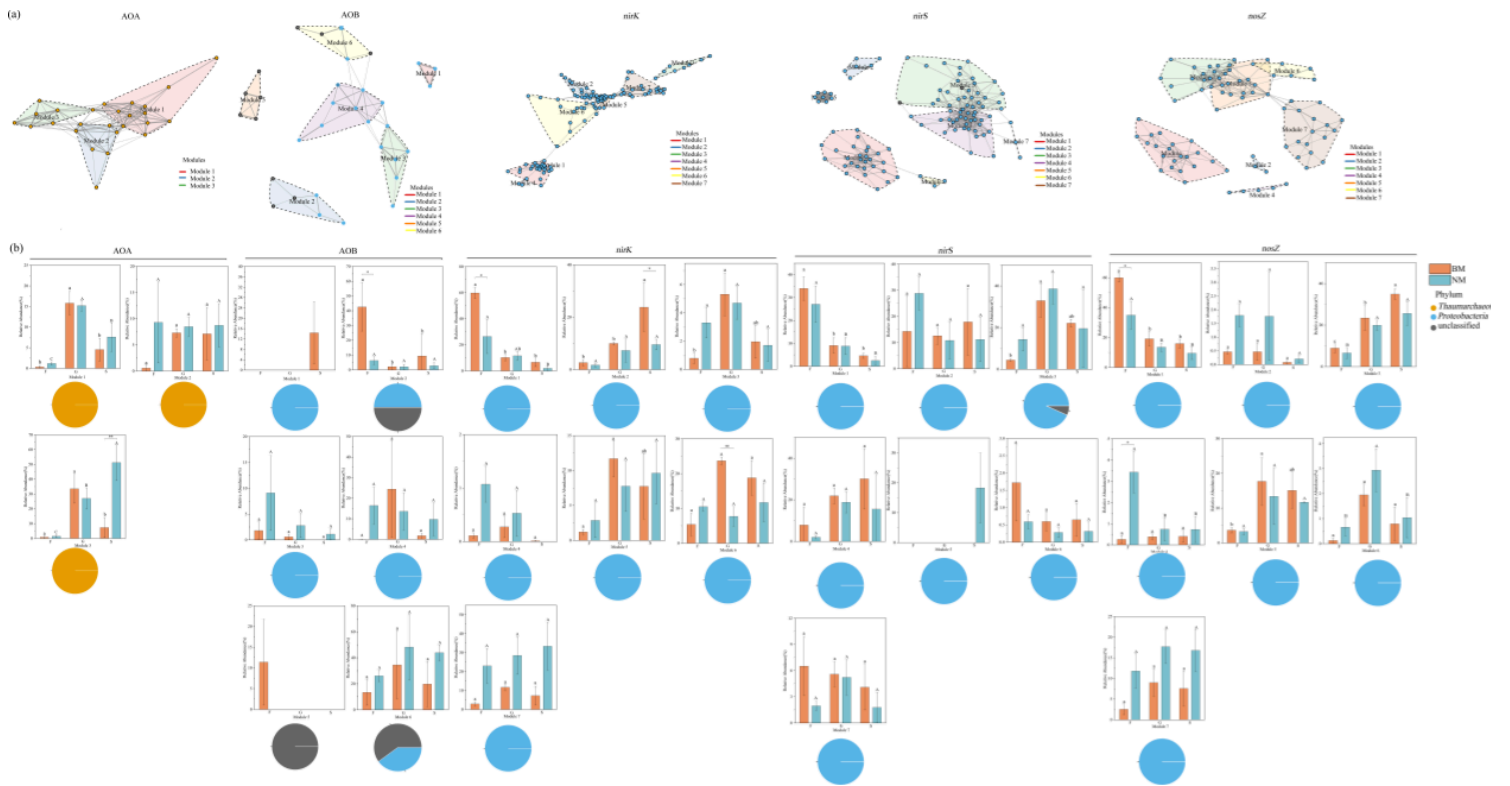


Figure 6

OTU co-occurrence networks of dominant ecological clusters in soil nitrogen-cycling microbial communities across BSC and slope-gradient treatments. **(a)** Networks are colored according to ecological modules. **(b)** Bar charts and pie charts show the relative abundances of modules across treatments and the phylum-level taxonomic composition within each module, respectively. Different lowercase letters indicate significant differences among BSC-covered meadows at different slope gradients, whereas different uppercase letters indicate significant differences among non-BSC meadows at different slope gradients ($P < 0.05$).

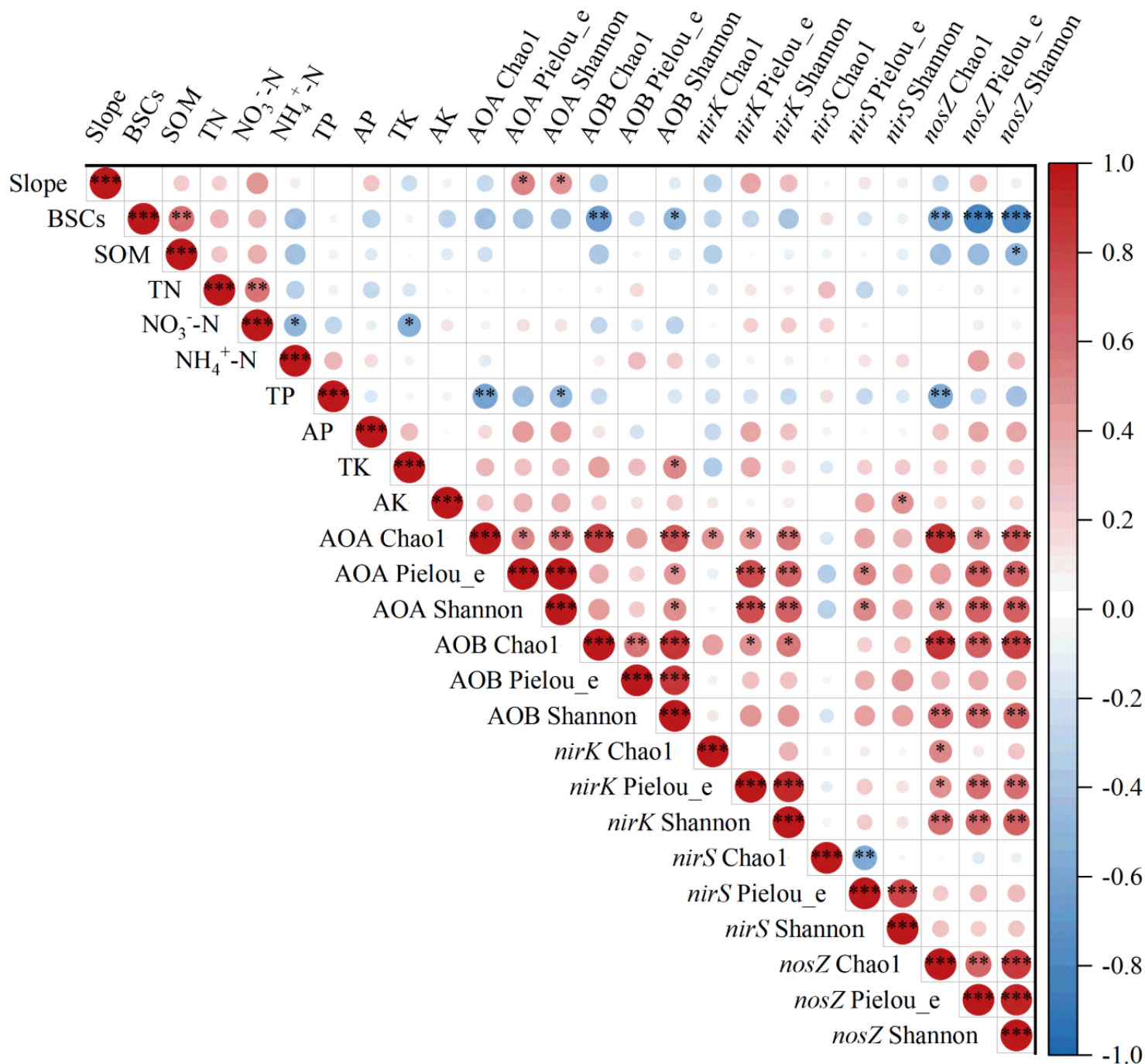


Figure 7

Pearson correlations between α -diversity indices of nitrogen-cycling functional microbial communities and soil fertility indicators, BSC status, and slope gradient.* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

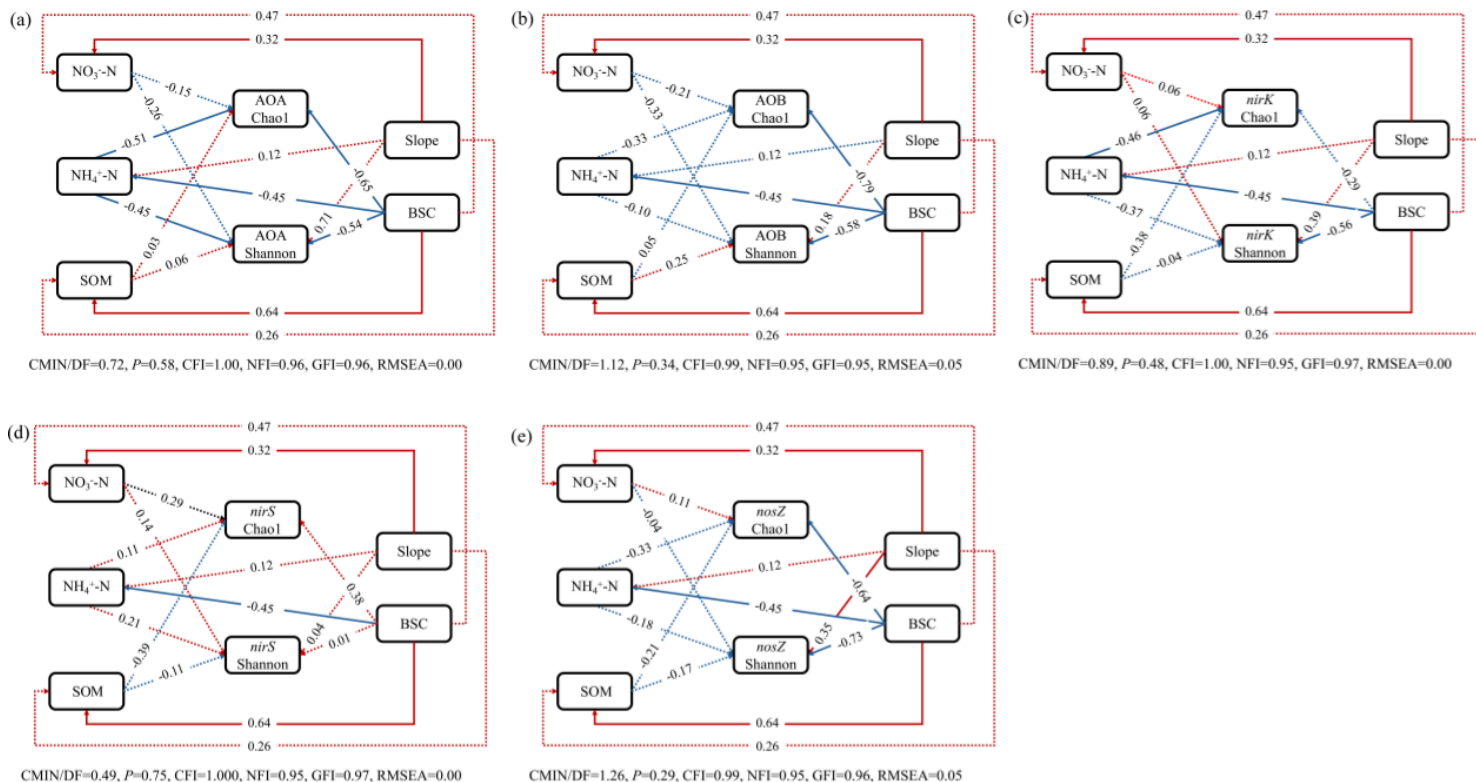


Figure 8

Structural equation models showing associations among BSCs, slope gradient, soil nutrients, and α -diversity indices of five nitrogen-cycling functional microbial communities. **(a)** AOA; **(b)** AOB; **(c)** *nirK*; **(d)** *nirS*; and **(e)** *nosZ*. Red lines represent positive effects, blue lines represent negative effects, solid lines represent significant path effects, and dashed lines represent insignificant path effects. Values along paths are standardized path coefficients. Model-fit indices (CMIN/DF, CFI, NFI, GFI, and RMSEA) are shown below each panel.

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