



Soil extracellular enzyme stoichiometry reflects the shift from P- to N-limitation of microorganisms with grassland restoration

Yang Yang ^{a,b,c}, Chao Liang ^d, Yunqiang Wang ^{a,b}, Huan Cheng ^{e,f}, Shaoshan An ^{c,*}, Scott X. Chang ^{g,h}

^a State Key Laboratory of Loess and Quaternary Geology, Institute of Earth Environment, Chinese Academy of Sciences, Xi'an, 710061, China

^b CAS Center for Excellence in Quaternary Science and Global Change, Xi'an, 710061, China

^c State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Northwest A&F University, Yangling, 712100, China

^d Key Laboratory of Forest Ecology and Management, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, 110016, China

^e Department of Biology, University of Maryland, College Park, MD, 20742, USA

^f College of Forestry Sichuan Agricultural University, Chengdu, 611130, China

^g Department of Renewable Resources, University of Alberta, Edmonton, T6G 2E3, Canada

^h State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Lin'an, 311300, Zhejiang, China



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ABSTRACT

Soil extracellular enzyme stoichiometry (EES), the ratio of extracellular enzyme activities (EEAs) related to the acquisition of nutrients such as carbon (C), nitrogen (N) and phosphorus (P), reflects the demand for resources by microorganisms. However, how grassland restoration shifts microbial nutrient limitation as indicated by soil EES remains unclear. Here, we evaluated microbial nutrient limitation by studying soil EES across a chronosequence of grassland restoration sites. The ratio of the natural logarithm of C-, N-, and P-acquiring enzymes in our studied system ranged from 1:1.47:1.05 to 1:0.82:1.38, and the average was 1:1.08:1.28, deviating from the global ratio of 1:1:1 and indicating that microorganisms were co-limited by N and P. Enzyme N:P ratio increased and vector angles decreased with time since restoration, suggesting that the restored grassland shifted from P-limitation (angles > 45°) to N-limitation (angles < 45°), and N limitation gradually exacerbated over time. Strong relationships between microbial biomass C:N:P ratios and soil EES suggest that soil EES was dependent on microbial resource availability. We also found that the variation in soil EES was better explained by biotic than by abiotic factors, and the total variation explained increased with time since restoration, indicating that biotic control over soil EES increased over time as the environmental conditions such as soil moisture availability and nutrients improved for microorganisms. Overall, this study highlights the utility of the ecoenzymatic stoichiometry approach in assessing relationships in microbial resource ecology, and such insights provide guidance in developing restoration strategies such as the need for nutrient management in grassland ecosystems.

1. Introduction

Soil microorganisms drive nutrient cycling by producing a variety of extracellular enzymes that regulate the breakdown of soil organic matter (Waring et al., 2014; Liu et al., 2019; Rosinger et al., 2019). The soil organic matter decomposition process provides the energy and nutrients needed for microbial metabolism (Sinsabaugh et al., 2008, 2009). During the process of soil organic matter decomposition, a few of the extracellular enzyme activities (EEAs) will account for most of the measurable enzyme activities in the soil, because those enzymes target

the most abundant substrates such as carbon (C), nitrogen (N), and phosphorus (P) in the environment (Zhao et al., 2018; Tian et al., 2020). These EEAs are often classified as “C-acquiring” (e.g., hemicellulase, cellulase, and glucosidase), “N-acquiring” (e.g., urease, chitinase, and peptidase), and “P-acquiring” (e.g., phosphatase) enzymes (Burns et al., 2013; Cenini et al., 2016) that can degrade high-molecular-weight organic compounds into assimilable molecules (Sinsabaugh et al., 2013; Lalanne et al., 2018).

Soil extracellular enzyme stoichiometry (EES), the ratio of EEAs related to the acquisition of nutrients such as C, N and P, reflects

* Corresponding author. State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Northwest A&F University, Rd. Xinong No. 26, Yangling, Shaanxi, 712100, China.

E-mail address: shan@ms.iswc.ac.cn (S. An).

microbial nutrient acquisition and the availability of limiting resources (Deng et al., 2019; Cui et al., 2019; Li et al., 2020; Zhou et al., 2020). Microorganisms produce a large amount of EEAs to target the most limiting nutrient from complex substrates, and the process is controlled by both abiotic and biotic environmental factors (Allison and Vitousek, 2005; Manzoni et al., 2017; Xu et al., 2017). Here, we propose a conceptual framework (Fig. 1a) to describe how soil EES regulates resource utilization for soil microorganisms and their nutrient limitation, which is highly responsive to abiotic and biotic factors. Abiotic factors such as soil pH and nutrient availabilities have been widely reported to affect soil EES (Allison and Vitousek, 2005; Manzoni et al., 2012; Stark et al., 2014; Bowles et al., 2014; Dong et al., 2019); however, the effects remain variable among different ecosystems. For example, abiotic factors had a greater effect on soil EES than biotic factors in Chinese temperate grasslands, with soil total C, N and P contents accounting for most of the variation in soil EES (Peng and Wang, 2016). In forest ecosystems in eastern China, soil pH was positively related to soil EEAs (Zheng et al., 2020). In contrast, soil physical properties such as soil texture and moisture availability drove changes of EES in degraded grasslands in arid northern Loess Plateau in China (Cui et al., 2018). A recent meta-analysis showed that both temperature and soil C:N ratio were crucial factors explaining the variation in soil EES in China's forest ecosystems (Zhou et al., 2020).

Biotic factors, such as plant traits and soil microbial properties, on the other hand, have also been frequently reported to affect soil EES across different ecosystems. For instance, Tapia-Torres et al. (2015) demonstrated that microorganisms were co-limited by both soil N and P availabilities in the Chihuahuan Desert in Coahuila, Mexico. Soil EES has also been reported to be modified by plant diversity and species richness in arid grasslands on the Loess Plateau in China (Cui et al., 2019). A recent study showed that vegetation type rather than climate modulated the variation in soil EES in subalpine forests on the eastern Tibetan

Plateau of China (He et al., 2020). Therefore, results on the impact of abiotic and biotic factors on soil EES across different ecosystems have been inconsistent, possibly due to differences in vegetation type, soil characteristics, and climate conditions among the ecosystems studied (Sinsabaugh et al., 2009; Sinsabaugh and Follstad Shah, 2012; Bowles et al., 2014; Dong et al., 2019).

Grasslands are one of the most widely distributed ecosystems and play a central role in the global C cycle (Scurlock and Hall, 1998). In recent decades, global changes induced by human activities (mainly land use change and fossil fuel combustion) have dramatically accelerated the decline of plant productivity and soil C sequestration, as well as altering microbial community structure and composition (Fang et al., 2018). Under this global change background, grassland ecosystems have widely been degraded due to unsustainable use and environmental change (Xiao et al., 2018). Restoration of grasslands has been proven to be an effective practice to reverse the decline in plant productivity by improving a series of ecosystem services and functions (Liu et al., 2019). Although the effects of abiotic and biotic factors on soil nutrient availability and microbial community structure have extensively studied, we still do not have a good understanding of factors controlling microbial nutrient limitation and their relationship with soil EES. To this end, we studied microbial nutrient limitation by assessing soil EES, and examined the effect of abiotic and biotic factors on soil EES during grassland restoration on the Loess Plateau, in northwestern China. Here, our objective was to test the following three hypotheses: (1) the activities of C-, N- and P-acquiring enzymes increase with year since grassland restoration, as the demand for nutrients increases with the development of the vegetation, and increased EEAs accelerates nutrient cycling to meet the increasing nutrient demand; (2) microorganisms are generally limited by soil N and P levels, and the N limitation will be exacerbated over time since grassland restoration, as N is often the most limiting nutrient which will be amplified with the development of the vegetation; and (3) the variations in soil EES are driven by abiotic and biotic

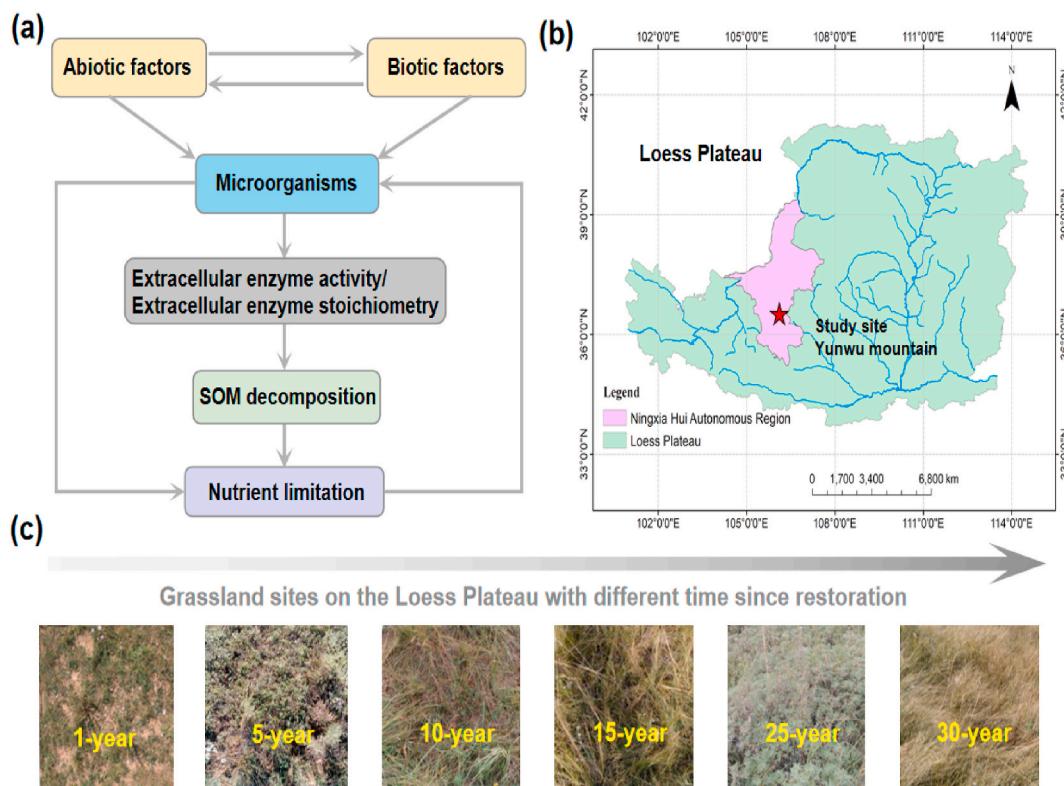


Fig. 1. a) Soil extracellular enzyme stoichiometry regulates soil resource utilization for microorganisms, that are affected by abiotic and biotic factors; b) Location of the study area in Yunwu Mountain, Ningxia Hui Autonomous Region, People's Republic of China; and c) Photos of representative vegetation at the six sites with different times since grassland restoration.

factors during grassland restoration.

2. Materials and methods

2.1. Study sites

This study was conducted in the Yunwu Mountain Nature Reserve ($106^{\circ}24' - 106^{\circ}28'E$, $36^{\circ}13' - 36^{\circ}19'N$), located in the Ningxia Hui Autonomous Region, the Loess Plateau of China (Fig. 1 b). The elevation of the Reserve ranges from 1800 to 2100 m. The annual precipitation ranges from 400 to 450 mm, with 60–75% occurs between July and September. The total amount of evaporation ranges between 1017 and 1739 mm per year. The average annual temperature is about $7.01^{\circ}C$. The annual total hours of sunshine is approximately 2500 h, on average, while there are 137 frost-free days in the year. The soil type is classified as a Calcaric Cambisol based on the soil classification system from the Food and Agriculture Organization (FAO). Historically, grasslands in this region had been degraded due to overgrazing (>50 sheep/ha) and cultivation for crop production. Since 1982, large areas of degraded grasslands have been fenced for natural ecological restoration, resulting in a 30-year chronosequence of restoration grasslands without human disturbance and grazing. The plant community in those restored sites is

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

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

In addition, the following abiotic factors were measured in this study: soil moisture content was determined using a WET-2 sensor (Delta-T Devices Ltd., Cambridge, UK), pH with a pH meter (Model PHS-2, INESA Instrument, Shanghai, China), organic C by the potassium dichromate external heating method (Nelson, 1996), total N by the Kjeldahl method (Belay-Tedla et al., 2009), and total P by the molybdenum antimony colorimetric method. Biotic factors measured include microbial biomass C (MBC), N (MBN) and P (MBP), all determined with the chloroform fumigation-extraction method (Brookes et al., 1985).

2.4. Data and statistical analyses

Vector analysis (vector length [L, unit less] and vector angle [A, °]) of soil EEAAs was conducted to evaluate microbial nutrient limitation. Vector length and angle were calculated as follows (Moorhead et al., 2016):

$$\text{Vector length (L)} = \sqrt{[\ln(\text{BG})/\ln(\text{NAG} + \text{LAP})]^2 + [\ln(\text{BG})/\ln(\text{AP})]^2} \quad (4)$$

$$\text{Vector angle (A)} = \text{Degrees}\{\text{ATAN2}[(\ln(\text{BG}) / \ln(\text{AP}), (\ln(\text{BG}) / \ln(\text{NAG} + \text{LAP}))]\} \quad (5)$$

generally dominated by *Stipa grandis* P. Smirn., *Stipa przewalskyi* Roshev., *Artemisia sacrorum* Ledeb. and *Thymus mongolicus* Ronn.

2.2. Sampling design

The field sampling was carried out in August 2019. Grassland sites with different year since restoration (1-, 5-, 10-, 15-, 25- and 30-year old sites) were established in this region (Fig. 1 c). These study sites had similar topographies, including elevation, slope aspect and position.

For each restoration site, a 60×60 m plot was established, and 15 sub-plots (1×1 m) were homogenized arranged in each plot. The samples in each sub-plot were collected with five soil cores from the 0–10 cm depth with a 5-cm diameter stainless-steel corer, and mixed to a composite soil sample. Samples were placed on ice in a cooler and transported back to the laboratory. The samples were processed by removing the roots and large debris and stored at $4^{\circ}C$ prior to the analysis of soil EEAAs and microbial biomass. A portion of the soil samples was passed through a 2-mm sieve and ground to fine powder using a ball grinder (RM200, Retsch, Haan, Germany); those samples were used for soil C, N, and P analyses. All analyses were carried out in triplicate.

2.3. Analysis of soil EEAAs and EES

We quantified the activities of soil C- (α -1,4-glucosidase, AG; β -1,4-glucosidase, BG; xylosidase, XS; and β -D-cellobiohydrolase, CB), N-leucine aminopeptidase, LAP and β -N-acetylglucosaminidase, NAG), and P-acquiring enzymes (acid phosphatase, AP). These EEAAs were measured using standard fluorometric techniques with highly fluorescent compounds 7-amino-4-methylcoumarin and 4-methylumbelliferylone (Sinsabaugh et al., 2008). Enzyme commission numbers, incubation times, and substrates used for each enzyme assay are in Table S1. Soil EEAAs were expressed in units of nmol activity g^{-1} dry soil h^{-1} ($\text{nmol g}^{-1} \text{soil } h^{-1}$), and soil EES was calculated as the ratio of C-, N-, and P-acquiring enzymes (Sinsabaugh et al., 2008).

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

Also, vector angles above 45° were considered to be relatively more limited by P than N with the opposite interpretation for lower angles (Sinsabaugh et al., 2009).

We tested the heterogeneity of variances, and the data were log-transformed or standardized prior to statistical analysis by SAS 9.3 version (<http://support.sas.com/>). All data were analyzed with one-way analysis of variance using Fisher's test. Pearson's correlation coefficients for correlations among abiotic and biotic factors, and soil EEAAs and EES were calculated. Redundancy analysis was performed in CANOCO 5.0 (<http://www.canoco5.com/>). We determined the relative contributions of abiotic and biotic factors on the variance of soil EES through variation-partitioning analysis. In addition, three variable groups—soil EES, abiotic factors and biotic factors—were decomposed in priori structural equation models (SEMs), and the final SEMs were fitted to present the pathways of the interaction effects among soil EES, abiotic and biotic factors. Structural equation models were conducted using Mantel R values in AMOS (<https://spssau.com/front/spssau/index.html>). Variation-partitioning analysis was performed and linear regression models were fitted using the R software v3.4.2 (<https://www.r-project.org/>) using the “vegan” and “gvlma” packages.

3. Results

3.1. Changes in soil EEA and EES over time in grassland restoration

Soil moisture, organic C and N, and microbial biomass C and N contents all increased with year since restoration, peaking in the 30-year old site, whereas soil pH, total P and microbial biomass P contents showed an opposite trend (Table S2). Most of soil EEAAs were higher in the 25- and 30-year old sites than those in the younger sites (Table 1). Soil BG (C-acquiring enzyme), NAG + LAP (N-acquiring enzymes), and AP (P-acquiring enzyme) activities increased with time since restoration, were highest in the 30-year old (25.06, 24.08, and 61.40 nmol $h^{-1} g^{-1}$, respectively) and lowest in the 1-year old site (13.18, 10.27, and

Table 1

Soil extracellular enzyme activities (EEAs) (of C-, N- and P-acquiring enzymes), extracellular enzyme stoichiometry (EES) ($\ln(\text{BG})$: $\ln(\text{NAG} + \text{LAP})$, $\ln(\text{NAG} + \text{LAP})$: $\ln(\text{AP})$ and $\ln(\text{BG})$: $\ln(\text{AP})$), and vector analysis to indicate soil microbial nutrient limitation with time since grassland restoration. Different lowercase letters indicate a significant difference ($p < 0.05$) between sites with different year since restoration according to the Fisher's test.

Parameter	1-year old	5-year old	10-year old	15-year old	25-year old	30-year old	Mean
Soil EEAs	AG (n mol g ⁻¹ h ⁻¹)	2.38 ± 0.34 d	3.60 ± 0.56 c	4.07 ± 0.32 c	8.39 ± 0.56 b	9.78 ± 0.77 b	13.00 ± 0.79 a
	BG (n mol g ⁻¹ h ⁻¹)	13.18 ± 2.13 d	14.59 ± 3.09 c	15.07 ± 2.45 c	18.39 ± 2.67 b	19.38 ± 3.11 b	25.06 ± 3.56 a
	AP (n mol g ⁻¹ h ⁻¹)	49.60 ± 5.22 e	52.98 ± 6.17 d	53.59 ± 5.38 c	54.58 ± 4.77 b	58.39 ± 6.16 b	61.40 ± 5.89 a
	CB (n mol g ⁻¹ h ⁻¹)	1.98 ± 0.24 e	3.59 ± 0.56 d	5.27 ± 0.35 c	6.59 ± 0.67 b	7.78 ± 0.38 ab	8.40 ± 0.82 a
	XS (n mol g ⁻¹ h ⁻¹)	2.78 ± 0.34 d	3.99 ± 0.45 c	4.13 ± 0.27 c	4.27 ± 0.48 b	5.11 ± 0.53 ab	5.45 ± 0.41 a
	LAP (n mol g ⁻¹ h ⁻¹)	7.88 ± 0.57 d	8.98 ± 0.74 d	12.60 ± 1.15 c	13.39 ± 1.56 b	14.98 ± 1.38 ab	15.69 ± 1.76 a
Soil EES	NAG (n mol g ⁻¹ h ⁻¹)	2.39 ± 0.26 d	3.79 ± 0.31 c	4.41 ± 0.38 b	7.67 ± 0.43 a	7.89 ± 0.57 a	8.39 ± 0.39 a
	Ln(BG):Ln(NAG + LAP)	0.68 ± 0.03 d	0.80 ± 0.06 c	0.87 ± 0.05 c	0.95 ± 0.07 b	1.06 ± 0.09 b	1.21 ± 0.07 a
	Ln(BG):Ln(AP)	0.95 ± 0.06 a	0.89 ± 0.07 ab	0.87 ± 0.05 ab	0.85 ± 0.04 b	0.78 ± 0.06 c	0.72 ± 0.05 d
Vectors	Ln(NAG + LAP):Ln(AP)	0.65 ± 0.04 c	0.70 ± 0.05 c	0.76 ± 0.08 a	0.81 ± 0.06 b	0.83 ± 0.05 b	0.87 ± 0.07 a
	Vector length (L)	1.88 ± 0.35 a	1.75 ± 0.24 b	1.76 ± 0.19 b	1.75 ± 0.20 b	1.70 ± 0.27 c	1.68 ± 0.32 c
	Vector angle (A, °)	51.2 ± 3.4 a	47.2 ± 2.9 ab	43.4 ± 3.8 b	40.6 ± 2.7 c	41.0 ± 2.5 c	41.6 ± 3.1 c

AG: α 1,4-glucosidase; BG: β-1,4-glucosidase; CB: β-Dcellobiohydrolase; XS: xylosidase; AP: acid phosphatase; LAP: leucine aminopeptidase; NAG: β-N-acetylglucosaminidase.

49.60 nmol h⁻¹ g⁻¹, respectively) (Table 1). The other C-acquiring enzyme (AG, CB and XB) activities showed a similar trend to that of BG, NAG + LAP and AP (Table 1).

Soil enzyme C:N and N:P ratios increased with time since restoration, peaking in the 30-year old site, and enzyme C:P ratio decreased over time, with enzyme C:N and N:P ratios in the 30-year old site significantly higher than those in the 1-year old site, while the enzyme C:P ratio was

significantly lower in the 30-year than in the 1-year old site (Table 1). Most of enzyme C:P and N:P ratios were less than 1, while enzyme C:N ratios were more than 1, and the ratio of the natural logarithm of C-, N-, P-acquiring enzymes ranged from 1:1.47:1.05 to 1:0.82:1.38, and the average was 1:1.08:1.28 (Table 1), deviating from the global mean ratio of 1:1:1, suggesting an overall P limitation.

Vector analysis (including vector length and angle) of soil EEAs that

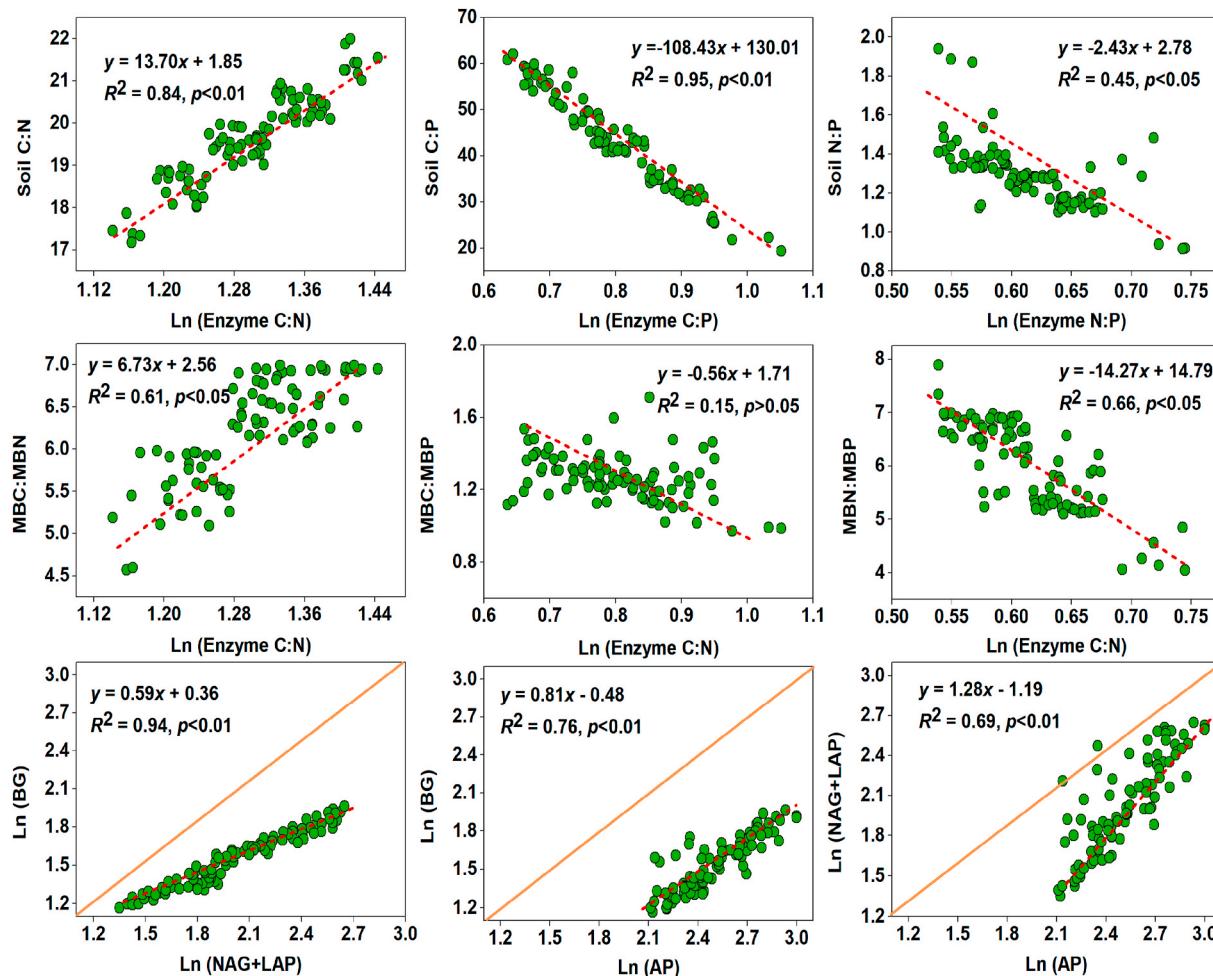


Fig. 2. Standard major axis regressions between soil extracellular enzyme stoichiometry and soil C:N:P or microbial biomass C:N:P stoichiometry ($n = 90$). All regression slopes were significant at $p < 0.01$. The dashed red lines represent the linear fitting equation, and reference lines with a slope of 1.0 are shown in the last three graphs.

was used to evaluate microbial nutrient limitation indicated that vector length gradually decreased with time since restoration (Fig. S1 a), and was at the highest in the 1-year old site (mean value: 1.88) and at the lowest in the 30-year old site (mean value: 1.68); the vector angle in the 1-, 5- and 10-year old sites was greater than 45°, while that in the 15-, 25- and 30-year old sites was less than 45° (Table 1).

3.2. Linkages between soil EES and abiotic-biotic factors

Enzyme C:N ratio had a positive linear relationship with soil C:N and MBC:MBN ratios (Fig. 2). Similarly, enzyme N:P ratio was positively related to soil N:P and MBN:MBP ratios, and enzyme C:P ratio was positively related to soil C:P and MBC:MBP ratios (Fig. 2).

Redundancy analysis revealed that abiotic and biotic factors together explained 92.8% (axis 1, 59.1%; axis 2, 33.7%) of the total variation in soil EES, and there were strong positive relationships between enzyme C:N, N:P ratios and soil moisture content, soil organic C, C:N, and N:P (Fig. 2), but strong negative relationships between enzyme C:N, N:P ratios and pH (Fig. S1 b). In the RDA figure, biotic factors accounted for more of the variation in soil EES than abiotic factors; the explanatory ratio of biotic factors increased from 30.1% to 48.2% and that of abiotic factors increased from 21.1% to 35.6%, respectively (Table 2).

Variation-partitioning analysis and structural equation models indicated that there was a high comparative fit index (CFI) (>0.90), low root mean square error of approximation (RMSEA <0.05), low chi-square (χ^2), low Akaike Information Criterion (AIC), and no statistical significance ($p > 0.05$). These parameters indicated that soil EES was strongly explained by abiotic and biotic factors. Our data showed that biotic factors accounted for a greater percentage of the variance in EES (39.1%) than abiotic factors (36.3%), consistent with results from the redundancy analysis.

4. Discussion

4.1. Changes in soil EEA with time since grassland restoration

Soil EEAs increased with time since restoration, peaking in the 30-year old site (Table 1), supporting our first hypothesis that C-, N- and P-acquiring enzyme activities increased during grassland restoration. This finding is consistent with results reported for forests (Xu et al., 2017; Shao et al., 2018; Feng et al., 2019), grasslands (Peng and Wang, 2016; Zhang et al., 2016; He et al., 2020; Wang et al., 2020), abandoned farmlands (Cui et al., 2018, 2019), and desert ecosystems (Liu et al., 2017). In the early stage of grassland restoration (i.e., in the 1- and 5-year old sites), the soil had low moisture and nutrient availabilities, and microbial biomass (Table S2), and most of the vegetation consisted of annual herbaceous plants with shallow roots. Thus, the capacity of microorganisms to perform exoenzymatic synthesis was low due to limited availability of organic C substrate, ultimately limiting soil EEAs (Li et al., 2020; Liu et al., 2018). In contrast, the 30-year old site had bigger plants with larger root systems, and more resources were

available to microorganisms, in the form of above- and below-ground litter input, for the synthesis of soil EEAs (Zhang et al., 2019). However, soil and microbial biomass P content declined with time since restoration (Table S2), most likely due to plant uptake and immobilization of P in plant biomass, which may limit P availability in the soil (Zhang et al., 2016, 2017). In addition, the increased input and decomposition (releasing organic acids) of plant litter caused the soil pH to change from alkaline to neutral (Table S2), and the more neutral soil pH increased the migration of P contents, ultimately leading to the decline of soil and microbial biomass P contents with time since restoration.

A global meta-analysis by Sinsabaugh et al. (2008) demonstrated that the ratio of C:N:P acquisition enzymes in the topsoil was 1:1:1 on a global scale, suggesting that there is a broad pattern for microorganisms to maintain stoichiometric homeostasis of C, N and P in different ecosystems. However, this ratio is highly variable depending on ecosystem type and regional environmental conditions. For example, the ratio of C:N:P acquisition enzymes was close to 1:1:1 in the topsoil in forest ecosystems in eastern China (Xu et al., 2017), but deviated from 1:1:1 in China's forests based on a nation-wide dataset (Zhou et al., 2020). In temperate grassland ecosystems in northern China, Peng and Wang (2016) reported a value of 1:1.2:1.4 for the ratio of C:N:P acquisition enzymes. In this study, the average ratio of C:N:P acquisition enzyme was 1:1.08:1.28, which also deviated from 1:1:1, indicating (1) relatively smaller C than N or P-acquiring enzyme activities and (2) relatively larger activities of P-acquiring enzyme activities than N or C.

4.2. Soil enzyme stoichiometry reveals microbial nutrient limitation in grassland restoration

The vector angles being greater than 45° in the 1-, 5- and 10-year old sites but less than 45° in the 15-, 25- and 30-year old sites (Table 1) support our second hypothesis that microorganisms were generally limited by soil N and P levels, and shifted from P-limitation to N-limitation with time since restoration (see summary in Fig. S1 a). Nitrogen-limitation aggravated in the late-stage of grassland restoration, leading to microbial metabolism secreting more N- than P-acquiring enzymes (Wang et al., 2020), thereby resulting in increased enzyme N:P ratio with time since restoration (Fig. 4). The greater root biomass in the 30-year old site likely provided more exogenous resources to microorganisms (Cui et al., 2018, 2019), as reflected by the increasing soil C and N contents, with a greater increase in soil C than in N, causing the soil C:N ratio to increase (Table S2), which likely decreased soil N availability (Waring et al., 2014; Zhao et al., 2018; Zhang et al., 2019; Liu et al., 2019; Rosinger et al., 2019; Zhou et al., 2020), and resulting in aggravating N-limitation of microorganisms.

In line with our third hypothesis, the abiotic and biotic factors explained most of the variations in soil EES based on the redundancy analysis (Fig. S1 b; Table 2) and variation-partitioning analysis (Fig. 3). The greater proportion of variation in soil EES accounted for by biotic than by abiotic factors was likely because biotic factors such as soil microorganisms are directly involved in the decomposition of soil organic matter and stimulate the production of EEAs (Chen et al., 2018). Interestingly, the total variance of soil EES explained by the abiotic and biotic factors increased with time since restoration (Table 2), linked with the improved environmental conditions, such as soil moisture availability, and microbial biomass generally increased with time since grassland restoration (Table S2), and such increases considerably enhanced the metabolism of microorganisms (Zhang et al., 2019; Zheng et al., 2020; Zheng et al., 2020), ultimately stimulating the synthesis of EEAs (Cui et al., 2019).

Finally, we built a theoretical framework to summarize the effects of grassland restoration on soil EES and microbial nutrient limitation (Fig. 4). We show that soil EEAs increased with time since restoration, which was linked with increased plant root biomass over time in grassland restoration; increased plant root biomass promoted the

Table 2

Variance explained by abiotic and biotic factors for soil extracellular enzyme stoichiometry based on redundancy analysis (RDA). Total variance consists of the respective contributions of each set of explanatory variables as well as their covariance. Significance was assessed using $n = 999$ permutations of the full RDA model or the appropriate partial redundancy analysis model, at $p < 0.001$.

Site	Biotic factor	Abiotic factor	Total variance (%)	<i>p</i> -value	<i>F</i>
1-year old	30.1	21.1	65.0	<0.05	45.1
5-year old	35.1	23.7	69.4	<0.05	62.1
10-year old	38.8	27.1	74.2	<0.05	71.1
15-year old	44.1	29.3	79.1	<0.01	83.4
25-year old	46.3	33.3	82.2	<0.01	91.8
30-year old	48.2	35.6	86.1	<0.01	97.3

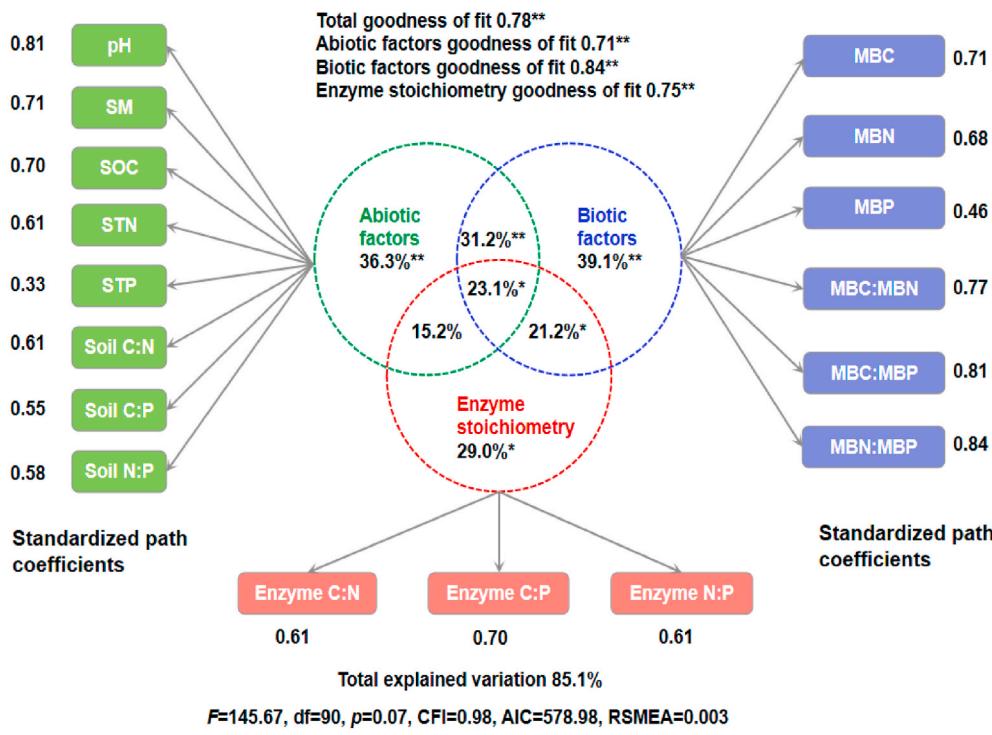


Fig. 3. Venn diagrams based on results from structural equation models (SEMs) and variation-partitioning analysis (VPA), explaining the contribution of abiotic and biotic factors to soil extracellular enzyme stoichiometry (EES). The standardized coefficients are from the SEMs. Parameters in rectangular frames denote the measured variables. Values in the green circle and rectangular frames denote abiotic factors, those in the blue circle and rectangular frames denote biotic factors, and those in red circle and rectangular frames denote EES. The numbers adjacent to the rectangular frames are standardized path coefficients, analogous to relative regression weights. From VPA, the following fractions were obtained: the effect of abiotic factors (green circle); effect of biotic factors (blue circle); effect of soil EES (red circle); and their combined effects and respective variations explained are in areas overlapping by the different colored circles. Note: The path coefficients and explained variability were calculated using 999 bootstrap replicates. Models with different structures were assessed using the goodness-of-fit statistic (a measure of the overall predictive performance). * $p < 0.05$, and ** $p < 0.01$.

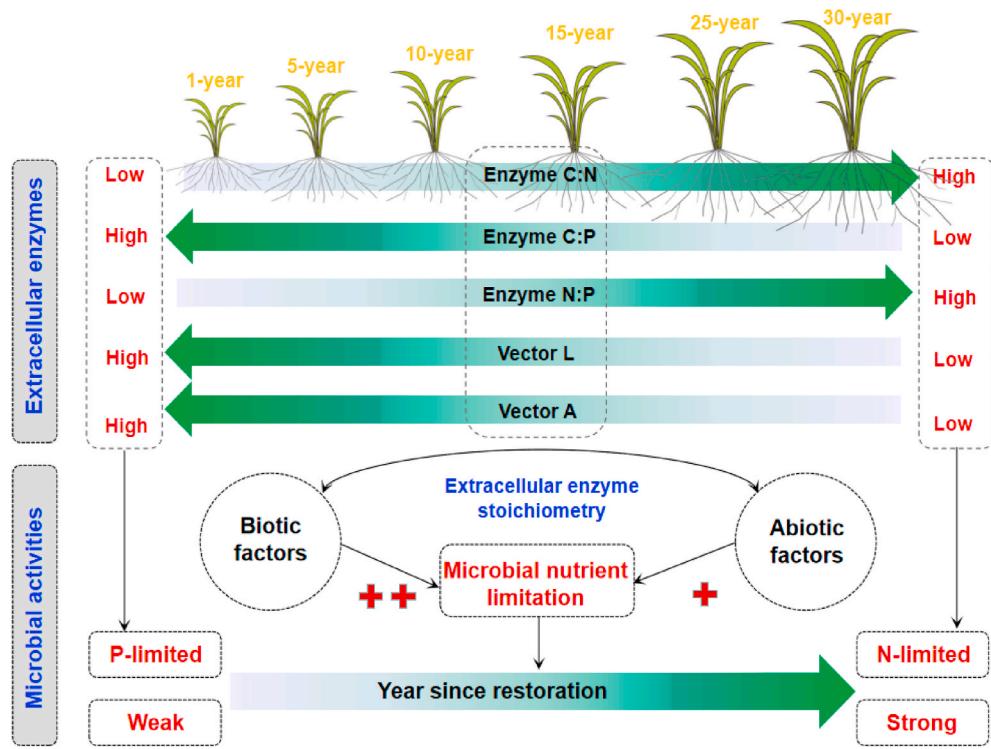


Fig. 4. A theoretical framework exploring the effects of grassland restoration on soil extracellular enzyme stoichiometry (EES) in relation to biotic and abiotic factors. Soil EES induced by microorganisms affect soil organic matter decomposition and facilitate plant growth. The key changes in soil EES and microbial nutrient limitations over time in the grassland restoration process are presented in the figure.

activities of protein- and chitin-degrading enzymes, resulting in increased C and N mineralization to cope with N limitation (Allison and Vitousek, 2005; Chen et al., 2014, 2018; Manzoni et al., 2017; Xu et al., 2017). Both soil enzyme C:N and N:P ratios increased, while enzyme C:P ratio, vector length and angle decreased with time since restoration,

indicating that microorganisms shifted from P- to N-limitation over time. More importantly, the variation in soil EES was better accounted by biotic factors than abiotic factors, and the joint influence of biotic and abiotic factors on soil EES strengthened with time since restoration.

We conclude that the ecoenzymatic stoichiometry approach is a

useful technique in assessing relationships in microbial resource ecology; in grassland restoration systems soil microorganisms can be co-limited by N and P levels, while the limitation shifted from P- to N-limitation as the N limitation exacerbated over time. This study provides evidence that soil EES can be used to interpret microbial nutrient limitation in grassland restoration. Our data should help in developing restoration strategies such as the requirement for nutrient management in grassland ecosystems on the Loess Plateau. We also conclude that both abiotic and biotic factors should be considered in future studies on microbial nutrient limitation in ecosystem restoration.

Author contributions

YY and HC conceived and designed this study. YY, YQW performed field work, sample and data analyses. YY wrote the first draft of the manuscript. SSA and CL provided constructive suggestions. YY and SXC revised the manuscript. All authors contributed to the final version of the manuscript.

Declaration of competing interest

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

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Appendix A. Supplementary data

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