Agriculture, Ecosystems and Environment 374 (2024) 109177

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

Nitrogen fertilization increases the niche breadth of soil nitrogen-cycling microbes and stabilizes their co-occurrence network in a karst agroecosystem

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ARTICLE INFO

Keywords:
Green manure
Conventional fertilization
N-cycling processes
Metagenomics
Community assembly

ABSTRACT

Microbes play a key role in mediating soil nitrogen (N) cycling in agroecosystems. However, it remains unknown how N management practices affect the taxonomic and functional structure of soil N-cycling microbes, and their community assembly and co-occurrence networks in karst agroecosystems. Here, we conducted a field experiment to examine the effects of mineral N addition (+N) and legume (*Medicago sativa*) intercropping (+L) on soil N-cycling functional taxa and genes in a karst forage (*Broussonetia papyrifera*) agroecosystem. Results showed that compared to the control and +L treatment, mineral N addition significantly increased the functional gene diversity of nitrification-related microbes and the abundance of *hao* gene, but slightly reduced the abundances of *nifD* and *nifH* genes related to N fixation by 33.3–56.0 %. The abundance of *nifK* gene was 3.7-fold higher in the +L treatment than in the control. The assembly of microbial communities involved in ammonification, assimilatory nitrate reduction (ANR) and denitrification was controlled by a homogeneous selection process. Stochastic processes played a dominant role in shaping the communities related to nitrification, dissimilatory nitrate reduction (DNR), N fixation, and N assimilation. High soil pH and total N stimulated microbial N assimilation and the related gene abundance (e.g., *GDH2*), but suppressed the abundances of genes involved in N fixation. Both mineral N addition and legume intercropping significantly increased the niche breadth of the whole community and the functional groups related to denitrification, ANR, DNR and N assimilation. Actinobacteria related to N assimilation dominated the co-occurrence networks across the treatments. Compared to the control, the network robustness was significantly increased in the +N and +L treatments. Our findings indicate that there are distinct responses to the two N management practices among N-cycling functional groups and highlight the importance of N fertilization in increasing the niche breadth of N-cycling microbes and stabilizing their co-occurrence network in a karst agroecosystem.

1. Introduction

Application of mineral nitrogen (N) fertilizers (e.g., urea) is a common measure to enhance agricultural productivity (Guo et al., 2024). Applied N fertilizers are transformed into bio-available N through a series of N-cycling processes catalyzed by soil microbes (Klimasmith and Kent, 2022). The taxonomic structure of soil N-cycling microbes and their functional potential directly determine soil N availability, and are greatly affected by N management practices (Ma et al., 2022; Yang et al., 2023; Wang et al., 2024). For instance, mineral N addition can increase ammonium (NH_4^+) and nitrate (NO_3^-) content by stimulating abundances of microbes related to mineralization, dissimilatory nitrate reduction (DNR) and nitrification, as well as their N transformation rates (Yang et al., 2023). However, less than 50 % of the transformed N is utilized by

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https://doi.org/10.1016/j.agee.2024.109177
Received 4 April 2024; Received in revised form 7 July 2024; Accepted 8 July 2024
Available online 13 July 2024

crops (Quan et al., 2021; Elrys et al., 2023). Most unexploited N resources are further transformed to gases (e.g., N_2O and NH_3) or leached (Bowles et al., 2018; Klimasmith and Kent, 2022), especially in fragile karst agroecosystems (Li et al., 2020; Ren et al., 2022). Currently, legume intercropping is generally recommended to control N losses in karst agroecosystems (Liao et al., 2023a; Zhao et al., 2023). In contrast with mineral N fertilizer, legumes can optimize soil N-fixing bacterial communities and their N fixation function (Castellano-Hinojosa et al., 2022). The fixed N by root-rhizobia symbiont can be transferred to roots and then be slowly released to soil as labile N (e.g., ammonium and amino acids) via root exudation (Paynel et al., 2001; Lesuffleur et al., 2007; Moe, 2013). Most previous studies mainly focus on how mineral N addition and legume intercropping affect the patterns of the taxonomic diversity and community structure of soil N-cycling microbes (Sun et al., 2021; Ma et al., 2022; Wang et al., 2024). However, how N management practices affect the underlying ecological processes is poorly understood in karst agroecosystems.

Community assembly is essential in describing how microbial community structure is shaped by different ecological processes, including both deterministic and stochastic processes (Ning et al., 2024). Deterministic processes (i.e., niche-based theory) shape community structure patterns by environmental filtering (e.g., pH and nutrients) and various biological interactions (e.g., competition and mutualisms) (Ning et al., 2020). Conversely, stochastic processes (i.e., neutral theory) control community structure patterns by random birth, death, and dispersal events (Ning et al., 2020). In agroecosystems, soil bacterial communities that play a dominant role in mediating soil N cycling (Sun et al., 2021), are shaped primarily by stochastic processes (Jiao et al., 2020). However, some specific N-cycling functional groups deviate from such community assembly pattern. For example, the assembly of microbial communities involved in N fixation, ammonification, nitrification and denitrification is governed by deterministic processes in the acid and saline soils (Fan et al., 2018; Li et al., 2021; Liu et al., 2022). Yet, we still lack comprehensive knowledge of community assembly patterns of soil N-cycling functional groups in the fragile karst agroecosystems where soils are featured by high pH, shallow depth, and low retention of water and nutrients (Wang et al., 2019; Jiang et al., 2020).

The balance between deterministic and stochastic processes is mediated by N management practices (Wang et al., 2022; Zhou et al., 2022). Mineral N addition and legume intercropping increase the relative importance of stochastic processes for soil microbial community assembly by increasing soil N availability (Zhou et al., 2022; Wang et al., 2023b). In contrast, long-term mineral N addition and legume intercropping can acidify soils (Bolan et al., 1991; Wang et al., 2023c), which can shift community assembly of soil N-cycling microbes from stochastic to deterministic processes (Fan et al., 2018; Jiao and Lu, 2020). Generally, soil microbial communities shaped by stochastic assembly processes have wider niche breadth, and allow more taxa that encode the same functions and occupy the same ecological niche to coexist, leading to more complex co-occurrence network relationships (Fan et al., 2018; Jiao et al., 2020; Zhou et al., 2022). The co-occurrence patterns of soil N-cycling microbes may be more sensitive to changes in some other functional groups because multiple N-cycling processes interact with each other (Klimasmith and Kent, 2022). Rare taxa as key N-cycling drivers have a relatively small niche breadth compared with abundant taxa and are easily affected by environmental factors (Yang et al., 2022; Cui et al., 2023). Furthermore, niche diversity of N-cycling microbes increases with formation of macro-aggregates by legume roots (Hartmann and Six, 2023). Whether increased niche breadth affects the stability of co-occurrence networks of soil N-cycling microbes is yet to be determined.

In the present study, we conducted a field experiment to investigate how two N management practices, mineral N addition and legume intercropping, affect the taxonomic and functional structure of soil N-cycling microbes, as well as their community assembly processes and co-occurrence networks in a karst forage (*Broussonetia papyrifera*) agroecosystem. The objectives of the present study were to 1) determine how the two N management practices affect soil N-cycling microbial communities and their community assembly processes; 2) determine whether wider niche breadth weakens competitive interactions among taxa and stabilizes the co-occurrence networks. We hypothesized that N fertilization-induced shifts in soil N availability and pH would alter the taxonomic and functional structure of soil N-cycling microbes, because mineral N addition and legume intercropping can effectively increase soil N availability and induce soil acidification during nitrification and N fixation processes (Bolan et al., 1991; Wang et al., 2023c). We further hypothesized that stochastic processes dominated soil N-cycling microbial community assembly, and their niche breadth and the stability of co-occurrence networks would increase with increased soil N availability. Mineral N addition and legume intercropping can create optimal environments for soil N-cycling microbes by providing more available N resources and diverse micro-habitats, reducing competition for limited resources and living spaces, and allowing more species to co-exist (Fan et al., 2018; Hartmann and Six, 2023). Consequently, the stability of co-occurrence networks is maintained when some taxa are lost due to environmental stresses (Hernandez et al., 2021).

2. Materials and methods

2.1. Study area and experimental design

The study area was located at the Huanjiang Observation and Research Station for Karst Ecosystems (107°51'−108°43' E, 24°44'−25°33' N), Chinese Academy of Sciences, Guangxi Zhuang Autonomous Region, China. The soil in the study area originates from a dolostone base and is characterized by a high calcium content (Xiao et al., 2020). The climate type in the region is subtropical monsoon. The wet season occurs from April to September, whereas the dry season lasts from October to March (Liao et al., 2023b). The mean annual temperature is 18.5℃, and the mean annual precipitation is 1234 mm (Liao et al., 2023a).

The experiment was conducted in a hybrid *B. papyrifera* monoculture ecosystem, which had been utilized previously as farmland and subsequently converted into grassland through environmental restoration initiatives. The dominant vegetation species in the study area included *Microstegium vagans*, *Apluda mutica*, and *Imperata cylindrica* (Xiao et al., 2020). In preparation for the experiment, all existing vegetation was cleared and replaced with hybrid *B. papyrifera* in April 2017.

In February 2018, a total of 18 experimental plots were established. Each plot consisted of 20 seedlings of *B. papyrifera*, spaced 30 cm apart in five rows. To ensure isolation and prevent the exchange of nutrients and water between the plots, PVC boards were used to enclose each plot. These PVC boards were 40 cm in height and 3.0 mm in thickness, extending 30 cm into the soil and rising 10 cm above the soil surface. The width of the buffer strip between adjacent plots was 50 cm. The experiment followed a totally randomized block design, with six replicate plots for each of the three treatments: control (CK), mineral N addition (+N), and legume (*Medicago sativa*) intercropping (+L). The total mineral N fertilizer was applied at rates of 20 g N m^{-2} yr^{-1} in the +N plots. Urea was used as the form of mineral N fertilizer. In March 2018, 2019, and 2020, four furrows (5 cm deep) were excavated manually using a shovel between two rows of *B. papyrifera* in the +L plots. Each furrow was then sown with two grams of *M. sativa* seeds acquired from Jiangsu Leerda Seed Industry Co., Ltd (Jiangsu, China). All experimental plots were subjected to the same management practice, including manual weed control without soil disturbance every two months.

2.2. Soil sampling

Soil sampling was conducted on July 5, 2020. Five random soil cores (5 cm in diameter, 0–10 cm in depth) were collected from each plot and

then were mixed to form one composite sample. The sampling places were away from plant roots. The composite sample was then passed through a 2-mm sieve and divided into three subsamples. One subsample was frozen at −80℃ for metagenomic analysis, while another subsample was stored at 4℃ for the determination of soil microbial biomass nitrogen (MBN), net mineralization rate, net nitrification rate, and net ammonification rate. The remaining subsample was air-dried for soil physico-chemical analysis.

2.3. Measurements of soil properties and net N transformation rates

Soil moisture content (SMC) was determined by oven-drying for 12 h at 105℃. Soil pH was measured using a pH meter with a soil-water suspension (1:2.5 w/v). Soil total nitrogen (TN) was measured with a continuous flow analyzer (Skalar, Breda, Netherlands) after Kjeldahl digestion. A continuous flow analyzer was used to determine soil ammonium N (NH_4^+-N) and nitrate N (NO_3^--N) after being extracted in 2 mol L^{-1} KCl. Soil MBN was determined by chloroform fumigation (Brookes et al., 1982, 1985; Wu et al., 1990). Non-fumigated and fumigated soil samples were extracted in 0.5 mol L^{-1} K_2SO_4 for dissolved nitrogen, which was determined using a continuous flow analyzer. MBN was calculated as the difference between nitrogen concentrations in fumigated and non-fumigated extractions with a conversion factor of 0.45.

A 14-day incubation experiment was conducted to determine soil net N mineralization, nitrification and ammonification rates. For each treatment, 50 g of fresh soil samples were put in glass bottles (250 ml) and soil moisture content was adjusted to 60 % of the water holding capacity. All bottles were sealed with polyethylene film to minimize soil moisture loss, while allowing for aerobic conditions through small pores. The glass bottles were then incubated at a constant temperature of 25℃. During the incubation period, soil moisture was maintained at 60 % of the water holding capacity by periodic weighing every three days. The NH_4^+-N and NO_3^--N concentrations of paired subsamples were extracted with 2 mol L^{-1} KCl at day 0 and 14, respectively, and quantified by a continuous flow analyzer. The soil net mineralization rate was calculated as the difference between the sum of NH_4^+-N and NO_3^--N at day 14 and at day 0. The soil net ammonification rate was calculated as the NH_4^+-N concentrations at day 14 minus the NH_4^+-N concentrations at day 0. Similarly, the soil net nitrification rate was calculated by subtracting the NO_3^--N concentrations at day 0 from the NO_3^--N concentrations at day 14.

2.4. Metagenomic analysis

According to the manufacturer's protocol, soil total DNA was extracted from 0.5 g of fresh soil using the PowerSoil spin kit (QIAGEN GmbH, Germany). The extracted DNA samples were then stored at −80℃ for library preparation and shotgun metagenomics sequencing which were finished by the BioMarker Co., Ltd (Qingdao, China). A Qubit 3.0 fluorometer (Thermo Fisher Scientifific, Waltham, MA, USA) combined with Qubit TM dsDNA HS Assay Kit (Thermo Fisher Scientifific, Waltham, MA, USA) was used to quantify the DNA concentrations. Then, the quality of DNA samples was evaluated by 1 % agarose gel. In this study, 10 ng of each DNA sample was used for library construction, following the manufacturer's protocol, using the VAHTS® Universal Plus DNA Library Prep Kit (NEB, USA). The DNA fragments (approximately 350 bp) were generated by sonication. The quality of the constructed libraries was evaluated by using Qsep-400 (Bioptic, Taiwan, China). Finally, high-quality libraries were sequenced on Illumina NovaSeq6000 combined with NovaSeq 6000 S4 Reagent Kit (Illumina, San Diego, CA, USA).

For data processing, Trimmomatic software (version 0.33) was employed to remove low-quality reads using the default settings. Contigs smaller than 300 bp were eliminated using MEGAHIT software. Gene prediction was performed using MetaGeneMark software (version 3.26) with the default settings (Zhu et al., 2010). Non-redundant genes were obtained using MMseq2 (version 11-e1a1c). The gene similarity and coverage cutoff values were set at 97 % and 90 %, respectively. Taxonomic assignment was achieved by blasting the non-redundant genes against the non-redundant protein database (Nr) of NCBI using Diamond software (threshold value with E≤ 1e-5) (Buchfink et al., 2015). Functional annotation was conducted by blasting the non-redundant genes against the KEGG database (Kanehisa et al., 2004). The gene abundances were normalized by dividing the gene length and rarefied to the smallest number of sequences among the metagenomic samples (Qin et al., 2012; Wu et al., 2022). In total, 28 marker genes were selected and classified into various functional groups including ammonification (*ureA*, *ureB* and *ureC*), nitrification (*amoA*, *amoB*, *amoC* and *hao*), denitrification (*narG* , *nirK*, *nirS*, *norB* and *nosZ*), N fixation (*nifD*, *nifH* and *nifK*), nitrogen assimilation (*GDH2*, *gdhA*, *glnA*, *gltB*, *GLU*, *GLUD1_2* and *gudB*), assimilatory nitrate reduction (ANR; *nasA*, *nasB*, *nirA* and *nirB*), and dissimilatory nitrate reduction (DNR; *napA* and *nrfA*).

2.5. Data analysis

Prior to the analysis, we assessed the normal distribution of the data, which were transformed using natural logarithm if they did not meet the assumption of normal distribution. One-way analysis of variance (ANOVA) was performed followed by least significant differences (LSD) test to determine pairwise differences in soil properties, gene abundance, gene diversity, taxonomic diversity and network topological properties between the treatments using the *lsdTest* function in the *PMCMRplus* package. A co-occurrence network analysis was performed to investigate the relationships among taxa involved in N cycling. Only non-redundant genes that were present in at least 75 % of the samples for each treatment were included in constructing the global network. The Spearman correlation matrix was constructed using the *corr.test* function in the *psych* package. The random matrix-based method was used to retain the strong correlations with a Spearman's coefficient $>$ 0.96 and a p-value $<$ 0.05 (false-discovery rate [FDR] adjusted) (Deng et al., 2012). Sub-networks were extracted from the global network using the *induced_subgraph* function in the *igraph* package (Wei et al., 2021). The sub-network topological properties were calculated using the *net_properties.4* function in the *ggClusterNet* package (Wen et al., 2022). The network natural connectivity was calculated by removing nodes in the static network randomly to evaluate the network stability (Wu et al., 2021).

Null model analysis was based on the framework described by Ning et al. to classify community assembly processes into homogeneous selection, heterogeneous selection, dispersal limitation, homogenizing dispersal, and drift (Ning et al., 2020). The analysis was performed using the *qpen* function in the *iCAMP* package. Levins' niche width index was calculated using the *niche.width* function in the *spaa* package to evaluate the niche breadth of N-cycling functional groups (Cui et al., 2023).

Permutational multivariate analysis of variance (PERMANOVA) was carried out to determine the pairwise dissimilarity of soil N-cycling microbial communities between the treatments using the *pairwise.adonis* function in the *pairwiseAdonis* package. Pearson's correlation analysis was conducted to examine the relationships between soil biotic-abiotic variables and functional gene abundance and diversity using the *corr.test* function from the *psych* package (Peng et al., 2022). Mantel test was conducted using the *mantel_test* function (999 permutations) in the *linkET* package to evaluate relationships between N-cycling microbial communities and soil variables (Li et al., 2022). All figures were created using the *ggplot* function in the *ggplot2* package, the *ggbarplot* function in the *ggpubr* package, and Adobe Illustrator CS6. All statistical analyses were conducted in R version 4.2.1.

3. Results

3.1. Soil properties and nitrogen transformation rates

Compared to the control (CK), mineral N fertilization (+N) and legume intercropping (+L) slightly increased soil pH, NH_4^+-N, MBN, and net ammonification rate by 1.1−1.8 %, 13.1−14.2 %, 3.8−17.7 %, and 5.8−28.3 %, respectively (Fig. S1). Soil NO_3^--N increased by 119−167 % in the +N treatment compared to the CK and +L treatments ($p < 0.05$, Fig. S1). The soil net mineralization rate and net nitrification rate exhibited a decrease in the +N treatment compared to the CK treatment ($p < 0.05$, Fig. S1). Legume intercropping marginally reduced SMC by 10.2 % compared to the +N treatment ($p = 0.074$, Fig. S1).

3.2. Soil N-cycling microbial community structure and taxonomic and functional diversity

The PERMANOVA analysis indicated a marginal difference in the community structure of N fixation-related microbes between the +N and +L treatments ($R^2 = 0.143$, $p = 0.080$, Table S1). Compared to the CK treatment, the diversity of the nitrification-related genes and the whole functional genes increased in the +N treatment ($p < 0.05$, Fig. 1). The taxonomic and functional diversity of microbes related to nitrification was greater in the +N treatment than in the +L treatment ($p < 0.05$, Fig. 1 and Fig. S2).

3.3. Abundances of soil N-cycling functional genes

The abundance of the *hao* gene related to nitrification was 10.4-fold higher in the +N treatment (mean = 5.2) compared to the CK (mean = 0.5) and +L treatments (mean = 0.5) ($p < 0.05$, Fig. 2). In the +L treatment, the abundances of *ureA* gene associated with ammonification ($p < 0.05$) and *nirA* gene related to ANR ($p = 0.076$) were reduced compared to the CK treatment (Fig. 2). Compared to the CK and +L treatments, the abundances of genes (*nifD* and *nifH*) related to N fixation were reduced by 33.3−56.0 % in the +N treatment (Fig. 2). The abundance of *nifK* gene involved in N fixation was 3.7-fold higher in the +L treatment (mean = 5.5) than in the CK treatment (mean = 1.5) ($p = 0.10$, Fig. 2). Mineral N fertilization and legume intercropping slightly increased the abundances of denitrification- (e.g., *narG*, *nirS*, *norB* and *nosZ*) and DNR-related genes by 5.1−210 % and 16.7−56.6 %, respectively (Fig. 2).

3.4. Assembly of soil N-cycling microbial communities and the key abiotic factors affecting taxonomic and functional structure of soil N-cycling microbes

The assembly of the soil N-cycling microbial communities was dominated by both stochastic (i.e., dispersal limitation, homogenizing dispersal, and drift) and deterministic (i.e., homogeneous and heterogeneous selection) processes, with varying relative importance among functional groups (Fig. 3). A relatively high proportion of homogeneous selection contributed to the assembly of microbial communities involved in ammonification (59.6−65.4 %), ANR (80.0−84.6 %), and denitrification (53.8−58.5 %) among treatments (Fig. 3). Stochastic processes played a dominant role in shaping the communities related to nitrification, DNR, N fixation, and N assimilation (Fig. 3). Mineral N addition increased the relative importance of drift (58.5 % vs. 51.9 %) and dispersal limitation (35.4 % vs. 15.4 %) on the community assembly of nitrification-related microbes compared to the CK treatment (Fig. 3). Additionally, compared to the CK treatment, the relative importance of dispersal limitation (50.8 % vs. 46.2 %) and homogenizing dispersal (15.4 % vs. 9.6 %) on the assembly of assimilation-related microbial communities was increased in the +N treatment (Fig. 3). Legume intercropping increased the relative importance of heterogeneous selection on the assembly of microbial communities involved in N fixation (3.8 % vs. 0.0 %) and N assimilation (15.4 % vs. 5.8 %) compared to the CK treatment (Fig. 3).

Soil pH, TN and MBN were significant deterministic factors controlling the structure of the N-cycling functional taxa (Fig. 4a). Additionally, soil pH, TN, NH_4^+-N and MBN were important factors influencing the structure of functional genes associated with ammonification, DNR, and N assimilation (Fig. 4b). Pearson's correlation analysis showed that higher soil pH and TN facilitated microbial N

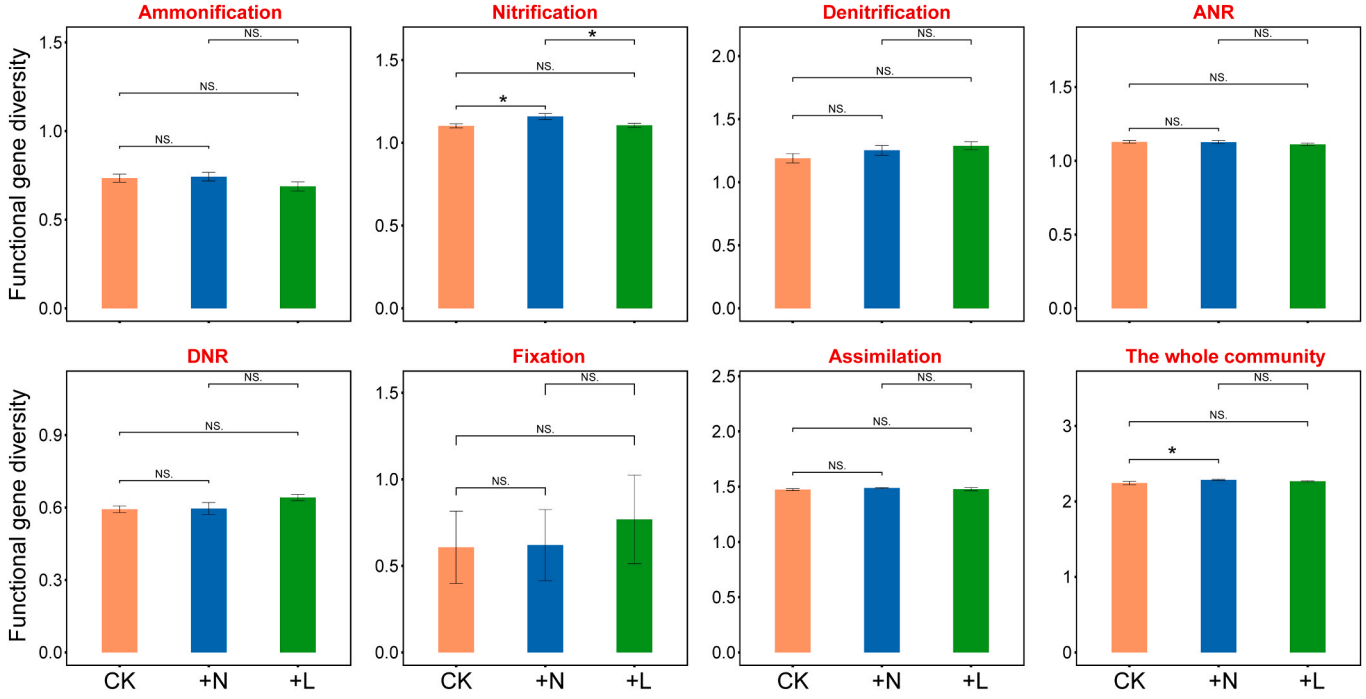

Fig. 1. Diversity of soil N-cycling functional genes as affected by different N management practices. ANR, assimilatory nitrate reduction; DNR, dissimilatory nitrate reduction; CK, control; +N, mineral nitrogen addition; +L, legume intercropping. Bars show means ± SE. NS., non-significant ($p > 0.10$); *$p < 0.05$.

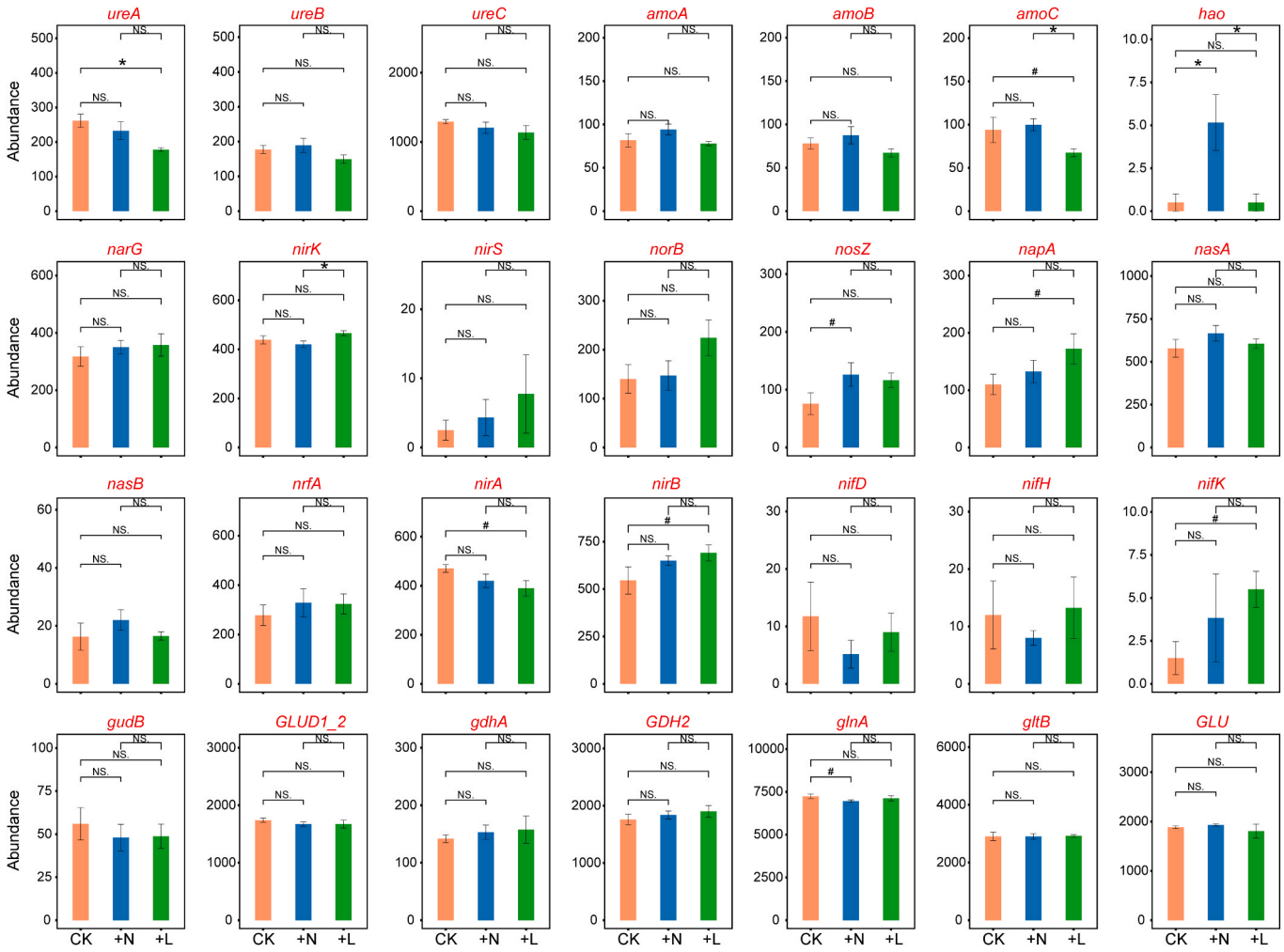

Fig. 2. Responses of soil N-cycling functional genes to different N management practices. CK, control; +N, mineral nitrogen addition; +L, legume intercropping. Bars show means ± SE. NS., non-significant ($p > 0.10$); #$0.05 \leq p \leq 0.10$; *$p < 0.05$.

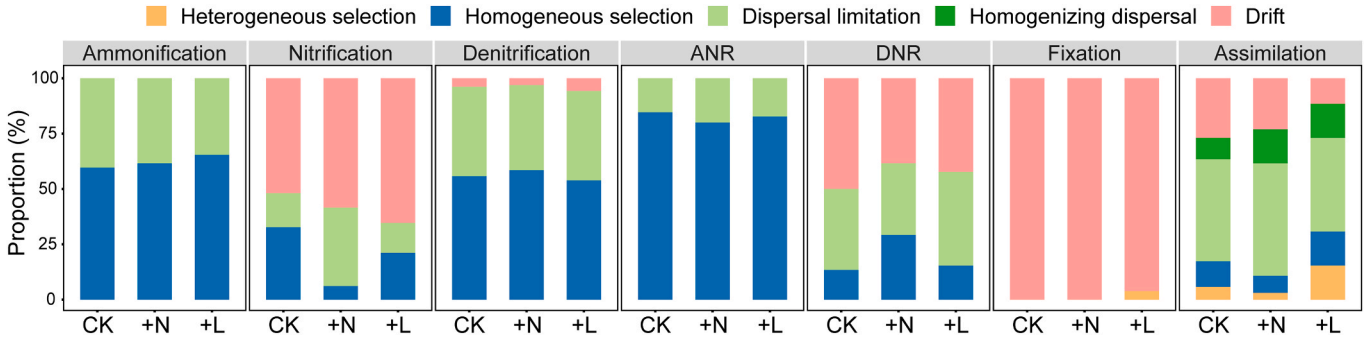

Fig. 3. Assembly processes of soil N-cycling microbial communities as affected by different N management practices. ANR, assimilatory nitrate reduction; DNR, dissimilatory nitrate reduction; CK, control; +N, mineral nitrogen addition; +L, legume intercropping.

assimilation ($p < 0.05$, Fig. 4a, b). Moreover, soil pH and TN were positively related to the abundances of genes related to DNR (*nrfA*) and assimilation (*GDH2*) ($p < 0.05$, Fig. 4c). However, TN was negatively correlated with the abundances of genes involved in soil microbial N fixation ($p < 0.05$, Fig. 4c). Increases in the abundances of *napA* and *nrfA* genes associated with DNR significantly enhanced the soil net ammonification rate ($p < 0.05$, Fig. 4c). In contrast, high abundance of genes related to N fixation as well as N ammonification corresponded with reduced net ammonification rate ($p < 0.05$, Fig. 4c).

3.5. Niche breadth of soil N-cycling microbes and co-occurrence network relationships among N-cycling taxa

Niche breadth of the whole N-cycling microbial community was greater in the +N and +L treatments compared to the CK ($p < 0.001$, Fig. 5). Mineral N addition increased the niche breadth of functional groups except the N fixation group ($p < 0.001$, Fig. 5). Legume intercropping increased the breadth of functional groups related to denitrification, ANR, DNR, and N assimilation ($p < 0.01$, Fig. 5), but reduced

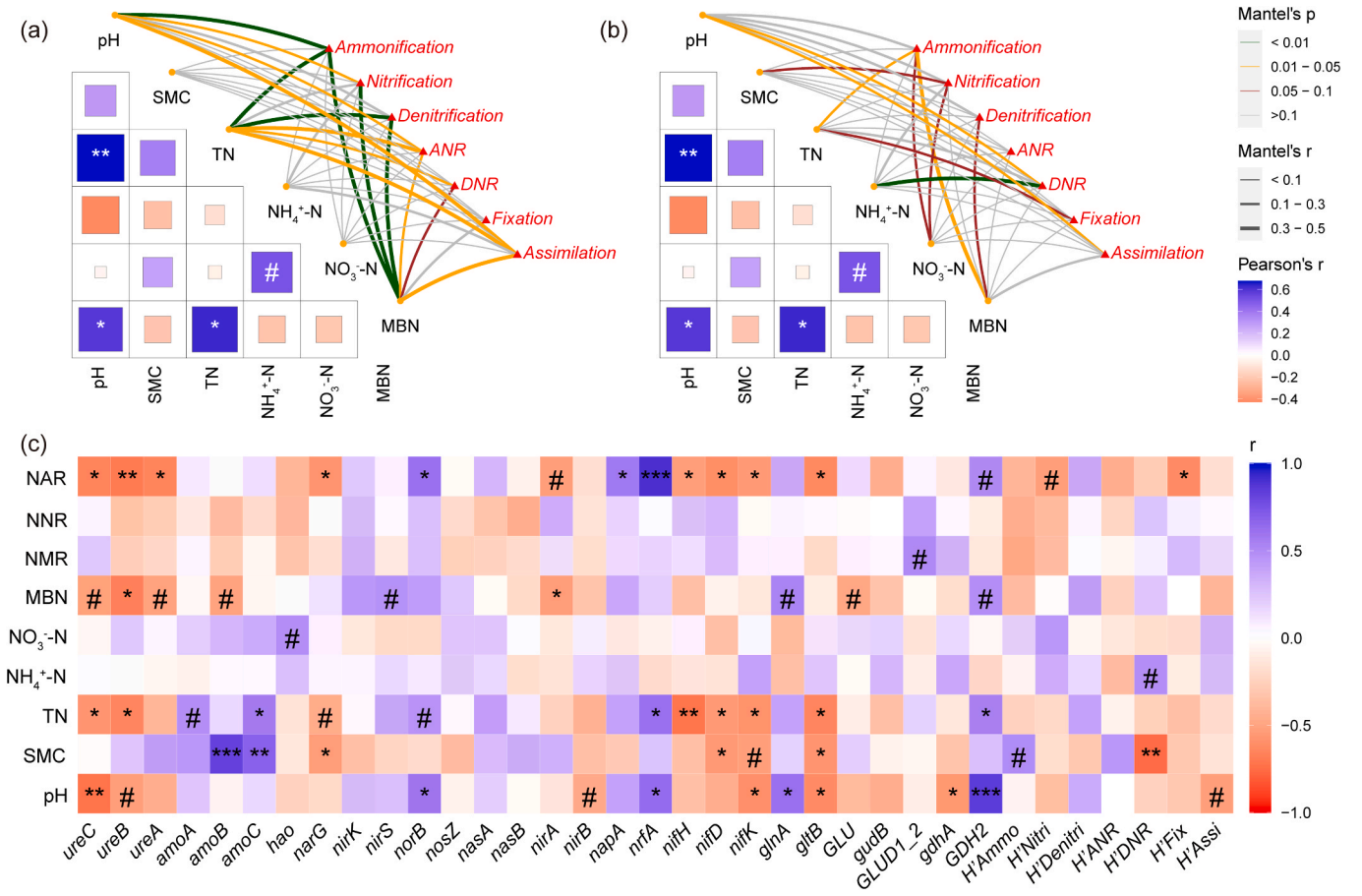

Fig. 4. Correlations between soil factors and soil N-cycling microbial communities and functional genes. The taxonomic (a) and functional (b) structure based on Bray-Curtis distance is related to each soil factor by partial Mantel test. The thickness of the lines indicates the strength of the partial Mantel's r statistic, while the color represents the statistical significance based on 999 permutations. Pairwise comparisons of soil factors are displayed with a color gradient, representing the Pearson's correlation coefficient. (c) Pearson's correlation analysis on the relationships between soil factors and functional genes. The correlation coefficient values are displayed with a color gradient. Significant correlations are indicated by symbols (#$0.05 \leq p \leq 0.10$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). pH, soil pH; SMC, soil moisture content; TN, soil total nitrogen; NH_4^+-N, soil ammonium nitrogen; NO_3^--N, soil nitrate nitrogen; MBN, microbial biomass nitrogen; NMR, net mineralization rate; NNR, net nitrification rate; NAR, net ammonification rate; ANR, assimilatory nitrate reduction; DNR, dissimilatory nitrate reduction; H'Ammo, Shannon diversity of genes related to ammonification; H'Nitri, Shannon diversity of genes related to nitrification; H'Denitri, Shannon diversity of genes related to denitrification; H'DNR, Shannon diversity of genes related to dissimilatory nitrate reduction; H'ANR, Shannon diversity of genes related to assimilatory nitrate reduction; H'Fix, Shannon diversity of genes related to nitrogen fixation; H'Assi, Shannon diversity of genes related to nitrogen assimilation.

the niche breadth of communities related to ammonification ($p < 0.05$, Fig. 5). Overall, the niche breadth of the whole N-cycling microbial community and all functional groups except the N-fixation group were greater in the +N treatment than in the +L treatment ($p < 0.001$, Fig. 5).

The co-occurrence network analysis revealed that Actinobacteria related to N assimilation dominated the networks (Fig. 6a, c). Compared to the CK, the complexity (i.e., nodes, edges and average degrees) of the co-occurrence networks was reduced under the two N fertilization practices, especially in the +N treatment ($p < 0.01$, Fig. 6b). The natural connectivity (i.e., robustness) of networks was reduced to a greater degree in the CK (slope = −41.4) than in the +N (slope = −18.4) and +L (slope = −28.5) treatments by removing the same proportion of nodes (Fig. 6d).

4. Discussion

4.1. Soil pH and TN altered the taxonomic and functional structure of soil N-cycling microbes and their N assimilation potential

Soil bacteria play a dominant role in mediating soil N cycling (Sun et al., 2021). Their communities are very sensitive to changes in soil pH and N levels (Geisseler and Scow, 2014; Fan et al., 2018). However, there were no significant changes in soil pH, TN and NH_4^+-N under the two N fertilization practices in our study. Previous studies have confirmed that both soil microbial nitrification and N fixation can result in soil acidification (Bolan et al., 1991; Wang et al., 2023c). In contrast, soil pH tended to slightly increase in the present study which may be explained by the buffering capacity offered by high calcium content in karst soil (Chen et al., 2023). Generally, urea rapidly dissolves and its' availability quickly decreases within 60 d (Wang et al., 2023a). However, soil sampling was conducted about 120 d after mineral N addition. Some available N resources (particularly NH_4^+-N) are rapidly utilized by plants and microbes, while the unexploited N resources are lost via leaching or gas emission (Dan et al., 2023). In addition, karst soil has a high pH which can drive NH_4^+-N reduction through NH_3 volatilization and nitrification (Rochette et al., 2013; Wang et al., 2023a). Nevertheless, soil pH and TN were key factors affecting the taxonomic and functional structure of soil N-cycling microbes, which is partially consistent with the first hypothesis. In return, shifts in N-cycling functional groups can affect soil N availability (Zhu et al., 2023).

It is well known that soil microbial nitrification and N fixation play an important role in mediating soil N availability (Frey et al., 2023). Mineral N addition can stimulate nitrification potential in karst soil (Yang et al., 2023). Our results suggested that the abundances of genes

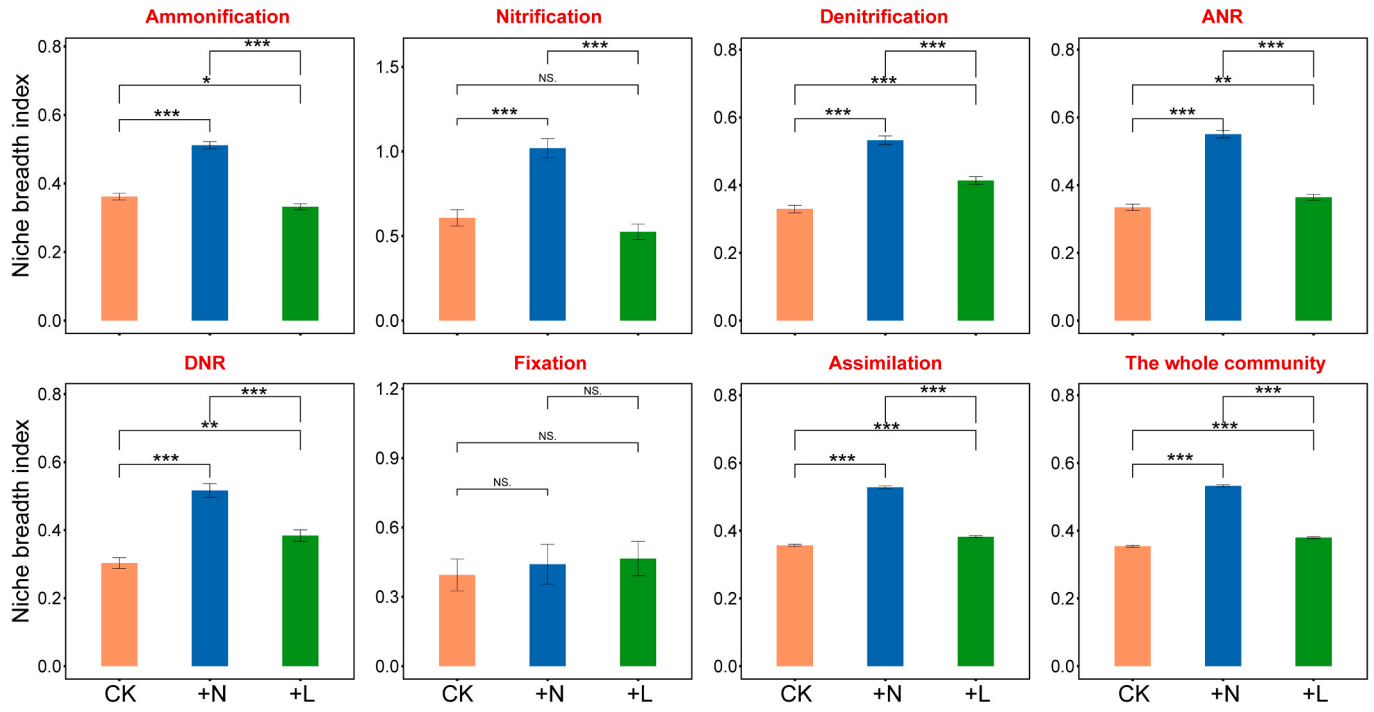

Fig. 5. Niche breadth of N-cycling functional groups and the whole community as affected by different N management practices. ANR, assimilatory nitrate reduction; DNR, dissimilatory nitrate reduction; CK, control; +N, mineral nitrogen addition; +L, legume intercropping. Bars show means ± SE. NS., non-significant ($p > 0.10$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

related to nitrification (except *hao*) did not significantly increase after mineral N addition because mineral N addition-disturbed microbial communities can rapidly return to the previous state (Geisseler and Scow, 2014). However, the taxonomic and functional diversity of nitrification-related microbes and nitrification potential (e.g., *amoA*, *amoC* and *hao*) were greater in the mineral N addition treatment than in the legume intercropping treatment. This can at least partially explain why soil NO_3^--N content increased in the mineral N addition treatment more than in the legume intercropping treatment. In our study, legume intercropping marginally increased microbial N fixation potential (e.g., *nifK*), which is an alternative pathway of increasing soil N availability (Dynarski and Houlton, 2018; Smercina et al., 2019). The fixed N by root-rhizobia symbiont can be transferred to soil as labile N (e.g., ammonium and amino acid) via root exudation, which can be directly exploited by microbes (Paynel et al., 2001; Lesuffleur et al., 2007; Moe, 2013). However, a meta-analysis study suggests that mineral N addition suppresses biological N fixation potential (Zheng et al., 2019). In the current study, there were significantly negative correlations between the abundances of N-fixation-related genes and soil TN content, despite the fact that soil TN only slightly increased with mineral N addition due to plant and microbial uptake, leaching or gas emission during a short period of time. It implies that N fertilization-induced increase in soil TN content may have a long-lasting negative effect on soil biological N fixation potential. These results suggest that there are distinct strategies to increase soil N availability between mineral N addition and legume intercropping. With soil N availability increasing, soil microbes gain access to more N resources, leading to more soil microbial biomass N accumulation (Elrys et al., 2023). Especially in karst soil, high soil NO_3^--N content stimulates microbial N assimilation (Zhu et al., 2023). This is why soil MBN and the abundance of *GDH2* related to N assimilation increased with higher soil pH and greater TN in our study.

4.2. Community assembly, ecological niche breadth and co-occurrence network of soil N-cycling microbes as affected by different N management practices

Partially consistent with the second hypothesis, the assembly of soil microbial communities involved in nitrification, DNR, N fixation, and N assimilation was dominated by stochastic processes. This finding contrasts with previous studies that deterministic processes dominate the assembly of microbial communities related to N fixation, and nitrification in acidic and saline soils (Fan et al., 2018; Li et al., 2021; Liu et al., 2022). Instead, soil pH levels in our study were in the neutral range of 7.1–7.2, which can alleviate environmental selection pressure (Fan et al., 2018). Regardless, the relative importance of stochastic assembly processes in microbes related to nitrification and N assimilation increased with mineral N addition, which is consistent with previous evidence that the importance of stochastic assembly processes is stimulated with N addition (Zhou et al., 2022). Nonetheless, the importance of deterministic assembly processes on these functional groups should not be overlooked. This phenomenon explains why the structure of microbial communities involved in nitrification, DNR, N fixation, and N assimilation was positively correlated with soil pH and TN. Deterministic assembly processes played a dominant role in shaping the communities associated with ammonification, denitrification, and ANR. Both soil pH and TN were key abiotic factors affecting the structure of microbes involved in ammonification, denitrification and ANR.

Consistent with the second hypothesis, mineral N addition and legume intercropping increased the ecological niche breadth of the whole N-cycling microbial community. Compared to the legume intercropping treatment, mineral N addition had a greater impact on the niche breadth of the whole N-cycling microbial community and all N-cycling functional groups except the N fixation-related group. Generally, microbial communities with wide niche breadth are more adaptable to environmental shifts (Jiao et al., 2020; Cui et al., 2023). Furthermore, wider niche breadth allows more taxa to coexist in agroecosystems, leading to an increase in the stability of co-occurrence networks (Jiao et al., 2020). N fertilization practices can supplement N resources for

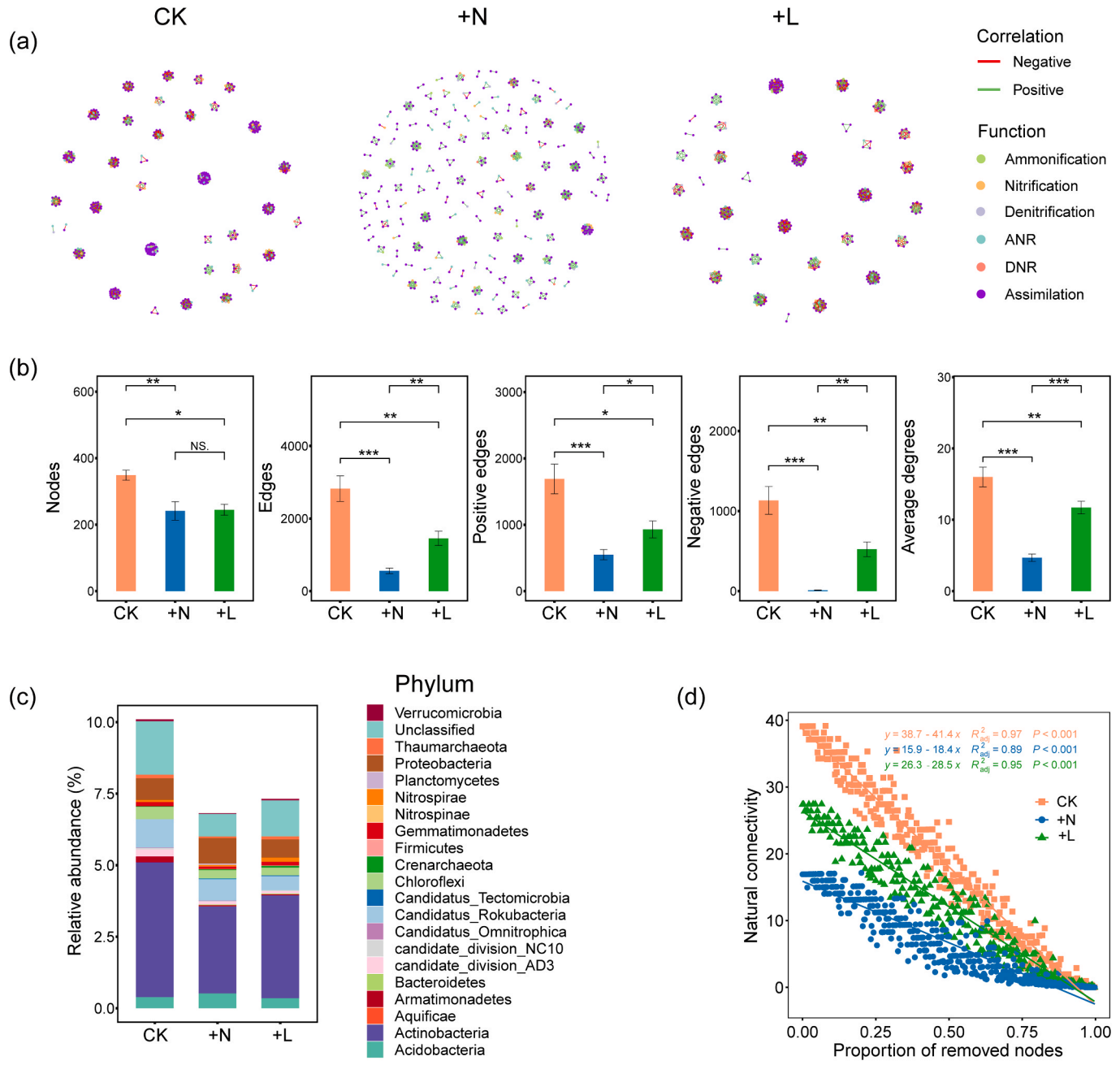

Fig. 6. Co-occurrence networks and robustness analysis for soil N-cycling microbial communities. (a) Global co-occurrence networks of N-cycling functional taxa. Red and green lines indicate significantly negative and positive correlations, respectively. ANR, assimilatory nitrate reduction; DNR, dissimilatory nitrate reduction. (b) Pairwise comparison of topological properties of the sub-networks between the treatments. Bars show means ± SE. NS., non-significant ($p > 0.10$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (c) The relative abundance of taxa involved in the network construction. (d) Robustness analysis on the relationships between natural connectivity and the proportion of removed nodes. Reduced robustness or stability within co-occurrence networks is indicated by a sharp decline in natural connectivity at the same percentage of node removal. CK, control; +N, mineral nitrogen addition; +L, legume intercropping.

microbial growth and metabolism, which facilitates reduction of both interspecific and intraspecific competition for limited available N resources (Liu et al., 2022). Likewise, the two N fertilization regimes both increased network stability with node removal compared to the control. Microbial taxa involved in N assimilation dominated the co-occurrence networks establishing strong positive relationships with other taxa, indicating both a potential collaboration with other functional taxa in N assimilation and resistance to environmental stresses. In the karst region, extreme climate events (e.g., drought) occur frequently (Zhang et al., 2019), which can lead to a reduction in soil microbial diversity, especially for prokaryotic microbes (de Vries et al., 2018; Preece et al., 2019). Thus, optimal N fertilization practices facilitate to maintain the stability of soil N-cycling microbial communities and N-cycling function in the karst agroecosystem.

5. Conclusions

Mineral N addition and legume intercropping altered the taxonomic and functional structure of soil N-cycling microbial communities in a karst forage ecosystem. Compared to the legume intercropping, mineral N addition presented a much stronger influence on taxonomic and functional diversity of nitrification-related microbes. The potential of

soil nitrate transformation (e.g., *hao*) increased with mineral N addition, resulting in greater soil NO_3^--N content. Legume intercropping facilitated an increase in soil N availability by increasing microbial N fixation potential (e.g., *nifK*). Soil N-cycling microbial community assembly was co-driven by both stochastic and deterministic processes. Soil pH and TN were the deterministic factors influencing the taxonomic and functional structure of soil N-cycling microbes and their N transformation potentials. Interestingly, high soil pH and TN stimulated microbial N assimilation, which facilitates to increase soil N retention in a karst agroecosystem. The two N fertilization practices could support wider niche breadth of soil N-cycling microbes and maintain the stability of co-occurrence networks, which benefits to improve resistance of soil N-cycling microbial communities to the elimination of some species. The current study provides insights into how mineral N addition and legume intercropping affect the taxonomic and functional structure of soil N-cycling microbial communities and underlying community assembly processes and co-occurrence patterns in a short period of time. In the future, long-term monitoring of functional groups related to nitrification, N fixation, and N assimilation will be required in conjunction with stable isotope labelling techniques to effectively manage soil N availability in karst agroecosystems.

CRediT authorship contribution statement

JIE ZHAO: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Jiangnan Li:** Investigation. **Jiachen Wang:** Writing – review & editing. **Wei Zhang:** Writing – review & editing. **Jun Xiao:** Writing – review & editing, Funding acquisition. **Dan Xiao:** Writing – review & editing. **Peilei Hu:** Writing – review & editing. **Kelin Wang:** Writing – review & editing, Project administration, Funding acquisition. **Tiangang Tang:** Data curation, Writing – review & editing. **Deborah A. Neher:** Writing – review & editing. **Xionghui Liao:** Writing – original draft, Visualization, Software, Methodology, Investigation, Funding acquisition, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

We appreciate greatly the Institutional Center for Shared Technologies and Facilities of Institute of Subtropical Agriculture, CAS, for providing supports to analyze soil properties. This study was supported by the National Key R&D Program of China (No. 2022YFD1901000); the Joint Funds of the Natural Science Foundation of China (Nos. U21A20189 and U23A20155); the Postdoctoral Fellowship Program of CPSF (No. GZB20230831); the National Natural Science Foundations of China (Nos. 42377284 and 42007432); the Science and Technology Innovation Program of Hunan Province (Nos. 2023RC1076 and 2022RC1016); the Guangxi Bagui Young Scholars Special Fund given to Jie Zhao; and the Natural Science of Foundation of Hunan Province (No. 2022JJ40536).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2024.109177.

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