SOIL ECOLOGY LETTERS

SEL RESEARCH ARTICLE
Volume 6 / Issue 1 / 230188 / 2024
https://doi.org/10.1007/s42832-023-0188-0

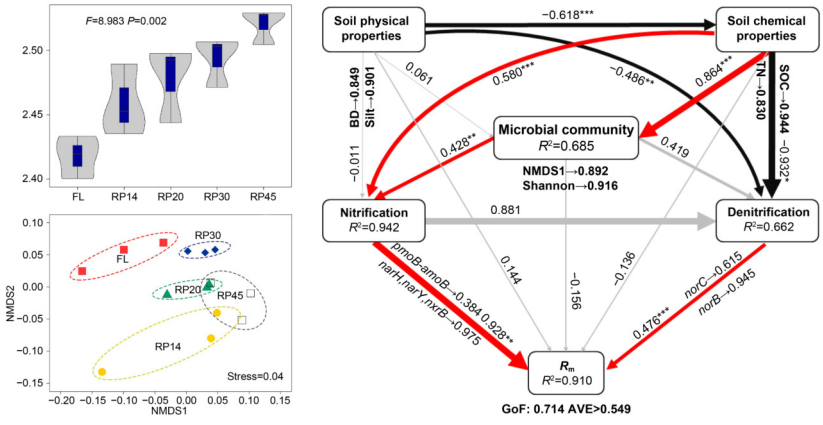
N-cycle gene abundance determination of N mineralization rate following re-afforestation in the Loess Plateau of China

Yaping Zhao[1,2], Yuqing Zhao[1,2], Shuohong Zhang[1,2], Yulin Xu[1,2], Xinhui Han[1,2], Gaihe Yang[1,2], Chengjie Ren[1,2,*]

1 College of Agronomy, Northwest A&F University, Yangling 712100, China
2 Shaanxi Engineering Research Center of Circular Agriculture, College of Agronomy, Northwest A&F University, Yangling 712100, China
* Corresponding author. E-mail: Rencj1991@nwsuaf.edu.cn (C. Ren)
Received January 11, 2023; Revised May 22, 2023; Accepted June 8, 2023

ABSTRACT

• Afforestation effectively improved soil microbial communities and significantly increased soil nitrogen mineralization rate (R_m).
• Soil microorganisms drive R_m by regulating soil N-cycling genes.
• Soil nitrification genes had a major effect on soil R_m than denitrification genes after afforestation.



Assessing the function of forest ecosystems requires an understanding of the mechanism of soil nitrogen mineralization. However, it remains unclear how soil N-cycling genes drive soil nitrogen mineralization during afforestation. In this study, we collected soil samples from a chrono-sequence of 14, 20, 30, and 45 years of *Robinia pseudoacacia* L. (RP14, RP20, RP30, and RP45) with a sloped farmland (FL) as a control. Through metagenomic sequencing analysis, we found significant changes in the diversity and composition of soil microbial communities involved in N-cycling along the afforestation time series, with afforestation effectively increasing the diversity (both alpha and beta diversity) of soil microbial communities. We conducted indoor culture experiments and analyzed correlations, which revealed a significant increase in both soil nitrification rate (R_n) and soil nitrogen mineralization rate (R_m) with increasing stand age. Furthermore, we found a strong correlation between soil R_m and soil microbial diversity (both alpha and beta diversity) and with the abundance of soil N-cycling genes. Partial least squares path modeling (PLS-PM) analysis showed that nitrification genes (*narH*,*narY*,*nxrB*, *narG*,*narZ*,*nxrA*, *hao*, *pmoC-amoC*) and denitrification genes (*norB*, *nosZ*, *nirK*) had a greater direct effect on soil R_m compared to their effect on soil microbial communities. Our results reveal the relationships between soil nitrogen mineralization rate and soil microbial communities and between the mineralization rate and functional genes involved in N-cycling, in the context of *Robinia pseudoacacia* L. restoration on the Loess Plateau. This study enriches the understanding of the effects of microorganisms on soil nitrogen mineralization rate during afforestation and provides a new theoretical basis for evaluating soil nitrogen mineralization mechanisms during forest succession.

Keywords afforestation, soil N mineralization rate, denitrification genes, nitrification genes, the Loess Plateau

1 Introduction

Soil nitrogen (N) is the main limitation factor of net primary productivity in terrestrial ecosystems (LeBauer and Treseder, 2008), and approximately 88% of global plant N requirements come from soil (Li et al., 2017). Soil N availability affects plant growth and productivity, thereby affecting terrestrial ecosystem functions and changing ecosystem processes (Li et al., 2014; Li et al., 2019a). Afforestation involves introducing trees into an area previously used for agriculture, this practice enhances net primary productivity and reduces soil disturbance (Cheng et al., 2013; Li et al., 2014), which in turn leads to improvement of soil N conversion and availability (Chen et al., 2009; Gurlevik and Karatepe, 2016; Li et al., 2018). Moreover, any fluctuation in soil available N can impact ecosystem functions (Li et al., 2014), and soil nitrogen mineralization and nitrogen potential strongly respond to vegetation succession and restoration (Grunzweig et al., 2003; Gelfand and Yakir, 2008). Therefore, exploring the mechanism of soil nitrogen mineralization plays an important role in assessing the direction of forest succession and

forest ecosystem functions.

During the past several decades, the global change research community has made considerable efforts to explore drivers of soil nitrogen mineralization. Some examples of research relate to seasonal precipitation (Chen et al., 2017), the inputs of litter (Corbin and D'Antonio, 2011), soil physicochemical properties (Booth et al., 2005; Li et al., 2014), soil microbial community (Ding and Wang, 2021; Kuypers et al., 2018), and soil microbial biomass (McMillan et al., 2007; Ren et al., 2016; Li et al., 2019a). However, the response of plantation ecosystems to these factors varies. It is known that land use affects the transformation of soil nitrogen (Attard et al., 2011; Domeignoz-Horta et al., 2018; Tang et al., 2019). Land-use legacies, particularly soil N and P concentration, have been shown in previous studies to affect the composition and activity of soil microbial communities (de la Peña et al., 2016). However, recent research suggests that the transformation of land-use patterns can also influence the composition of vegetation and soil properties, thereby impacting the structure of the soil microbial community including N-cycling microbes (Zhang and Lv, 2021; Wang et al., 2023). Functional genes of soil microorganisms are related to the nitrogen cycle and are affected by land use changes (Xu et al., 2019). Research has shown that the abundances of nitrification and denitrification genes decrease with the transition from paddy fields to orchards due to different preferences of N-cycling genes for oxygen supply (Wu et al., 2017). In contrast, in forest ecosystems, the abundances of nitrification and denitrification genes significantly increases with the development of forest chronosequences (Sun and Badgley, 2019). These studies only focus on changes in the microbial community compositions of nitrification and denitrification during land use changes, and pay less attention to the nitrogen mineralization rate changes caused by soil N-cycling microbes. The relationships between nitrification and denitrification functional gene changes and soil nitrogen mineralization rate, due to differences in soil characteristics in the context of afforestation, are not clear. This uncertainty brings huge uncertainty regarding the transformation of soil nitrogen and the availability of nitrogen in terrestrial ecosystems.

The study focused on the Loess Plateau as the research location, where soil samples were gathered from *Robinia pseudoacacia* L. and from farmland sites, with chronosequences of 14, 20, 30, and 45 years. Metagenomics sequencing was utilized to investigate the properties of soil microbial communities and the functional genes of the nitrogen cycle. Through the combination of an indoor mineralization cultivation experiment and a partial least squares analysis model, the relationship between soil mineralization rate and the microbial community, as well as functional genes, in the context of forestland restoration, was explored. Based on the analyses conducted, one hypothesis was confirmed: Nitrification and denitrification genes have direct impacts on soil nitrogen mineralization during the farmland-to-forest restoration process on the Loess Plateau.

2 Materials and methods

2.1 Study site and sampling

Our experimental site, the Wuliwan watershed, is located in the Ansai region of northern Shaanxi, China (36°51′–36°52′ N, 109°20′–109°21′ E). It is in the middle of the Loess Plateau, with an average annual precipitation of 510 mm and an average annual temperature of 8.8°C, belonging to a semi-arid climate. According to the world soil type distribution map (Selcer, 2015), the area is characterized by highly erodible Calcic Cambisols. Since the 1970s, most of the land in this area has been planted with trees to avoid soil erosion. The main crops before the afforestation were maize (*Zea mays*) and millet (*Setaria italica*), and no fertilization or irrigation were applied during the growth of the plantation.

We collected samples during the plant growth period in August 2019. In this study, four recovery stages (14 a, 20 a, 30 a, 45 a) of *Robinia pseudoacacia* L. were used as reference (Xu et al., 2021), and three replicates of 20 m × 30 m plots were set for each recovery stage. At the same time, the selected site provided cultivated land, with traditional cultivation techniques, as the control group. All plots were essentially the same in elevation, slope, and soil texture, the only difference being different reafforestation years. After the litter layer was completely removed, soil samples from 0 to 10 cm were collected using a stainless-steel sampler with a diameter of 5 cm. Ten soil cores were harvested, and mixed into a composite sample, from each plot. The soil samples were then divided into two subsamples after removal of visible plant roots, stone, litter and debris. One of the subsamples was stored at −80°C for DNA sequencing analysis for microbial biomass. Another sample was air-dried for physical and chemical analysis.

2.2 Analysis of soil physicochemical properties

Soil moisture contents were determined by gravimetric analysis. Soil bulk density (BD) was calculated by dividing the dry soil mass by the core volume after drying the soil at 105°C for 24 h, following the method of (De Vos et al., 2005). The soil pH was determined with a pH meter (PHS-3C, Shanghai, China) in a 1:2.5 soil-water mixture. We used the percentage of clay (< 2 μm) and silt (50–2 μm) to describe the soil particle size distribution, which was measured by a Malvern MS 2000 (Malvern Instruments, Malvern, UK) (Jin et al., 2013). Soil organic carbon (SOC) was measured by the dichromate oxidation method. Total nitrogen (TN) was extracted by Kjeldahl digestion previously described (Zhang

et al., 2011). Total inorganic nitrogen (TIN) in soil was established using the hydrometer method as described by Yanu and Jakmunee (2015). Soil Ammonium nitrogen (NH_4^+) and nitrate nitrogen (NO_3^-) were determined by using a continuous flow analyzer (AutoAnalyzer-AA3, Seal Analytical, Norderstedt, Germany) after extraction with 2 mol L^{-1} KCl. The chloroform fumigation extraction method was utilized to measure the soil microbial biomass nitrogen (MBN). Instruments used in these procedures included a hydrometer for the texture analysis, an oven for drying the soil, and a fumigation chamber for the MBN measurement.

2.3 Data calculation

A subset of fresh soil samples were adjusted to 60% water-holding capacity (on a 10 g dry soil basis) was extracted with 50 mL KCl (2 mol L^{-1}) solution to determine the initial dissolved inorganic N concentrations. Another subset of fresh samples was incubated in the dark at 25°C for one week. The water content of the soil was maintained at 60% water-holding capacity by a regular supply of deionized water during incubation. Soil net N ammonization, nitrification and mineralization rates were calculated as the changes in NH_4^+-N, NO_3^--N and total inorganic N (NH_4^+-N + NO_3^--N) concentrations before and after incubation. The potential net mineralization rates were calculated as the changes in inorganic N (NH_4^+, NO_3^-) before and after incubation.

For an interval of time, $\Delta t = t_{i+1} - t_i$

$$R_a = [c(NH_4^+)_{i+1} - c(NH_4^+)_i] / \Delta t$$

$$R_n = [c(NO_3^-)_{i+1} - c(NO_3^-)_i] / \Delta t$$

$$R_m = R_a + R_n$$

Where t_i and t_{i+1} are the initial and end dates, $c\left(NH_4^+\right)_i$ and $c\left(NH_4^+\right)_{i+1}$ are the concentrations of ammonization before and after incubation, respectively. $c\left(NO_3^-\right)_i$ and $c\left(NO_3^-\right)_{i+1}$ are the concentrations of ammonization before and after incubation, respectively. R_a, R_n and R_m are net N ammonization, net N nitrification and mineralization rates, respectively.

2.4 DNA extraction and sequencing

Soil DNA was extracted in triplicate from 0.5 g of fresh soil sample using the FastDNA spin kit for soil (MP Biomedicals), following the manufacturer's instructions. The DNA quality and integrity of the samples were assessed with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Three replicates on each soil sample were carried out to ensure adequate representation of forest soil and to obtain sufficient DNA for shotgun metagenomic sequencing. The metagenome libraries were sequenced on an Illumina HiSeq 2000, generating 150 bp paired end reads at a greatersequencing depth. Readings that mapped to the human genome were eliminated, and the lengths were trimmed using Sickle. All DNA sequencing can be found on the National Center for Biotechnology Information (NCBI) website.

2.5 Metagenomics analysis

To improve the reliability and quality of subsequent analysis, the sequencing reads were filtered as previously described (Zhang et al., 2017), which included removal of the adapter sequences, trimming the reads, and discarding the quality-trimmer reads that were below 50 bp or containing N (ambiguous bases). Cutadapt (v1.2.1) was used to remove sequencing adapters from sequencing reads (Martin, 2011). For the utilization of high-quality reads, the megahit software (Li et al., 2015) was used to assemble the mixed sequence of all samples, and thus a larger database of contigs and scaffolds were generated. The MetaGeneMark was used to predict the genes in the contigs (longer than 200 bp), and the per-base coverage depth across all contigs was calculated by mapping raw reads from each sample (Zhu et al., 2010). In accordance with the KEGG database, the functional annotation and taxonomic assignment from each sample were obtained for further analysis.

2.6 Statistical analyses

The microbial community diversity index (Shannon) and non-metric multidimensional scaling (NMDS) were used to analyze microbial community structures among different samples via the “vegan” and “picante” package in R (version 4.2.0). Heatmaps for Spearman correlation analysis were plotted using the “pheatmap” package in R. To examine differences between means, we performed one-way ANOVA using SPSS version 26.0 (SPSS Inc., Chicago, USA). The least significant difference (LSD) was computed to examine differences between mean values at a value of $P < 0.05$. Correlations between soil N-cycle genes and soil properties were determined using redundancy analysis (RDA) with CANOCO version 5.0.

In this study, we utilized a partial least squares path analysis model (PLS-PM) to investigate the interactions among soil properties, microbial communities, soil N-cycle genes, and soil mineralization rates, in the context of afforestation restoration. To avoid issues with internal collinearity of latent variables, we removed factors with a strong collinearity (VIF < 3) during the screening process. Next, we removed variables with low internal load coefficients of the latent variables, based on the model fitting results. We used the loading coefficient between the latent variables and the observable variables to determine the relative contribution of each

observable variable to the latent variables (Table S4).

In the model, soil physical properties include soil bulk density (BD) and soil texture (silt and clay). Soil chemical properties include soil organic carbon (SOC) and soil total nitrogen (TN). The soil microbial community is represented by the Shannon index and NMDS1. Nitrification is represented by gene abundances of *pmoB-amoB* and *narH,narY, nxrB*, while denitrification is represented by *norB* and *norC* abundances. Using R-4.0.5, the plspm algorithm of the "gastonstat" package was used to construct the PLS-PM. We constructed the best model for path analysis by selecting the above indicators (Fig. 4, see below). The path coefficient represents the linear relationship between the various latent variables. The size of the path coefficient reflects the direction of its relationship. R^2 reflects the interpretation rate of other variables to the latent variable. The goodness of fit (GoF > 0.4) and the average variance (AVE > 0.5) are listed below. Random forest analysis was used with the "tidyverse" package in R to screen the genes that determine R_m.

3 Result

3.1 Changes in soil properties and N mineralization rate following afforestation

Along the entire afforestation chrono-sequence, BD, Clay, and pH of sample RP45 are comparable to those of FL, with ranges from 1.28 to 1.06 g cm^{-3}, from 14.59% to 18.15%, and from 8.44 to 8.35, respectively. Compared with the FL, the SOC, TN, C/N of RP45 were significantly higher by 159.4%, 69.2% and 47.2%, respectively. Similarly, soil NH_4^+, NO_3^-, TIN (total inorganic nitrogen) and MBN increased along the afforestation chrono-sequence ($P < 0.05$, Table 1). Soil R_n and R_m also significantly increased with increasing stand age ($P < 0.05$, Table 1), whereas soil R_a was lower at the 30- and 45-year sites, compared with other sites.

3.2 Changes in soil microbial N-cycling genes following afforestation

Soil microbial diversity (alpha and beta diversity) involved in N-cycling significantly changed along the afforestation chrono-sequence ($P < 0.05$, Fig. 1). Specifically, significant increase in alpha diversity of N-cycle genes was observed with increasing stand age (Fig. 1A). The NMDS plot (Fig. 1B) indicated that the weighted UniFrac distance between soil N-cycling microbial communities in different age stands was well separated, and that there was a relationship between the relative abundance of each stand age and time. The distribution of the nitrogen-cycling microbial communities at the 45- and 20-year sites tended to group together, while all the afforested sites were clearly separated from the farmland sites.

The functional microbes involved in N-cycling varied with afforestation stage (Fig. 2 and Fig. S1). For nitrification pathway, six functional genes (*hao, pmoA-amoA, pmoB-amoB, pmoC-amoC, narG,narZ,nxrA* and *narH,narY,nxrB*) were

Table 1 The soil physical-chemistry properties in the different ages after afforestation.

Sample characteristics	Farmland (FL)	*Robinia pseudoacacia* L. 14 (RP14)	*Robinia pseudoacacia* L. 20 (RP20)	*Robinia pseudoacacia* L. 30 (RP30)	*Robinia pseudoacacia* L. 45 (RP45)	*P
BD(g cm^{-3})	1.28 ± 0.03a	1.21 ± 0.04a	1.10 ± 0.11a	1.16 ± 0.05a	1.06 ± 0.06a	0.210
Clay (0−2μm)	14.59 ± 0.41b	19.16 ± 0.35a	18.43 ± 0.66a	18.60 ± 0.36a	18.15 ± 0.68a	**0.001**
Silt	43.74 ± 0.09b	48.47 ± 0.87a	42.75 ± 1.52bc	41.23 ± 0.80bc	40.64 ± 0.28c	**0.001**
SM	12.47 ± 0.14ab	13.57 ± 0.64a	12.10 ± 0.33b	11.94 ± 0.46b	12.75 ± 0.42ab	0.134
pH	8.44 ± 0.02a	8.44 ± 0.02a	8.42 ± 0.04a	8.42 ± 0.04a	8.35 ± 0.07a	0.567
SOC (g kg^{-1})	2.98 ± 0.07d	4.30 ± 0.19c	4.74 ± 0.30c	5.84 ± 0.40b	7.73 ± 0.32a	**0.000**
TN (g kg^{-1})	0.26 ± 0.02b	0.42 ± 0.05a	0.53 ± 0.05a	0.51 ± 0.04a	0.44 ± 0.01a	**0.005**
C/N	11.86 ± 1.26b	10.46 ± 0.94b	9.06 ± 0.83b	11.66 ± 1.19b	17.46 ± 1.00a	**0.002**
NH_4^+ (mg kg^{-1})	1.73 ± 0.08c	2.14 ± 0.10c	3.45 ± 0.15b	3.60 ± 0.21b	4.62 ± 0.32a	**0.000**
NO_3^- (mg kg^{-1})	2.89 ± 0.21b	2.75 ± 0.20b	2.84 ± 0.29b	2.58 ± 0.17b	4.40 ± 0.43a	**0.005**
TIN (mg kg^{-1})	4.62 ± 0.19c	4.89 ± 0.14c	6.29 ± 0.44b	6.18 ± 0.20b	9.01 ± 0.33a	**0.000**
MBN (mg kg^{-1})	7.39 ± 1.36c	16.24 ± 1.85c	33.13 ± 4.33b	46.43 ± 7.91b	68.00 ± 3.65a	**0.000**
R_a (mg N kg^{-1}soil d^{-1})	0.16 ± 0.012a	0.09 ± 0.01ab	0.11 ± 0.04ab	0.08 ± 0.02b	0.06 ± 0.01b	0.085
R_n (mg N kg^{-1}soil d^{-1})	0.07 ± 0.01c	0.22 ± 0.03b	0.43 ± 0.05a	0.54 ± 0.02a	0.57 ± 0.07a	**0.000**
R_m (mg N kg^{-1}soil d^{-1})	0.23 ± 0.02d	0.27 ± 0.03cd	0.42 ± 0.05bc	0.56 ± 0.04ab	0.61 ± 0.08a	**0.001**

BD, bulk density; SM, soil moisture; SOC, total soil organic C; TN, total N; C/N, the ratio of SOC and TN; TIN, total inorganic nitrogen; MBN, microbial biomass nitrogen; R_a, net ammonification rate; R_n, net nitrification rate; R_m, net mineralization rate; All data are presented as mean ± SD (n = 3). *P values are based on ANOVA. Different letters indicate significant ($P < 0.05$) differences among treatments. Numbers in bold font indicate significant changes*.

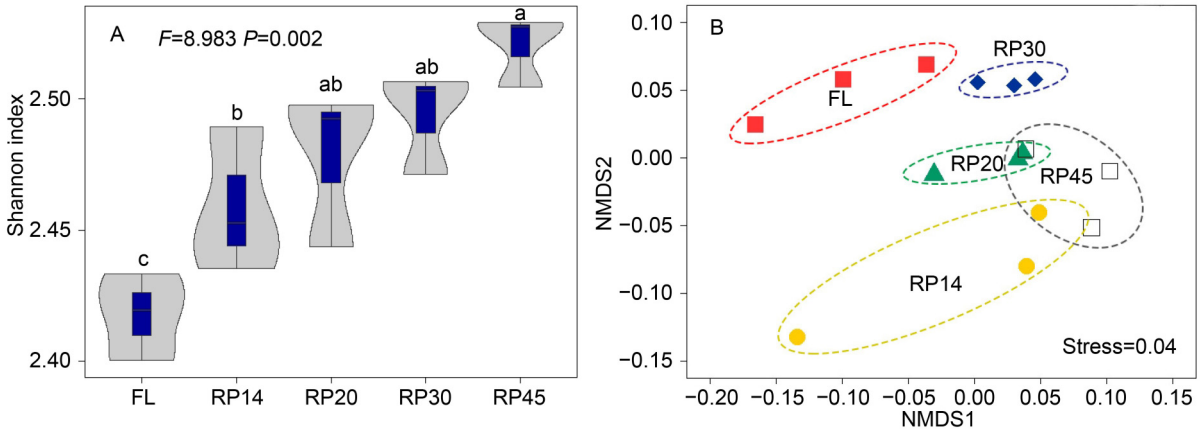


Fig. 1 Diversity of microbial N-cycling potential. (A) Variations of microbial alpha diversity along the years after afforestation. (B) Beta diversities, based on the Bray-Curtis distances. FL: farmland; RP14, RP20, RP30, and RP45 represent that the *Robinia pseudoacacia* plantations had been restored for approximately 14-, 20-, 30-, and 45-year, respectively.

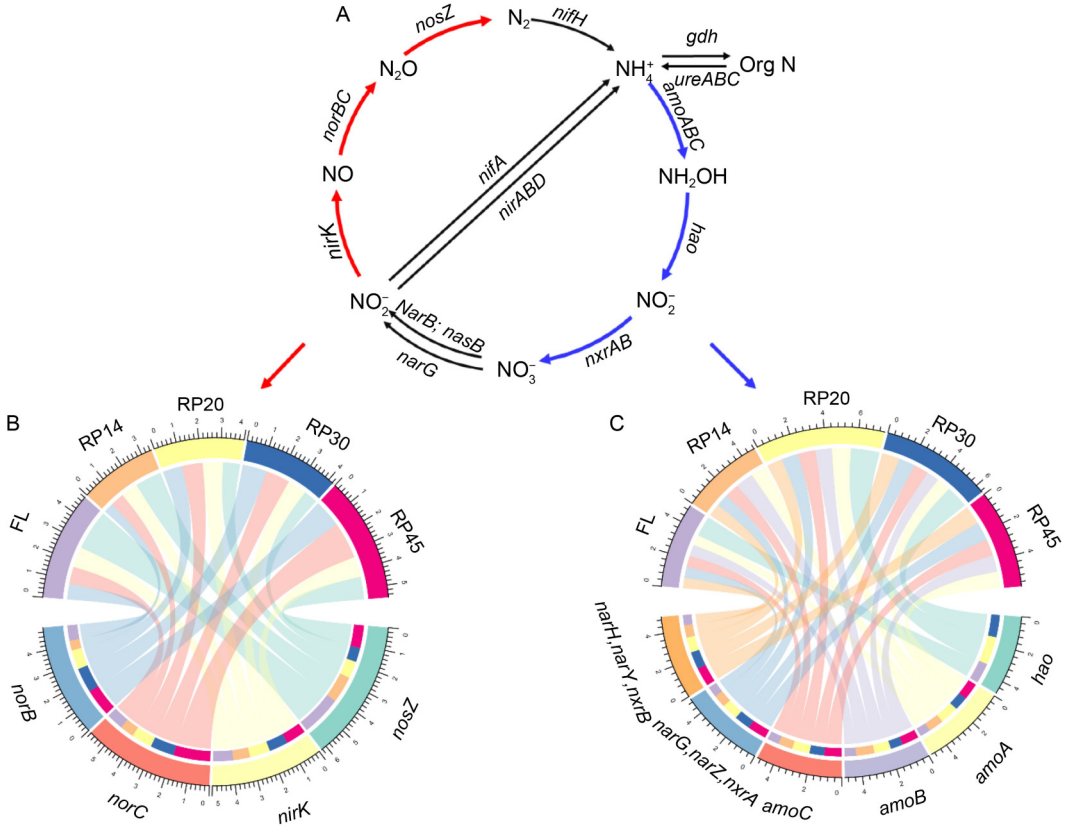


Fig. 2 Conceptual diagrams show the key microbial functional genes involved in the nitrogen cycle. Microbial nitrogen-transforming gene level (A); changes in denitrification main functional genes at different treatment levels (B); changes in nitrification main functional genes at different treatment levels (C).

detected. The abundance of all genes except *pmoC-amoC* significantly increased after afforestation ($P < 0.05$, Table S5). In particular, the *narG,narZ,nxrA* and *narH,narY,nxrB* exhibited a dominant status and were more common in afforested lands than in farmland (Fig. 2C); these dominant genes significantly increased as the stand age increased ($P < 0.05$, Table S5). As for denitrification pathway, the dominant genes were *nosZ, nirK, norC,* and *norB*; the *norB* at the 45-year sites was significantly increased, by 79.35% in excess of the farmland level ($P < 0.05$, Table S5 and Fig. 2B).

3.3 Effects of changes in soil properties and N-cycle genes abundance on soil mineralization rates

Both Pearson correlation and regression analysis revealed significant correlations between the microbial N-cycling

genes abundance and soil net mineralization rate ($P < 0.05$, Fig. 3, Fig. S2). Specifically, the microbial diversity indices, nitrification and denitrification genes abundance were all positively correlated with R_n and R_m, but were negatively correlated with the ammoniation rate. The PLS–PM analysis (GoF = 0.727) was also used to identify the relationships between the soil properties, related functional microbial community structure, nitrification genes abundance, denitrification genes abundance and the rate of soil mineralization (Fig. 4 and Tables S2−S4). The results revealed that microbial metabolic pathways (i.e., denitrification and nitrification processes) explained 91.0% of the total change in soil mineralization rate (Fig. 4A). The results showed that soil microbial community had an indirect effect on soil mineralization rate through influencing nitrification (path coefficient = 0.428) and denitrification process (path coefficient = 0.419; Fig. 4A); all considered associations are shown in Table S2. Furthermore, the random forest analysis showed that denitrification genes abundance (*norB*, *norZ* and *nirK*) and nitrification genes abundance (*narH,narY,nxrB*, *narG,narZ,nxrA*, *hao*, *pmoC-amoC*) were the most important drivers for soil R_m across forested biomes (Fig. 4B). The relationship between soil physicochemical variables (i.e., MBN, clay, silt, TIN, SOC, TN, C/N, BD, NO_3^- and pH) and soil nitrogen functional genes abundance were examined based on redundancy analysis (Table S1, Fig. S3). The results showed that soil physicochemical variables significantly affected soil nitrogen functional gene abundance (explaining together 72.65% of the total variation), especially for MBN and clay ($P < 0.01$; Table S1). After screening with a random forest model, NH_4^+ was identified as the property with the greatest impact on soil mineralization rate (Fig. S4).

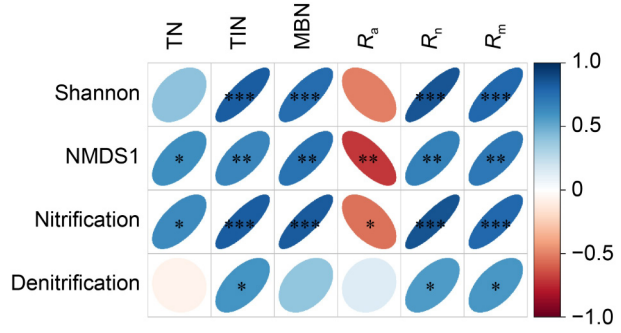


Fig. 3 Linkages between diversity and soil N status. The Pearson's rank correlation coefficients for the diversity indices (Alpha and NMDS) with the measured soil chemical characteristics. TN, total N; TIN, total inorganic nitrogen; MBN, microbial biomass nitrogen; R_a, R_n and R_m are net N ammonization, net N nitrification and mineralization rates, respectively. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

4 Discussion

Through metagenomics analysis, our study investigated the contributions of microbial N-cycle genes to N mineralization rates, while accounting for multiple soil properties. Our study

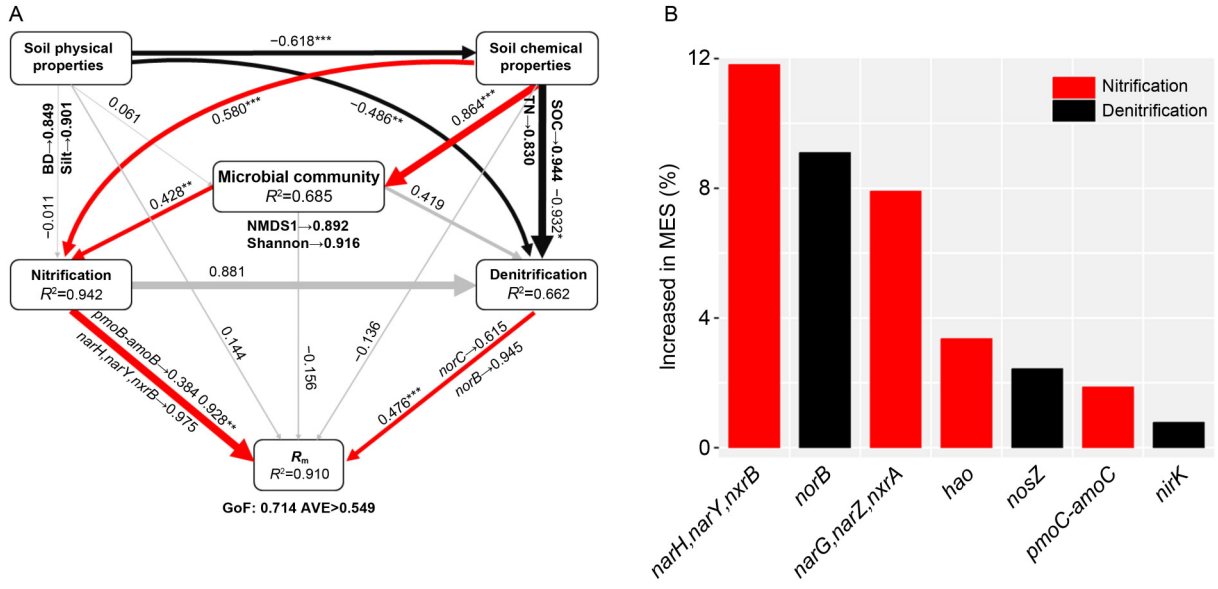


Fig. 4 Partial least squares path model (PLS-PM). (A) Each box in the schematics represents latent variables. Soil physical properties are latent variables that include soil bulk density (g cm^{-3}) and soil texture (Silt, %); while soil chemical properties include total nitrogen (TN) and soil organic carbon (SOC); The bacterial community involved in N-cycling can be analyzed using the Shannon diversity index and NMDS1; nitrification including the gene of *pomB-amoB* and *narH,narY,nxrB* gene abundance; denitrification is a latent variable, including *norB* and *norC* gene abundance. Larger path coefficients are reflected by the width of the arrow, with red solid lines indicating a significantly positive effect, black solid lines indicating a significantly negative effect, and gray lines indicating that the effect was not significant. *$P \leqslant 0.05$, **$P \leqslant 0.01$, or ***$P \leqslant 0.001$. (B) shows the significant ($P < 0.05$) gene of soil R_m, identified by random forest analysis.

highlights the importance of microbial N-cycle genes in determining the N mineralization rate of afforested ecosystems. Given the importance of N availability for plant productivity (Li et al., 2019b; Ding and Wang, 2021), our finding provides a scientific framework about soil N transformation in afforested areas.

4.1 Changes of soil microbial diversity and functional genes

Our results showed that soil microbial community alpha diversity increased with stand age, corresponding to changes in soil properties, such as MBN and clay content. The increased carbon input from plant sources after afforestation can provide soil microorganisms with materials for growth and metabolism, resulting in enhanced microbial diversity (Li et al., 2018). According to the study by Deng et al. (2016), afforestation has the potential to increase soil microbial biomass, leading to improved nitrogen fixation and retention. This, in turn, can influence the composition of the soil microbial community and enhance microbial nitrogen metabolism. In addition, clay affects soil water-holding capacity and the total volume of water-filled pores (Rakhsh et al., 2020), which can increase soil permeability that is a factor in microbial growth, leading to changes in soil microbial composition.

Our study found that the abundance of soil nitrification and denitrification genes gradually increased with the increasing age of forests, consistent with previous research findings (Deng et al., 2019; Zhang and Lv, 2021), illustrating that an increase in SOC contents could promote the abundance of N-cycle functional genes after afforestation restoration. The findings of Wu et al. (2017) differ from our findings, which reported that converting rice paddy land to citrus orchard resulted in an increase in the abundance of ammonia-oxidizing archaea (AOA), had no significant effect on ammonia-oxidizing bacteria (AOB) abundance, and led to a decrease in the abundance of *nirK, nirS,* and *nosZ*. The differences found in this study can be explained by the following two considerations. First, difference in tree species could lead to difference in soil microbial structure and function. In our study, *Robinia pseudoacacia* is a leguminous woody plant with strong nitrogen-fixing ability, which can replenish the soil nitrogen pool through the decomposition of litter or root systems. This process may result in the enhancement of soil available nitrogen and a reduction in nitrogen loss and N_2O emission (Canfield et al., 2010). A previous study by Wu et al. (2017) observed a significant increase in microbial N-cycle genes in *Robinia pseudoacacia* forests compared to non-nitrogen-fixing tree species such as rice and citrus.

Additionally, Wang et al. (2019) found that climatic factors, such as temperature and moisture, may be the primary drivers of soil N-cycle changes after land use conversion, based on their research in the red soil hilly regions of the mid-subtropical China. That region is characterized by abundant water and heat resources, which can result in a hypoxic state that inhibits microbial metabolism. A similar phenomenon can also be seen in other studies. Seasonal heavy rainfall can cause the soil water content to be high, gradually reducing the soil's nitrogen supply capacity (Chen et al., 2009). However, in the Loess Plateau, limited water availability directly affects the activity of microorganisms (He et al., 2016). Afforestation may promote changes in water absorption, enhancing the metabolic intensity of microorganisms and promoting the utilization of nitrogen (Rong et al., 2021).

4.2 Changes in microbial N-cycle genes abundance and soil properties regulate R_m

PLS-PM analysis revealed that the soil microbial community did not directly determine R_m but had a significant and indirect effect on the mineralization rate through influencing the microbial nitrification and denitrification process. This indicates that soil microbes drive the soil N-cycle after afforestation. Such a conclusion is consistent with a previous study (Zhang et al., 2018), who found that both R_m and R_n were influenced by microbes under prescribed burning conditions in forests. In particular, in the current study, soil nitrification genes had a major effect on soil R_m compared to denitrification genes after afforestation. These findings are consistent with previous studies that highlighted the importance of nitrification as a key process in the terrestrial nitrogen cycle, and that showed that the changes in the nitrifying bacteria community have a greater impact on soil nitrification than other nitrogen cycle processes (Seneca et al., 2020).

Chen et al. (2021) and Yoon et al. (2014) provided additional explanations on this topic. They suggested that an increase in the C/N ratio could lead to a reduction in denitrification, whereas a high soil C/N ratio may facilitate nitrification. Furthermore, they found that at pH levels above 7.0, ammonium formation could be favored during incubation. Therefore, the significantly increased C/N and pH values greater than 7 (Table 1) after afforestation on the Loess Plateau provided favorable conditions for soil nitrification, thereby enhancing the effect of nitrification genes on R_m. However, our findings contradict a previous study that suggested denitrification is responsible for a larger proportion of N_2O production (Banerjee et al., 2018; Kuypers et al., 2018). These differences may be influenced by two factors. On the one hand, denitrification is known to be more favorable in warm and humid anaerobic conditions, while forest restoration in arid and semi-arid ecosystems may slow down N_2O emission rates and weaken denitrification (Deng et al., 2019). On the other hand, it has been observed that denitrifi-

cation is limited where nitrate nitrogen fixation is restricted by carbon availability. With increased exogenous carbon input after afforestation, nitrate nitrogen fixation becomes more significant than denitrification (Chen et al., 2021). Additionally, due to the high C:N caused by afforestation, the denitrification rate in nitrogen-limited soil is reduced (Chen et al., 2021), resulting in a weaker effect of denitrification genes on R_m compared to nitrification genes. Overall, differences in climate and soil environment can lead to diverse responses of N-cycling genes to R_m. Our study further highlights the importance of microbial functional traits in vegetation restoration in arid and semi-arid ecosystems.

We further aimed to establish a link between specific N-cycling-driven species and N mineralization rates. The random forest analysis, a computational technique, revealed changes in several abundant genes after afforestation, exhibiting different responses to soil R_m. In particular, the presence of numerous well-established microbial nitrification genes, such as *narH,narY,nxrB*, *narG,narZ,nxrA*, *hao*, *pmoC-amoC*, and denitrification genes *norB*, *nosZ*, *nirK,* was identified as the dominant factor influencing soil mineralization rate. Among these, the relative abundance of nitrification genes *narH,narY,nxrB* emerged as the most significant determinant, confirming their crucial role in the soil N cycling. The *narH,narY,nxrB* genes encode nitrite oxidoreductases responsible for the oxidization of nitrite to nitrate. The *norB* gene encodes nitric oxide reductase, which catalyzes the reduction of NO to N_2O (Wang et al., 2021). Therefore, we speculate that the increase in organic matter and soil total nitrogen resulting from afforestation may stimulate the activities of nitrite oxidase and nitric oxide reductase, thereby enhancing N-NO_3^- leaching and N_2O production, and consequently accelerating the soil nitrogen mineralization rate.

5 Conclusion

Based on metagenomic sequencing, laboratory culture, and partial least squares methods, this study unveils the significance of soil nitrification and denitrification functional genes in regulating the availability of soil nitrogen during the process of re-afforestation on the Loess Plateau. The findings demonstrate, following the afforestation, that microorganisms influence soil R_m by modulating nitrogen cycle-related genes, with nitrification genes exerting a stronger direct impact on soil R_m than denitrification genes. In conclusion, alterations in soil N-cycling functional genes can have a profound influence on ecosystem processes via changes in soil R_m during afforestation and vegetation restoration, thereby advancing our current understanding of the role of soil N-cycling functional genes in mineralization rates during vegetation restoration.

Declaration of competing interest

The authors declare no competing financial interest.

Acknowledgments

This work were financially supported by the National Natural Science Foundation of China (No. 41907031), the China Postdoctoral Science Foundation (No. 2021T140565) and the China Postdoctoral Science Foundation (No. 2019M650276).

Electronic supplementary material

Supplementary material is available in the online version of this article at https://doi.org/10.1007/s42832-023-0188-0 and is accessible for authorized users.

References

Attard, E., Recous, S., Chabbi, A., De Berranger, C., Guillaumaud, N., Labreuche, J., Philippot, L., Schmid, B., Le Roux, X., 2011. Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. Global Change Biology 17, 1975–1989.

Banerjee, S., Schlaeppi, K., van der Heijden, M.G.A., 2018. Keystone taxa as drivers of microbiome structure and functioning. Nature Reviews. Microbiology 16, 567–576.

Booth, M.S., Stark, J.M., Rastetter, E., 2005. Controls on nitrogen cycling in terrestrial ecosystems: A synthetic analysis of literature data. Ecological Monographs 75, 139–157.

Canfield, D.E., Glazer, A.N., Falkowski, P.G., 2010. The evolution and future of Earth's nitrogen cycle. Science 330, 192–196.

Chen, F.S., Zeng, D.H., Fahey, T.J., 2009. Changes in soil nitrogen availability due to stand development and management practices on semi-arid sandy lands, in northern China. Land Degradation & Development 20, 481–491.

Chen, J., Xiao, G.L., Kuzyakov, Y.K., Jenerette, G.D., Ma, Y., Liu, W., Wang, Z.F., Shen, W.J., 2017. Soil nitrogen transformation responses to seasonal precipitation changes are regulated by changes in functional microbial abundance in a subtropical forest. Biogeosciences 14, 2513–2525.

Chen, Z., Tu, X., Meng, H., Chen, C., Chen, Y., Elrys, A.S., Cheng, Y., Zhang, J., Cai, Z., 2021. Microbial process-oriented understanding of stimulation of soil N_2O emission following the input of organic materials. Environmental Pollution 284, 117176.

Cheng, X.L., Yang, Y.H., Li, M., Dou, X.L., Zhang, Q.F., 2013. The impact of agricultural land use changes on soil organic carbon dynamics in the Danjiangkou Reservoir area of China. Plant and Soil 366, 415–424.

Corbin, J.D., D'Antonio, C.M., 2011. Abundance and productivity mediate invader effects on nitrogen dynamics in a California grassland. Ecosphere 2, 1–20.

de la Peña, E., Baeten, L., Steel, H., Viaene, N., De Sutter, N., De Schrijver, A., Verheyen, K., 2016. Beyond plant–soil feedbacks: mechanisms driving plant community shifts due to land-use legacies in post-agricultural forests. Functional Ecology 30,

1073–1085.

De Vos, B., Van Meirvenne, M., Quataert, P., Deckers, J., Muys, B., 2005. Predictive quality of pedotransfer functions for estimating bulk density of forest soils. Soil Science Society of America Journal 69, 500–510.

Deng, N., Wang, H., Hu, S., Jiao, J., 2019. Effects of afforestation restoration on soil potential N_2O emission and denitrifying bacteria after farmland abandonment in the Chinese Loess Plateau. Frontiers in Microbiology 10, 262.

Deng, Q., Cheng, X., Hui, D., Zhang, Q., Li, M., Zhang, Q., 2016. Soil microbial community and its interaction with soil carbon and nitrogen dynamics following afforestation in central China. Science of the Total Environment 541, 230–237.

Ding, L.L., Wang, P.C., 2021. Afforestation suppresses soil nitrogen availability and soil multifunctionality on a subtropical grassland. Science of the Total Environment 761, 143663.

Domeignoz-Horta, L.A., Philippot, L., Peyrard, C., Bru, D., Breuil, M. C., Bizouard, F., Justes, E., Mary, B., Leonard, J., Spor, A., 2018. Peaks of in situ N_2O emissions are influenced by N_2O-producing and reducing microbial communities across arable soils. Global Change Biology 24, 360–370.

Gelfand, I., Yakir, D., 2008. Influence of nitrite accumulation in association with seasonal patterns and mineralization of soil nitrogen in a semi-arid pine forest. Soil Biology & Biochemistry 40, 415–424.

Grunzweig, J.M., Lin, T., Rotenberg, E., Schwartz, A., Yakir, D., 2003. Carbon sequestration in arid-land forest. Global Change Biology 9, 791–799.

Gurlevik, N., Karatepe, Y., 2016. Long-term effects of afforestation on soil characteristics and net nitrogen mineralization in sandy soils. Austrian Journal of Forest Science 133, 187–202.

He, F., Tang, M., Zhong, S.L., Yang, R., Huang, L., Zhang, H.Q., 2016. Effects of soil and climatic factors on arbuscular mycorrhizal fungi in rhizosphere soil under *Robinia pseudoacacia* in the Loess Plateau, China. European Journal of Soil Science 67, 847–856.

Jin, Z., Dong, Y., Qi, Y., Liu, W., An, Z., 2013. Characterizing variations in soil particle-size distribution along a grass-desert shrub transition in the Ordos Plateau of Inner Mongolia, China. Land Degradation & Development 24, 141–146.

Kuypers, M.M.M., Marchant, H.K., Kartal, B., 2018. The microbial nitrogen-cycling network. Nature Reviews. Microbiology 16, 263–276.

LeBauer, D.S., Treseder, K.K., 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 89, 371–379.

Li, D., Liu, C.M., Luo, R., Sadakane, K., Lam, T.W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics (Oxford, England) 31, 1674–1676.

Li, D., Wen, L., Zhang, W., Yang, L., Xiao, K., Chen, H., Wang, K., 2017. Afforestation effects on soil organic carbon and nitrogen pools modulated by lithology. Forest Ecology and Management 400, 85–92.

Li, M., Zhou, X., Zhang, Q., Cheng, X., 2014. Consequences of afforestation for soil nitrogen dynamics in central China. Agriculture, Ecosystems & Environment 183, 40–46.

Li, X.J., Yang, H.T., Shi, W.L., Li, Y.F., Guo, Q., 2018. Afforestation with xerophytic shrubs accelerates soil net nitrogen nitrification and mineralization in the Tengger Desert, Northern China. Catena 169, 11–20.

Li, X.K., Li, Y.A., Peng, S.Z., Chen, Y.M., Cao, Y., 2019a. Changes in soil phosphorus and its influencing factors following afforestation in Northern China. Land Degradation & Development 30, 1655–1666.

Li, Z.L., Tian, D.S., Wang, B.X., Wang, J.S., Wang, S., Chen, H.Y. H., Xu, X.F., Wang, C.H., He, N.P., Niu, S.L., 2019b. Microbes drive global soil nitrogen mineralization and availability. Global Change Biology 25, 1078–1088.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet Journal 17, 10.

McMillan, R., Quideau, S.A., MacKenzie, M.D., Biryukova, O., 2007. Nitrogen mineralization and microbial activity in oil sands reclaimed boreal forest soils. Journal of Environmental Quality 36, 1470–1478.

Rakhsh, F., Golchin, A., Beheshti Al Agha, A., Nelson, P.N., 2020. Mineralization of organic carbon and formation of microbial biomass in soil: Effects of clay content and composition and the mechanisms involved. Soil Biology & Biochemistry 151, 108036.

Ren, C.J., Zhao, F.Z., Kang, D., Yang, G.H., Han, X.H., Tong, X.G., Feng, Y.Z., Ren, G.X., 2016. Linkages of C:N:P stoichiometry and bacterial community in soil following afforestation of former farmland. Forest Ecology and Management 376, 59–66.

Rong, G., Zhang, X., Wu, H., Ge, N., Yao, Y., Wei, X., 2021. Changes in soil organic carbon and nitrogen mineralization and their temperature sensitivity in response to afforestation across China's Loess Plateau. Catena 202, 105226.

Selcer, P., 2015. Fabricating unity: The FAO-UNESCO soil map of the world. Historical Social Research-Historische Sozialforschung 40, 174–201.

Seneca, J., Pjevac, P., Canarini, A., Herbold, C.W., Zioutis, C., Dietrich, M., Simon, E., Prommer, J., Bahn, M., Potsch, E.M., Wagner, M., Wanek, W., Richter, A., 2020. Composition and activity of nitrifier communities in soil are unresponsive to elevated temperature and CO_2, but strongly affected by drought. ISME Journal 14, 3038–3053.

Sun, S., Badgley, B.D., 2019. Changes in microbial functional genes within the soil metagenome during forest ecosystem restoration. Soil Biology & Biochemistry 135, 163–172.

Tang, Y.Q., Yu, G.R., Zhang, X.Y., Wang, Q.F., Tian, J., Niu, S.L., Tian, D.S., Ge, J.P., 2019. Different strategies for regulating free-living N-2 fixation in nutrient-amended subtropical and temperate forest soils. Applied Soil Ecology 136, 21–29.

Wang, W.Y., Hou, Y.T., Pan, W.H., Vinay, N., Mo, F., Liao, Y.C., Wen, X.X., 2021. Continuous application of conservation tillage affects in situ N_2O emissions and nitrogen cycling gene abundances following nitrogen fertilization. Soil Biology & Biochemistry 157, 108239.

Wang, Y., Li, C., Hu, S., 2023. Land use effects on the dynamics of soil carbon, nitrogen and microbes in the water-wind erosion crisscross region of the northern Loess Plateau. Pedosphere. doi: 10.1016/j.pedsph.2023.03.019.

Wang, Y., Zheng, H., Chen, F., Yang, Y., Zeng, J., Van Nostrand, J. D., Zhou, J., Ouyang, Z., 2019. Artificial reforestation produces less diverse soil nitrogen-cycling genes than natural restoration. Ecosphere 10, e02562.

Wu, X., Liu, H., Fu, B., Wang, Q., Xu, M., Wang, H., Yang, F., Liu, G., 2017. Effects of land-use change and fertilization on N_2O and NO fluxes, the abundance of nitrifying and denitrifying microbial communities in a hilly red soil region of southern China. Applied Soil Ecology 120, 111–120.

Xu, M., Jian, J., Wang, J., Zhang, Z., Yang, G., Han, X., Ren, C., 2021. Response of root nutrient resorption strategies to rhizosphere soil microbial nutrient utilization along *Robinia pseudoacacia* plantation chronosequence. Forest Ecology and Management 489, 119053.

Xu, Y., Wang, T., Li, H., Ren, C., Chen, J., Yang, G., Han, X., Feng, Y., Ren, G., Wang, X., 2019. Variations of soil nitrogen-fixing microorganism communities and nitrogen fractions in a *Robinia pseudoacacia* chronosequence on the Loess Plateau of China. Catena 174, 316–323.

Yanu, P., Jakmunee, J., 2015. Flow injection with in-line reduction column and conductometric detection for determination of total inorganic nitrogen in soil. Talanta 144, 263–267.

Yoon, S., Cruz-Garcia, C., Sanford, R., Ritalahti, K., Löffler, F., 2014. Denitrification versus respiratory ammonification: Environmental controls of two competing dissimilatory NO_3^-/NO_2^- reduction pathways in *Shewanella loihica* strain PV-4. ISME Journal 9, 1093–1104.

Zhang, C., Xue, S., Liu, G.B., Song, Z.L., 2011. A comparison of soil qualities of different revegetation types in the Loess Plateau, China. Plant and Soil 347, 163–178.

Zhang, L., Lv, J., 2021. Land-use change from cropland to plantations affects the abundance of nitrogen cycle-related microorganisms and genes in the Loess Plateau of China. Applied Soil Ecology 161, 103873.

Zhang, M., Wang, W., Wang, D., Heenan, M., Xu, Z., 2018. Short-term responses of soil nitrogen mineralization, nitrification and denitrification to prescribed burning in a suburban forest ecosystem of subtropical Australia. Science of the Total Environment 642, 879–886.

Zhang, X., Johnston, E.R., Barberán, A., Ren, Y., Lü, X., Han, X., 2017. Decreased plant productivity resulting from plant group removal experiment constrains soil microbial functional diversity. Global Change Biology 23, 4318–4332.

Zhu, W., Lomsadze, A., Borodovsky, M., 2010. Ab initio gene identification in metagenomic sequences. Nucleic Acids Research 38, e132.