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Soil extracellular enzyme activity reflects the change of nitrogen to phosphorus limitation of microorganisms during vegetation restoration in semi-arid sandy land of northern China

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Soil extracellular enzyme activity (EEA) and enzyme stoichiometry are indicators of soil nutrient availability and microbial substrate limitation. However, the patterns in soil enzyme activities and stoichiometry, and their key drivers remain unclear during vegetation restoration in degraded sandy land. Here, soil ecosystems at five restoration stages of degraded sandy land were selected for investigation and we quantified the microbial nutrient limitation using EEA stoichiometry. The average C:N:P ratio for the nutrient-acquisition enzymes was 1:0.88:0.86, which differed from the global ratio of 1:1:1, indicating stronger microbial C limitation than N or P limitation. With vegetation restoration, the enzyme N:P ratio decreased, but the angle term used in vector analysis increased, indicating that the restored grassland transitioned from N-restricted (angle < 45°) to P-restricted (angle > 45°). Plant C inputs increased the soil nutrient content and significantly decreased the microbial C and N limitation but increased P limitation due to nutrient competition between plants and soil microorganisms. Decreased soil water levels caused by vegetation transpiration may have shifted the microbial limitation from N to P. The decreased fungi:bacteria ratio strengthened the microbial C and N or P limitations. On the basis of these findings, as measures to alleviate the associated nutrient limitations, we would recommend supplementation with phosphorus in the potential and slight stages of rocky desertification and the supplementary application of nitrogen in the moderate and severe stages during the restoration of degraded sandy ecosystems.

KEYWORDS

sandy ecosystem, soil extracellular enzyme stoichiometry, nutrient limitation, microbial biomass, microbial community

1 Introduction

Mineralization of this soil organic matter (SOM) by heterotrophic microorganisms has the potential to affect the cycling of elements such as carbon (C), nitrogen (N), and phosphorus (P), as well as plant production and the atmosphere's composition on a global scale (Sinsabaugh et al., 2008). Soil extracellular enzymes are the proximate agents of SOM decomposition, as they catalyze a series of chemical reactions related to SOM degradation while also obtaining the required energy and nutrient resources for enzyme producers (Sinsabaugh et al., 2009). Changes in soil extracellular enzyme activity (EEA) levels result from changes in cellular metabolism during C, N, and P cycling (Peng and Wang, 2016), reflecting the biogeochemical balance between the microbial community's metabolism and nutrient requirements and a site's nutrient availability (Moorhead et al., 2013; Fanin et al., 2016).

Soil EEA stoichiometry is applied to predict nutrient availability in the environment and microorganism metabolic activity using EEA ratios and stoichiometric invariance (Sinsabaugh et al., 2009; Moorhead et al., 2013; 2016). Sinsabaugh et al. (2008) proposed that the metabolic and stoichiometric needs of soil microorganisms are reflected by the production of enzymes that target the principal C, N, and P sources. The most widely measured enzyme activities usually include C-acquiring enzymes: β -1,4-glucosidase (BG) and β -D-cellobiohydrolase (CBH); N-acquiring enzymes: β -1,4-N-acetylglucosaminidase (NAG) and leucine aminopeptidase (LAP); and P-acquiring enzymes: alkaline phosphatase (AP) (Sinsabaugh et al., 2008; Burns et al., 2013; Cenini et al., 2016; Mori, 2020).

Since these enzyme activities are higher than but correlated with the activities of other related enzymes (Sinsabaugh, 1994), these extracellular enzymes can be used as proxies for the allocation of microbial resources acquired by C, N, and P (Schimel and Weintraub, 2003; Fanin et al., 2016). Sinsabaugh et al. (2008) suggested that the EEA ratios, including BG:NAG (or BG [NAG + LAP]), BG:AP, and NAG:AP (or [NAG + LAP]:AP), reflect the relative activities of the enzymes that acquire carbon (C) vs. nitrogen (N), C vs. P, and N vs. P. Alternatively, if the ratios of BG:NAG (or BG [NAG LAP]), BG:AP, and NAG:AP (or [NAG + LAP]:AP) all rise, it indicates a microbial C limitation or shortage relative to N, a C limitation or a shortage relative to P, and an N limitation or shortage relative to P (Moorhead et al., 2016; Chen et al., 2018). In this context, Moorhead et al. (2016) developed a vector analysis method to calculate the "vector length" and "vector angle" that represent the enzymatic activity of the microbial community by connecting the origin of the plot to a point represented by the ratio of C:P to C:N. This approach assumes that the acquisition of relatively restricted elements by microorganisms is accomplished through the secretion of corresponding extracellular enzymes (Sinsabaugh and Follstad Shah, 2012). It is not affected by a single enzyme, and can comprehensively reflect the relative resources (C, N and P) of microbial metabolism as a whole. In addition, it can identify whether microbial metabolism in a given environment is limited by N or P alone, rather than by both together (Moorhead et al., 2016). This approach quantifies the acquisition of C, N, and P as vector lengths and the relative P and N acquisition as vector angles. Yang et al. (2020) adopted this method to confirm a shift from P

limitation to N limitation of microorganisms during grassland restoration on the Loess Plateau in northwestern China. Vector analysis also showed that all temperate grassland sites in northern China are nutrient-limited, with the most serious limitation being P (Deng et al., 2022). Thus, EEA stoichiometry can help us better understand the metabolic constraints of soil microorganisms. This knowledge can provide insights into the fate of SOC and other nutrients.

EEA responses to soil resource availability are primarily regulated by soil microbes to meet their nutrient requirements (Sinsabaugh et al., 2009; Burns et al., 2013; Fanin et al., 2016); that is, EEA modifies enzymatic allocations to extract energy (C) and nutrients (N, P) from the soil as soil nutrient availability changes (Deng et al., 2019b). The EEA is generally affected by the quality of the available organic matter and the nutrient demands of the microbial biomass (Sinsabaugh et al., 2015). Litter input is the primary source of soil organic matter, and changes in its quantity and quality can affect the soil C content as well as the N and P stoichiometry (Cross and Schlesinger, 2001; Córdova et al., 2018). Changes in C and nutrient requirements, as well as changes in organic matter decomposition, can alter the composition and activity of microorganisms, leading to corresponding changes in EEA (Kaiser et al., 2014; Li et al., 2019). Recent studies have widely reported that the EEA is highly responsive to abiotic factors such as pH and nutrient availability (Allison and Vitousek, 2005; Manzoni et al., 2012; Bowles et al., 2014; Stark et al., 2014; Dong et al., 2019; Yang et al., 2020). EEA also responds to soil texture and moisture availability (Cui et al., 2018), precipitation (Zheng et al., 2020), temperature and the C:N ratio (Zhou et al., 2020).

However, the impact of biotic factors on the EEA cannot be ignored. Given the relatively limited data on the C:N:P ratio of microbial biomass (Cleveland and Liptzin, 2007), enzymatic activity is expected to change to increase the availability of the most limited nutrients to meet microbial metabolic needs (Bowles et al., 2014). Fungi have lower nutrient requirements than bacteria and higher carbon-use efficiency in poor-quality substrates (Keiblinger et al., 2010; McGuire et al., 2010), whereas bacteria require more N and P to maintain a high concentration of these elements during biomass turnover (Elser et al., 2003). As a result, changes in soil nutrients can affect the soil microbial community composition (Schneider et al., 2012; Kaiser et al., 2014). Soil microbial communities adjust their physiological metabolism accordingly, which is reflected in varying degrees of exoenzyme expression (Sinsabaugh et al., 2009). However, the impact of the soil microbial community on EEA stoichiometry has received little attention, and their relative contributions to stoichiometry and the driving mechanisms have received little attention.

In many places around the world, restoring vegetation in degraded landscapes is an effective way to improve soil quality (Paul et al., 2002; Berthrong et al., 2009; Li et al., 2013; 2015; 2017; Deng and Shangguan, 2017; Zhu et al., 2018). On the other hand, soil quality changes relatively slowly, whereas soil biological characteristics, such as the soil EEA, are sensitive to changes in the soil's internal and external environments (Burns et al., 2013; Ren et al., 2016). As a result, soil EEA is frequently used as an indicator to assess soil recovery in various degraded ecosystems (Lino et al., 2016; Ren et al., 2016; Raiesi and Salek-Gilani, 2018; Deng et al., 2019a). Vegetation restoration can improve soil quality (Zhu et al., 2018;

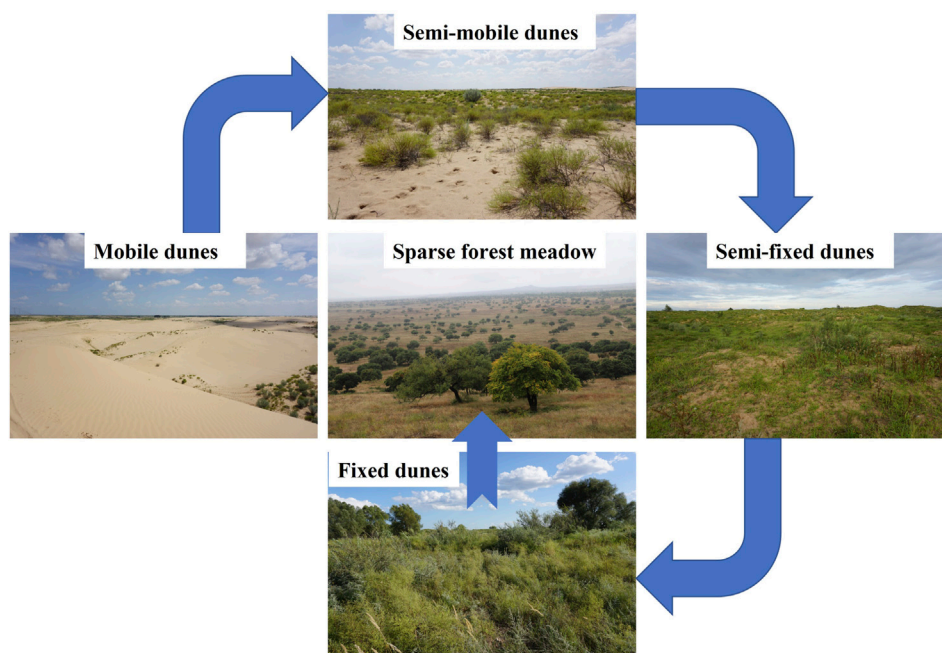


FIGURE 1

Five landscape types in different restoration stages in the Horqin Sandy Land, including (1) mobile dunes with successional pioneer species, which gradually recover to (2) semi-mobile dunes colonized primarily by small sand-fixing shrubs, (3) semi-fixed dunes dominated by annual dominant herbaceous species, (4) fixed dunes dominated by perennial and annual herbs, and (5) sparse forest meadows, which represent the climax community in the study area. The blue arrows represent the direction of vegetation restoration.

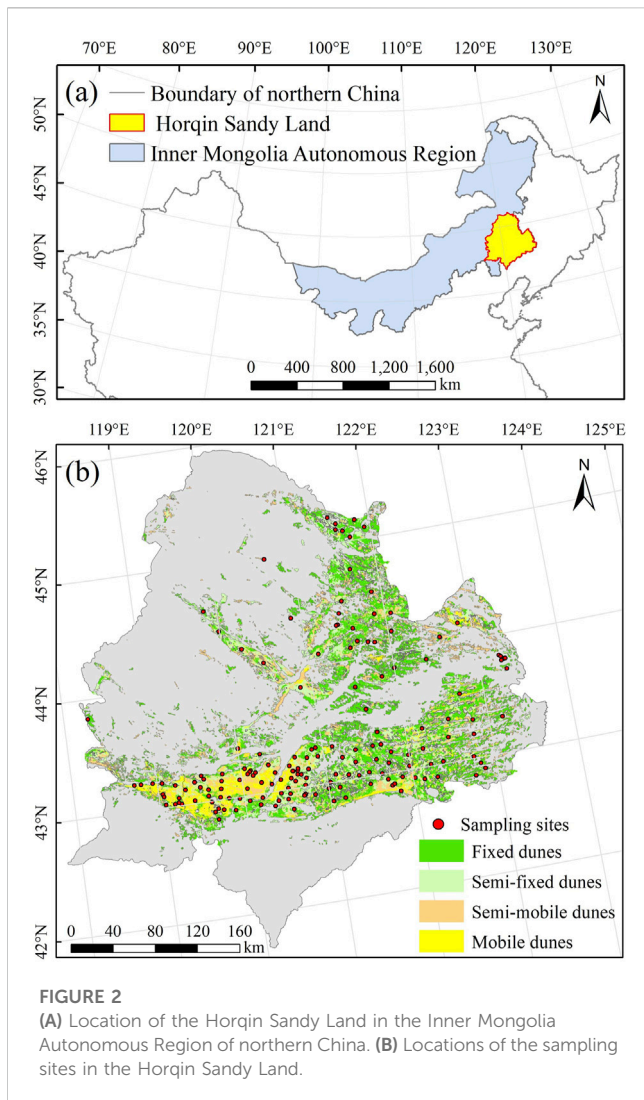
Deng et al., 2019a) by creating a favorable growth environment for soil microorganisms, thereby increasing soil microbial biomass and EEA (Ren et al., 2016; Raiesi and Salek-Gilani, 2018). Deng et al. (2019b) found that in the process of vegetation restoration in the Loess Plateau of China, soil microbial community was mainly limited by C and P, especially in the early stage of vegetation restoration (<13 years) soil microbial metabolism was mainly limited by C, while in the later stage of vegetation restoration (>13 years) soil microbial metabolism was mainly limited by P. Yang et al. (2020) found that with the increase of restoration years of degraded grassland, $\ln(BG):\ln(NAG + LAP):\ln(AP)$ gradually increased, while $\ln(NAG + LAP):LN(AP)$ gradually decreased. Soil microorganisms were jointly limited by N and P, but gradually changed from P limitation to N limitation. Moreover, the intensity of N limitation increases with the increase of recovery years. However, the regulation of limitations on soil microbial metabolism, as well as the mechanisms of metabolic responses to biotic and abiotic factors during the restoration of degraded ecosystems, remain poorly understood because of the complexity of soil microbial metabolic limitations.

The Horqin Sandy Land in northern China was formerly a grassland with abundant water and lush vegetation dominated by palatable grass species and sparsely scattered woody species. However, desertification began at the end of the 19th century, when the area was reclaimed for agriculture and livestock during the Qing Dynasty (1,644–1912), which resulted in a population boom that drove widespread grazing and crop cultivation (Zhou et al., 2008). Especially since the 1950s–1970s, more severe desertification has occurred due to a combination of the region's

fragile ecology and inappropriate human activities, such as overgrazing, large-scale intensive farming, firewood cutting, and excessive groundwater extraction (Li et al., 2014).

To control desertification, a variety of restoration practices have been implemented since the 1980s, primarily through the establishment of grazing prohibition and returning degraded farmland to forests and grasslands. Desertification has gradually reversed since 2000 as land's degraded vegetation has recovered, resulting in a variety of landscape types (Figure 1). These sites illustrate a landscape evolution gradient: 1) mobile dunes with successional pioneer species, which gradually recover to 2) semimobile dunes colonized primarily by small sand-fixing shrubs, 3) semifixed dunes dominated by annual herbaceous species, 4) fixed dunes dominated by perennial and annual herbs, and 5) sparse forest meadows, which represent the climax community in the Horqin Sandy Land (Zhang et al., 2005; Zuo et al., 2012; Lv et al., 2021). This region's unique features provide an ideal platform for studying microbial nutrient limitations after vegetation restoration. However, most previous studies focused on soil nutrients (Li et al., 2015; Zuo et al., 2015; Lv et al., 2021), soil microbial composition (Wang et al., 2016), or plant functional traits (Zuo et al., 2016a; b) along a gradient of degraded land restoration, with little information on microbial metabolic limitations or the combined effects of soil elements (e.g., C, N, P), extracellular enzymes, and microorganisms along a sandy land restoration gradient.

We treated each succession stage as a separate landscape type and investigated soil microbial nutrient limitations and their influencing mechanisms during grassland restoration in northern



China's Horqin Sandy Land. The goals of the study were to test three hypotheses. We hypothesized that (1) the activities of C-, N-, and P-acquiring enzymes would increase as nutrient demand increases along the vegetation restoration gradient and that increased EEA would accelerate nutrient cycling to meet the increasing nutrient demand; (2) the microbial C and N limitation would decrease in response to increasing plant C input with vegetation restoration; and (3) variations in soil EEA stoichiometry would result from differences in the vegetation, soil, microorganism communities, and other factors.

2 Materials and methods

2.1 Study area

The Horqin Sandy Land study area is a typical semiarid sandy land in northern China (Figure 2A). It is a temperate steppe of the Central Asian steppe ecosystem, with a continental semiarid monsoon temperate climate. The elevation ranges from 90 m to 1,625 m above sea level. The mean annual temperature ranges from 3°C to 7°C. Monthly mean temperatures range from -12.6°C

to -16.8°C in January and 20.3°C–23.5°C in July. The frost-free period lasts 140–160 days per year. The annual average precipitation is 350–500 mm, with the maximum precipitation during the summer, from June to August (Duan et al., 2014).

The zonal soils are classified as Kastanozems, Chernozems, and Luvisols according to the Food and Agriculture Organization of the United Nations (FAO, 2006). However, due to desertification, Arenosols are currently the dominant soil. The native vegetation are sparse forest meadows, mainly dominated by meso-xerophytes, which include delicate herbs and sparse woody plants (Li et al., 2017). However, extensive desertification has severely degraded the vegetation community over the past century because of a combination of climate change and unsustainable land use. As a result, xerophytes and psammophytes have come to dominate the area (Liu et al., 1996). Supplementary Table S1 presents the key species in each stage of restoration (Yu and Chen, 2007).

2.2 Experimental design and sampling

We used the “space for time” method to investigate the evolution of vegetation restoration. This chronosequence scheme has been proposed for application in vegetation restoration research to monitor plants and soils under similar climatic conditions by following the sequence of vegetation development (Bhøjvaid and Timmer, 1998). We selected five landscape types (mobile dunes, semimobile dunes, semifixed dunes, fixed dunes, and sparse forest meadows) to represent different restoration stages of the Horqin Sandy Land (Figure 1). Based on a classification map for the degree of desertification of the Horqin Sandy Land, we defined the specific classification criteria for desertification shown in Supplementary Table S1. We obtained RGB images of the UAV plots by using a Sony EXMOR Sensor mounted on a DJI Phantom 3 Professional (DJI Innovation Company Inc., Shenzheng, China) and used ImageJ software to measure vegetation cover (Ricotta et al., 2014; Lian et al., 2022). The samples of the surface soil (0–10 cm) were collected at 163 sites (to a depth of 10 cm) from July to August 2021 (Figure 2B). At each site, we first established a 10 m × 10 m plot, removed surface debris and then collected the soil samples randomly at 15 sampling points within each plot using a 5-cm diameter soil auger. The soils collected from each plot were combined to form a single composite sample. The collected soil samples were stored at 4°C in a portable refrigerator during field sampling. When sent to the lab we used a 2 mm screen to remove roots and other debris from all samples. Then the samples were separated into two subsamples: One subsample was used to measure soil microbial and enzyme activity, and the other was air-dried at room temperature to determine soil physical and chemical properties. To estimate the dry bulk density, we obtained three undisturbed soil cores using a soil bulk density sampler with a stainless steel cylinder (100 cm³ volume) at each site, which we dried for 48 h at 105°C for the bulk density calculation.

In addition, five quadrats (1 m × 1 m) were separately selected in each of the four corners and center of the plot (10 m × 10 m). In each quadrat, all the aboveground parts of the green plants were chopped off, gathered from the ground, placed in envelopes, and marked with tags. Because the biomass samples were large, they were weighed fresh, and a part of each sample was dried and weighed. The ratio of

dry weight to fresh weight was multiplied by the fresh weight to determine the samples' aboveground biomass (AGB).

2.3 Environmental variables

A suite of 9 environmental variables were collected to clarify the regional-scale factors controlling the spatial pattern of soil microbial metabolic limitations in the sandy ecosystems. Environmental variables include meteorological factors, topographic factors and vegetation index, specifically include the mean annual temperature (*MAT*) and mean annual precipitation (*MAP*) data with a spatial resolution of 1 km for 2021, the normalized difference vegetation index (*NDVI*) dataset during the growing season (April to September) in 2021, and the corresponding elevation, slope, ground roughness, and profile curvature. The source of the above dataset can be found in our previous article (Li et al., 2023; Wang et al., 2023). All the environmental variables mentioned above were uniformly converted into raster data with a spatial resolution of 500 m and extracted to our actual sampling points using the Extract Multi Values to Points geoprocessing tool, which extracts cell values from one or more rasters to a point feature class, in version 10.3 of ArcMap GIS software (<http://www.esri.com>).

2.4 Soil physical and chemical properties

The Walkley–Black method (Nelson and Sommers, 1982), Kjeldahl method (Bremner, 1996) and Olsen method (Olsen and Sommers, 1982) was used to determine the SOC, total N (*TN*) and total P content (*TP*), respectively. We determined the soil pH and electric conductivity (*EC*) at soil:water ratios of 1:2.5 v/v and 1:5 v/v, respectively, using pH and *EC* meters (PHS-3C, Puchun, Shanghai, China). The bulk soil samples were dried at 105°C for 24 h to determine the bulk density (*BD*) (Blake and Hartge, 1986). Wet sieving was used to separate the soil sample into four fractions: coarse sand (>0.25 mm), fine sand (0.10–0.25 mm), very fine sand (0.05–0.10 mm), and clay and silt (<0.05 mm) (Li et al., 2012). We used the chloroform fumigation–extraction method to determine the C, N, and P contents of the microbial biomass (Brookes et al., 1982; 1985; Vance et al., 1987). The amounts of bacteria and fungi were determined by the quantitative real-time polymerase chain reaction (qPCR) amplification technique to identify the abundance and diversity of soil community. The abundance and diversity calculations of soil microbial communities are described in our previous study (Duan et al., 2022).

2.5 Measurement of soil enzyme activity

Using 7-amino-4 methylcoumarin and 4-methylumbelliferone as fluorescent substrates, and standard fluorometric technique was used to measure the activities of the soil C-acquiring enzymes (BG and CBH), N-acquiring enzymes (NAG and LAP), and P-acquiring enzyme (AP) (Saiya-Cork et al., 2002; Sinsabaugh et al., 2008; 2009; Steinweg et al., 2012). The enzymatic activity was expressed in units of nmol activity g⁻¹ dry soil h⁻¹, and the ratios of C-, N-, and P-acquiring enzymes were calculated to express the soil EEA stoichiometry (Sinsabaugh et al., 2008):

$$\text{Soil enzyme C: N ratio} = \ln(BG + CBH) / \ln(LAP + NAG) \quad (1)$$

$$\text{Soil enzyme C: P ratio} = \ln(BG + CBH) / \ln(AP) \quad (2)$$

$$\text{Soil enzyme N: P ratio} = \ln(LAP + NAG) / \ln(AP) \quad (3)$$

2.6 Microbial metabolic limitation

Sinsabaugh et al. (2008) noted that the relative activities of C- vs. P-acquiring enzymes and C- vs. N-acquiring enzymes may reflect soil microbial nutrient limitations (N or P). Vector analysis of the soil EEA stoichiometry was proposed by Moorhead et al. (2016) to evaluate microbial nutrient limitations. In this method, the vector length quantifies relative C vs. N or P nutrient limitation, and the angle quantifies the relative P vs. N limitation. The vector length and angle were calculated as follows:

$$\text{Vector length} = \sqrt{x^2 + y^2} \quad (4)$$

$$\text{Vector angle} = \text{atan} 2(x, y) \quad (5)$$

where *x* represents the relative C- vs. P-acquiring enzyme activities (i.e., [BG + CBH]/AP) and *y* represents the relative C- vs. N-acquiring activities (i.e., [BG + CBH]/[LAP + NAG]). Soil microbial C limitation is thought to increase with increasing vector lengths, whereas soil microbial N or P limitation is represented by vector angles: angles less than 45° indicate N limitation, and angles greater than 45° indicate P limitation (Moorhead et al., 2013). The P limitation increases as the angle increases, whereas the N limitation increases as the angle decreases (Moorhead et al., 2016).

2.7 Statistical analysis

First, we tested for heterogeneity of variance, and if necessary, we log-transformed or standardized the data (using *z* scores) before statistical analysis. We used one-way analysis of variance to test for significant differences in soil enzyme activities and enzymatic stoichiometry among the sandy landscapes, followed by least-significance-difference (LSD) tests for multiple comparisons (*p* < 0.05). We used Pearson's correlation coefficient (*r*) to evaluate the relationships among the influencing factors and soil EEA. The redundancy analysis (RDA) was applied to determine the relative contributions of the influencing factors to the microbial metabolic limitation. RDA analysis was performed in version 5.0 of Canoco software (<http://www.canoco5.com/>).

3 Results

3.1 Soil C, N, and P content and microbial biomass C, N, and P

The SOC, *TN*, *TP*, and the soil microbial biomass C, N, and P (*MBC*, *MBN*, and *MBP*, respectively) increased significantly (*p* < 0.05) along the landscape evolution gradient from mobile dunes to sparse forest meadows (Table 1), whereas the soil C:N decreased significantly (*p* < 0.05). In addition, the C:P and N:P ratios increased significantly from mobile dunes to fixed dunes and then decreased

TABLE 1 Changes in soil characteristics brought on by vegetation restoration during the evolution of a landscape. Land types: MD, mobile dunes; SMD, semi-mobile dunes; SFD, semi-fixed dunes; FD, fixed dunes; SFM, sparse forest meadows.

Soil properties ^a	MD	SMD	SFD	FD	SFM
SOC	1.66 ± 1.80d	2.48 ± 1.57d	4.14 ± 1.82c	6.11 ± 2.43b	8.93 ± 3.50a
TN	0.09 ± 0.05d	0.16 ± 0.06d	0.35 ± 0.15c	0.60 ± 0.24b	0.97 ± 0.35a
TP	0.07 ± 0.03d	0.08 ± 0.02d	0.12 ± 0.05c	0.15 ± 0.04b	0.29 ± 0.10a
C:N	26.84 ± 32.77a	17.84 ± 16.38b	13.39 ± 7.18bc	10.49 ± 2.25bc	9.27 ± 1.71c
C:P	26.47 ± 28.6b	34.04 ± 25.45ab	37.97 ± 17.6ab	40.93 ± 13.14a	33.58 ± 15.87ab
N:P	1.29 ± 0.50d	2.10 ± 0.83c	2.97 ± 0.84b	3.95 ± 1.20a	3.51 ± 1.17a
MBC	10.88 ± 14.07d	41.99 ± 71.73cd	70.95 ± 52.69c	138.63 ± 105.56b	264.42 ± 152.82a
MBN	2.25 ± 1.03c	7.40 ± 13.77c	10.4 ± 7.96c	22.28 ± 17.61b	36.32 ± 21.24a
MBP	3.85 ± 1.97b	6.11 ± 6.99b	8.70 ± 5.93b	17.78 ± 9.55a	17.61 ± 16.96a
MBC:MBN	4.25 ± 3.52b	6.30 ± 4.25ab	8.16 ± 4.33a	8.08 ± 4.71a	8.18 ± 4.18a
MBC:MBP	2.82 ± 3.35b	7.06 ± 6.67b	9.19 ± 6.53b	7.78 ± 4.19b	26.04 ± 23.57a
MBN:MBP	0.75 ± 0.47b	1.16 ± 0.82b	1.27 ± 0.75b	1.17 ± 0.71b	3.10 ± 2.29a

^aThe terms SOC, TN, and TP stand for soil organic carbon, soil total nitrogen, and soil total phosphorus, respectively. C:N, C:P, and N:P stand for the ratios of SOC to TN, SOC to TP, and SOC to TP, respectively. Between stages of landscape restoration, values of a parameter denoted by various lowercase letters differ significantly (ANOVA followed by LSD test; $p < 0.05$).

(but not significantly) from fixed dunes to sparse forest meadows. Compared with mobile dunes, SOC content in semi-mobile dunes, semi-fixed dunes, fixed dunes and sparse forest meadows was increased by 49.40%, 149.40%, 268.07% and 437.95%, respectively. MBC content was increased by 285.94%, 552.11%, 1,174.17% and 2,330.33%, respectively. In addition, the MBC:MBN, MBC:MBP, and MBN:MBP ratios were lowest in mobile dunes and highest in sparse forest meadows.

3.2 Variations in soil EEA and EEA stoichiometry along the landscape restoration gradient

Figure 3 shows that the restoration of sandy land significantly (ANOVA followed by LSD test, $p < 0.05$) affected soil EEA (BG + CBH, NAG + LAP, AP). With increasing vegetation restoration, the activities of the C-acquiring enzymes (BG + CBH) increased significantly from mobile dunes (131.31 nmol g⁻¹ h⁻¹, mean ± SE) to sparse forest meadows (1992.01 nmol g⁻¹ h⁻¹). The N-acquiring enzymes (NAG + LAP) and P-acquiring enzyme (AP) followed the same pattern. The activity of the N-acquiring enzymes (NAG + LAP) was lowest in mobile dunes (243.39 nmol g⁻¹ h⁻¹) and highest in sparse forest meadows (724.33 nmol g⁻¹ h⁻¹). The activity of the P-acquiring enzyme (AP) was lowest in mobile dunes (15.57 nmol g⁻¹ h⁻¹) and highest in sparse forest meadows (1,036.86 nmol g⁻¹ h⁻¹). The enzyme C:N ratio calculated by ln (BG + CBH)/ln (NAG + LAP) was significantly lower in mobile dunes than at the other stages of restoration, which did not differ significantly. The enzyme C:N ratio in mobile dunes was less than 1 versus values greater than 1 at the other stages. In contrast, both the enzyme C:P ratio calculated by ln (BG + CBH)/ln (AP) and the N:P ratio calculated by ln (NAG + LAP)/ln (AP) were highest in the mobile dunes.

3.3 Effects of vegetation restoration on soil microbial metabolic limitations

We used vector analysis (vector length and vector angle) of the soil EEA stoichiometry data to evaluate microbial nutrient limitations. The microbial C limitation was represented by the vector length, with the microbial C limitation decreasing significantly (ANOVA followed by LSD test; $p < 0.05$) along the vegetation restoration gradient (Figure 4A). The vector angles in mobile dunes and semimobile dunes were less than 45°, indicating an N deficiency, but more than 45° in semifixed dunes, fixed dunes, and sparse forest meadows, indicating a P deficiency. Thus, during vegetation restoration in the Horqin Sandy Land, N limitation gradually weakened and P limitation gradually increased. Soil microorganisms suffer from N limitation in the initial stages of vegetation restoration (mobile dunes and semimobile dunes), but soil microorganisms in semifixed dunes, fixed dunes, and sparse forest meadows were mainly limited by P. That is, there was a transition from N limitation to P limitation for microorganisms during vegetation restoration in the Horqin Sandy Land.

Similarly, the majority of the mobile dune and semimobile dune study sites appeared on the lower right side of the graph of the ratios of C to N enzyme activities to the ratios of C to P enzyme activities (Figure 5); that is, the angles are <45°, indicating that soil microbial activity was primarily limited by N rather than P during these restoration stages. In contrast, most of the semifixed dune, fixed dune, and sparse forest meadow study sites appeared on the upper left side of the graph, at angles >45°, indicating that soil microbial activities were primarily limited by P rather than N during these restoration stages. Furthermore, we found a statistically significant negative correlation ($r = -0.64$, $p < 0.001$) between the two ratios; that is, as the soil microbial C limit increased, the soil microbial N or P limit decreased (Figure 6).

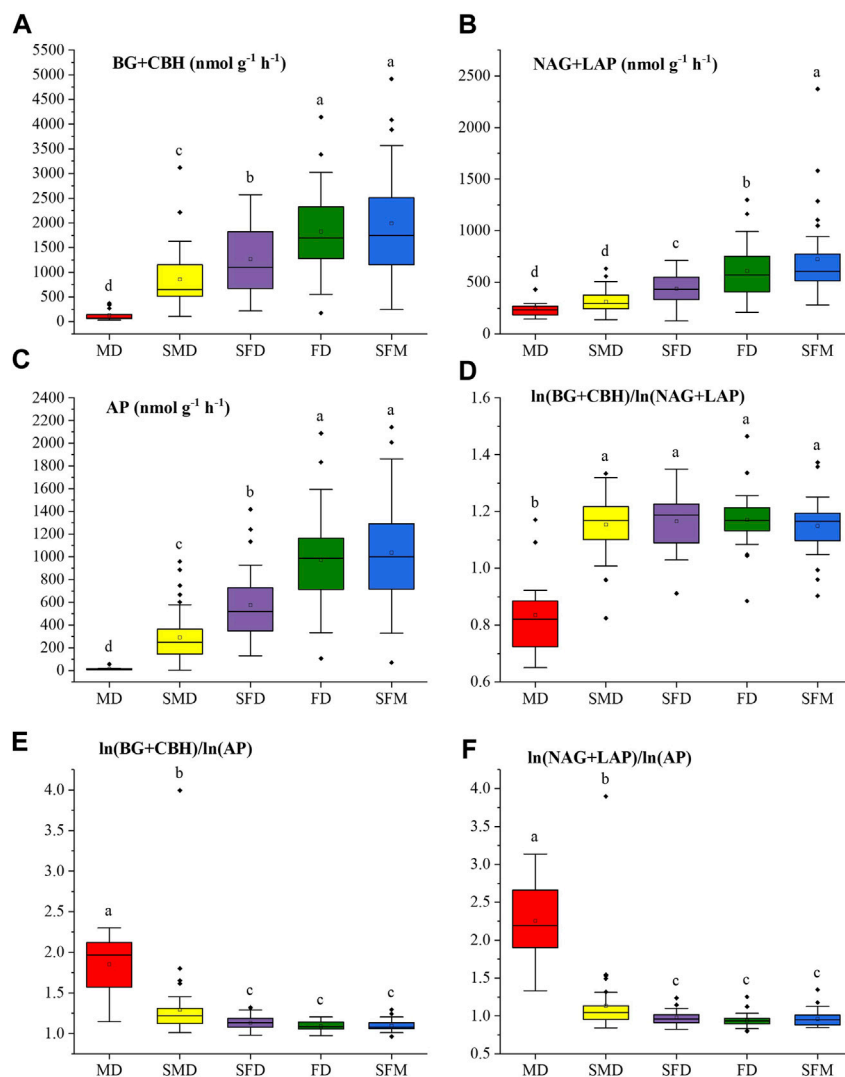


FIGURE 3

Variations of soil enzymatic activity and enzymatic stoichiometry at different stages of vegetation restoration: (A) C-acquiring enzymes (BG + CBH); (B) N-acquiring enzymes (NAG + LAP); (C) the P-acquiring enzyme (AP); and the logarithmic ratios (D) (BG + CBH)/(NAG + LAP); (E) (BG + CBH)/AP; (F) (NAG + LAP)/AP. Values of a parameter followed by different lowercase letters differ significantly among landscape restoration stages (ANOVA followed by LSD test; $p < 0.05$).

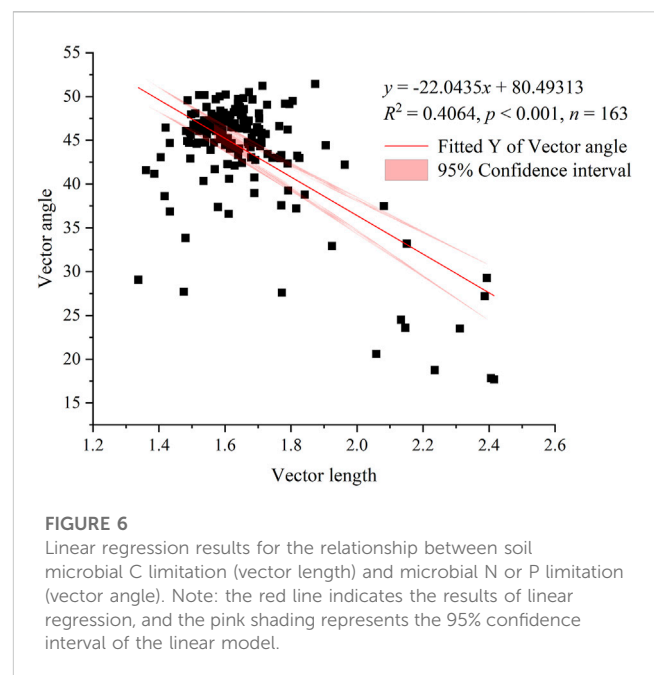
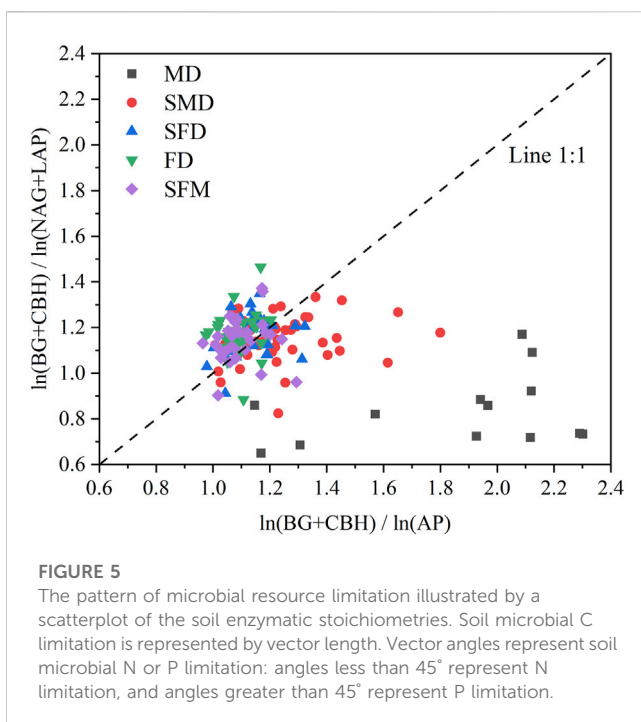
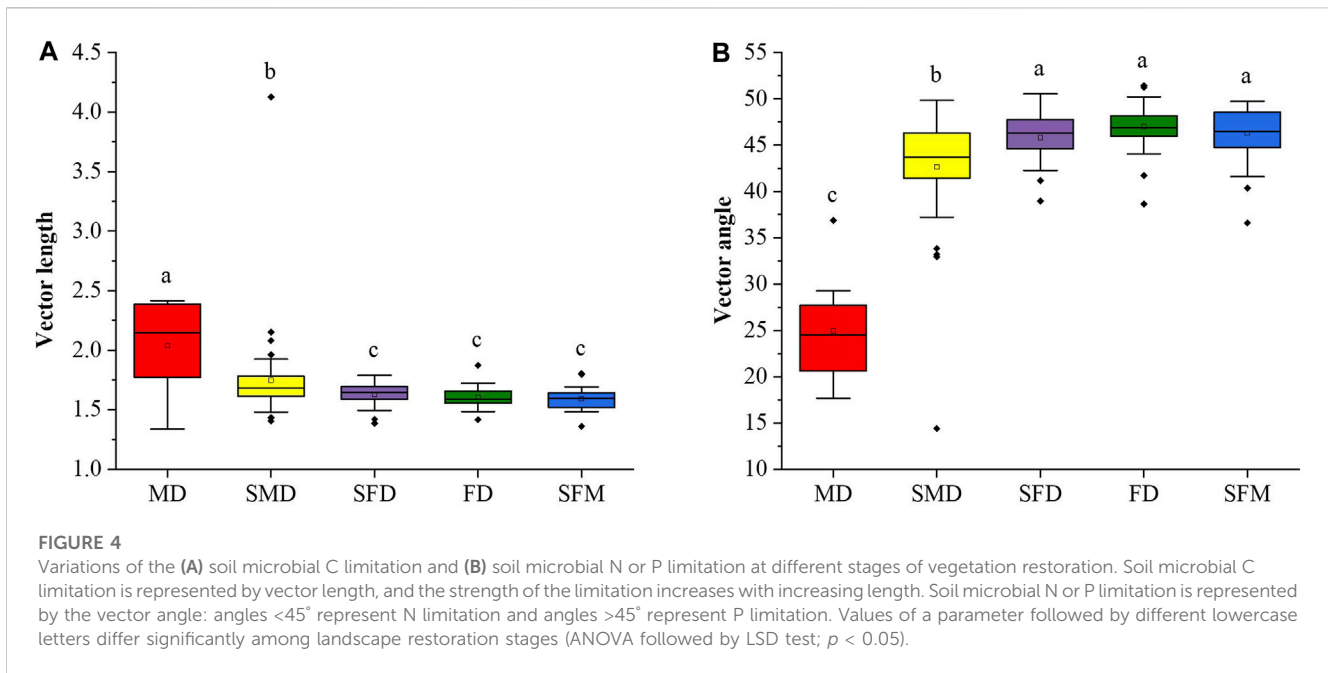
4 Discussion

4.1 Changes in soil enzyme activities during vegetation restoration

Soil extracellular enzymes are thought to be sensitive and reliable indicators of vegetation restoration (Yu et al., 2019). In our study, the activities of C-acquiring enzymes (BG + CBH), N-acquiring enzymes (NAG + LAP), and the P-acquiring enzyme (AP) increased during the restoration of the sandy grassland from mobile dunes to sparse forest meadows, which supports our first hypothesis that the activities of the C-, N-, and P-acquiring enzymes would increase as the nutrient demand increases with increasing vegetation restoration. This finding is consistent with previous findings for grasslands (Peng and Wang, 2016; Zhang et al., 2016; He et al., 2020; Wang et al., 2020), abandoned farmland (Cui et al., 2018; Cui et al., 2019; Yang et al., 2020), and

desert ecosystems (Liu et al., 2017). The soil at our sites had low levels of nutrients and microbial biomass in the early stages of grassland restoration (i.e., mobile and semimobile dunes) (Table 1), and the majority of the vegetation was annual herbaceous plants with shallow roots. As a result of the limited availability of an organic C substrate, microorganisms' ability to perform exoenzymatic synthesis was low, ultimately limiting soil EEA (Liu et al., 2018; Li et al., 2020). However, as vegetation restoration progressed, plant biomass increased significantly and increasingly competed with microorganisms for soil resources (Bardgett et al., 2005). Furthermore, fungal activity increased significantly, necessitating more nutrients to meet fungal growth requirements (Fanin et al., 2016). As a result, soil microorganisms began to secrete more C-, N-, and P-acquiring enzymes.

The C:N:P ratios of soil enzymes represent the biochemical balance between soil microbial nutrient allocation and resource



availability (Xiao et al., 2018). A meta-analysis of data from 40 ecosystems around the world confirmed that the ratio of C:N:P acquisition enzymes in the topsoil was close to 1:1:1 (Sinsabaugh et al., 2009), indicating that microorganisms have a broad pattern of maintaining stoichiometric homeostasis of C, N, and P across a range of ecosystems. However, due to the wide variation in ecosystems and regional environmental conditions, this equilibrium pattern is easily disrupted (Xiao et al., 2020; Zhou et al., 2020). For instance, the average C:N:P ratio of soil enzymes was approximately 1:1:1 in forest ecosystems along the

north-south transect in eastern China (Xu et al., 2017), but subsequent national-scale analysis results revealed that the ratio of C:N:P acquisition enzymes in Chinese forests averaged 1:1.4:2.6, deviating from the 1:1:1 ratio (Sinsabaugh et al., 2009; Zhou et al., 2020). The ratios of C:N:P acquisition enzymes were 1:1.2:1.4 and 1:1.08:1.28 in temperate grassland ecosystems of northern China and the Loess Plateau of China (Peng and Wang, 2016; Yang et al., 2020), respectively, indicating that C-acquiring enzyme activities were lower than N- and P-acquiring enzyme activities. In the present study, the average ratio of C:N:P acquisition enzymes was 1:0.88:0.86, suggesting (1) higher C-acquiring enzyme activities than N- or P-acquiring enzyme activities and (2) lower P-acquiring enzyme

activity than that of N- or C-acquiring enzymes, which is inconsistent with the abovementioned results.

4.2 Microbial nutrient limitations revealed by soil enzyme stoichiometry

We found that the vector length decreased with vegetation restoration from mobile dunes to sparse forest meadows, indicating that microbial C limitation decreased during grassland restoration, whereas the activity of C-acquiring enzymes (BG + CBH) increased significantly with increasing vegetation restoration, which suggests that there was an opposite trend between microbial C limitation and the activity of soil C-acquiring enzymes. This is mainly due to increasing plant C inputs to the soil (e.g., litter, roots), which mitigated the microbial C limitation that developed during vegetation restoration (Ruiz-Jaén and Aide, 2005; Deng et al., 2013) but did not reduce the demand for soil carbon. In addition, when soil C availability is severely limited, soil microorganisms are likely to be dormant until C limitation is alleviated, implying that soil microorganisms generally do not produce more extracellular enzymes associated with C acquisition until the limitation is alleviated (Sinsabaugh and Follstad Shah, 2012; Blagodatskaya and Kuzyakov, 2013).

In addition, mobile dunes had a significantly lower vector angle than the other restoration stages (Figure 4B), which suggests that soil microorganisms are more highly limited by N in the mobile dunes. The vector angles were less than 45° in the mobile and semimobile dunes but greater than 45° in the semifixed dunes, fixed dunes, and sparse forest meadows, which supports our second hypothesis that grassland restoration would reduce the microbial C and N limitation but cause a shift from N limitation to P limitation from semi-mobile dunes to semi-fixed dunes. It is worth noting that the shift from N limitation to P limitation of microorganisms between semimobile dunes and semifixed dunes indicates that vegetation restoration requires more P than N at this stage, which leads to the secretion of more P-acquiring enzymes than N-acquiring enzymes, resulting in a reduction in the enzyme N:P ratio as vegetation restoration progresses in sandy land (Figure 2).

Due to the sparse vegetation cover of mobile and semimobile dunes and the dominance of annual herbaceous plants with shallow roots in their vegetation communities, fewer exogenous nutrients were available for microorganisms. In contrast, plants growing on semifixed and fixed dunes or in sparse forest meadows have relatively large root biomass, which provides more exogenous resources for microorganisms (Cui et al., 2018; Cui et al., 2019 YX.). This is reflected by increasing soil C and N contents with increasing vegetation restoration (Table 1), but this increase was not synchronous; that is, the increase in soil C lagged behind the increase in soil N, resulting in a continuous decrease in the soil C:N ratio. This may increase soil N availability (Waring et al., 2014; Zhao et al., 2018; Liu et al., 2019; Rosinger et al., 2019; Zhang et al., 2019; Zhou et al., 2020), eventually leading to weakening of the soil microbial nitrogen limitation in the later stages of vegetation restoration in sandy land.

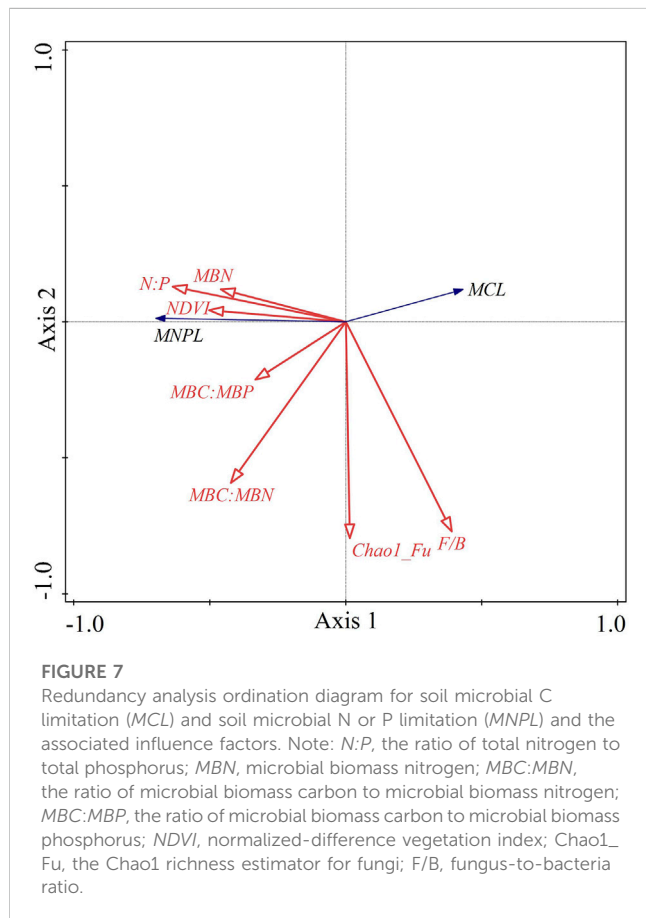
On the other hand, as restoration proceeded from semimobile dunes to sparse forest meadows, the soil P content increased significantly (Table 1), and the soil microbial biomass

significantly improved (Table 1) along with the obvious increase in plant biomass accumulation, which increased the competition for soil P. Soil microbial activity increases nutrient requirements to sustain the high growth rates (Sterner and Elser, 2002), but the limited input of soil P due to the relatively low plant biomass resulted in an increased microbial P limitation. Previous research has also found increased P limitation in soils during the late stages of vegetation restoration on the Loess Plateau (Jiao et al., 2013; Ren et al., 2016; Deng et al., 2019b), particularly in areas where soil erosion has accelerated soil P loss (Feng et al., 2013). Based on the discussion in this section, our results demonstrate a shift from N to P limitation during vegetation restoration in sandy land.

4.3 Factors affecting soil microbial metabolic limitation during vegetation restoration

Both aboveground biomass and the normalized difference vegetation index (NDVI) were significantly negatively correlated with microbial C limitation but significantly positively correlated with microbial N or P limitation (Supplementary Table S2), mainly due to improvement of the soil C pool by increased plant C inputs, such as litter and root turnover, during vegetation restoration, thus reducing the C demand of the soil microorganisms (Zhang et al., 2011; Deng and Shangguan, 2017; Deng et al., 2018). Similarly, the SOC, TN, and TP exhibited a significant negative correlation with microbial C limitation but a significant positive correlation with microbial N or P limitation, confirming that vegetation restoration can provide more C and nutrient sources for soil microorganisms (Zhu et al., 2010), thereby reducing the microbial communities' C limitation (Cui YX. et al., 2019).

In addition, the soil microbial community structure and composition significantly affected microbial metabolic limitations. We found that MBC, MBN, and MBP were significantly negatively correlated with microbial C limitation but significantly positively correlated with microbial N or P limitation (Supplementary Table S2). According to the growth rate hypothesis (Sterner and Elser, 2002), the increase in soil microbial activity is related to the high demand for P to support the synthesis of ribosomal RNA, so MBP had a strong direct positive impact on microbial phosphorus limitation. Previous research has also confirmed that the relative abundance of soil microorganisms is highly sensitive to changes in the soil C:N:P ratio (Turner and Haygarth, 2005; van der Heijden et al., 2008). Fundamental differences in bacterial and fungal growth forms and living strategies can influence C:N:P demand, and the ratio responds to changes in microbial community diversity (Güsewell and Gessner, 2009; Strickland and Rousk, 2010). The physiological metabolisms of these microorganisms can be further altered by the secretion of extracellular enzymes to acquire nutrients to support the organism's growth (Sinsabaugh et al., 2009; Schneider et al., 2012). For instance, EEA may be higher in soils with larger fungal populations due to the long hyphal lengths (Burke et al., 2011; Kivlin and Treseder, 2014). In our study, we discovered that both bacterial community diversity indices were significantly negatively correlated with C limitation but significantly positively correlated with N or P limitation, whereas the fungal community diversity



index was only significantly negatively correlated with N or P limitation (Supplementary Table S2). It is unknown whether the production of some enzymes is differentially controlled by different groups of soil microorganisms, and answering this question is critical to understanding the role of the microbial community structure in ecosystem functioning (Fanin et al., 2016); this will require additional research.

Figure 7 shows the RDA ordination results of the soil microbial C limitation and microbial N or P limitation and the influencing factors. The cumulative percentage of the variance for the dependent variables, including the soil microbial C limitation (*MCL*) and

microbial N or P limitation (*MNPL*), explained by the influencing factors totaled 44.6% for the first two axes. Soil microbial N or P limitation was positively associated with *N:P*, the soil microbial factors (i.e., *MBN*, *MBC:MBN* and *MBC:MBP*) and *NDVI*. The soil microbial C limitation exhibited the opposite relationships. Both *MCL* and *MNPL* were negatively correlated with the Chao1 richness estimator for fungi, and the fungus-to-bacteria ratio (*F/B*) was positively associated with *MCL* but negatively correlated with *MNPL*. Furthermore, we used a partial Monte Carlo permutation test to evaluate the contributions of each factor to the variation in *MCL* and *MNPL* (Table 2). *N:P* explained the largest proportion of the variation in soil microbial metabolic limitations (18.0%) and had the highest contribution rate (35.7%). The second-most important factor was *MBC:MBN*, which explained 5.5% of the variation and contributed 11.0%. The influences of the other factors were statistically significant but much smaller ($\leq 5\%$ of the variation explained). This indicates that *N:P* was the dominant driver for the soil microbial metabolic limitation in the semiarid sandy study area.

5 Conclusion

Through analyses of microbial metabolism and plant-microbe nutrient competition, we confirmed that during vegetation restoration in the Horqin Sandy land, the soil microbial C limitation gradually decreased. In contrast, soil microbial N or P limitation increased over time, and the limitation shifted from N limitation to P limitation as the P limitation increased in strength from mobile to semimobile dunes. The effect of vegetation restoration on microbial C limitation and N or P limitation resulted from a combination of effects induced by plants, soils, extracellular enzymes, and microorganisms. Plant C input significantly increased the soil nutrient content and decreased the microbial C limitation but increased the microbial N or P limitation due to competition between plants and soil microorganisms for nutrients. In addition, the decrease in the fungi:bacteria ratio (to a value less than 1) strengthened the microbial N or P limitation. This suggests that the number of fungi could be increased artificially in future restoration projects to alleviate microbial nutrient limitations during vegetation restoration. Our results provide

TABLE 2 The percentages of variation explained (the explanation rate) and contributions (based on redundancy analysis) of the influencing factors to variation of the soil microbial N or P constraint as well as soil microbial C limitation in the redundancy analysis. Abbreviations: *N:P* stands for the total nitrogen to total phosphorus ratio; *MBC*, *MBN*, and *MBP* stand for microbial biomass carbon, nitrogen, and phosphorus, respectively; *NDVI*, the normalized-difference vegetation index; *Chao1_Fu*, the Chao1 richness estimator for fungi; *F/B*, fungus-to-bacteria ratio.

Factors	Explanation (%)	Contribution (%)	Pseudo-F	P
<i>N:P</i>	18.0	35.7	35.4	0.002
<i>MBC:MBN</i>	5.5	11.0	11.6	0.002
<i>F/B</i>	5.0	9.9	11.1	0.004
<i>MBN</i>	4.4	8.8	10.5	0.004
<i>Chao1_Fu</i>	5.1	10.2	13.0	0.002
<i>NDVI</i>	3.8	7.5	10.2	0.002
<i>MBC:MBP</i>	2.7	5.4	7.6	0.012

an indirect theoretical basis for the sustainable management of ecological restoration. In the process of vegetation restoration in semiarid sandy land, it is necessary to replenish vegetation C input in the mobile dune stage. Due to N limitation in semimobile dunes, attention should be given to nitrogen fertilizer supplementation in this stage. To accelerate and maintain the vegetation restoration of semifixed dunes and fixed dunes, phosphorus fertilizer should be added in a timely manner. However, our study will be replicated in a different area of sandy land in subsequent research to confirm whether the results are widely applicable.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding authors.

Author contributions

BY: Formal Analysis, Writing—original draft, Data curation, Investigation, Methodology, Software, Visualization. XW: Methodology, Writing—original draft, Data curation, Formal Analysis, Funding acquisition, Investigation, Project administration, Visualization. YgL: Funding acquisition, Methodology, Writing—review and editing. JL: Data curation, Methodology, Writing—review and editing. YnL: Data curation, Writing—review and editing. YLuo: Funding acquisition, Methodology, Writing—review and editing. YIL: Methodology, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2023.1298027/full#supplementary-material>

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