Natural grassland as the optimal pattern of vegetation restoration in arid and semi-arid regions: Evidence from nutrient limitation of soil microbes

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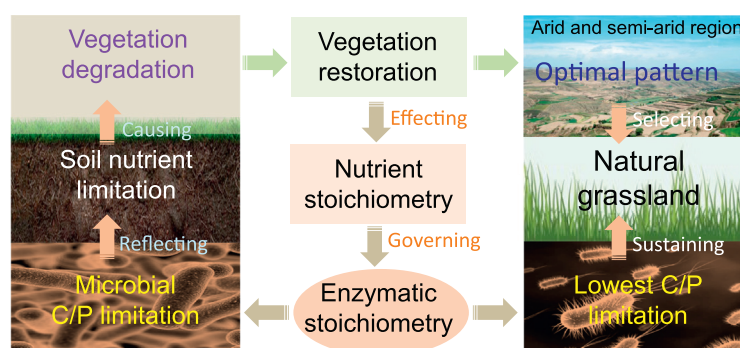[b] University of Chinese Academy of Sciences, Beijing, 100049, PR China
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HIGHLIGHTS

- Microbial metabolisms were co-limited by C and P in arid and semi-arid regions
- Vegetation restoration affected the characteristics of soil microbial metabolism
- Microbial C and P limitations were the lowest in the natural grassland
- Microbial metabolic limitations depend on the balance of nutrient stoichiometry

GRAPHICAL ABSTRACT

ABSTRACT

Soil microbial metabolism is vital for nutrient cycling and aboveground ecosystem stability. A general understanding of microbial metabolism and nutrient limitation under human disturbance in arid and semi-arid regions, which are the largest and most fragile oligotrophic ecosystems globally, however, is still limited. We quantified and compared the characteristics of nutrient limitation of soil microbes under natural/artificial grassland and shrubland, an ecological forest, an economic forest, and sloped cropland in typical arid and semi-arid ecosystems on the Loess Plateau, China. Vegetation restoration significantly affected the activities of extracellular enzymes and ecoenzymatic stoichiometry mainly by affecting soil nutrients and nutrient stoichiometry. A vector analysis of enzyme activity indicated that microbial communities were co-limited by carbon (C) and phosphorus (P) in all types of vegetation restoration. Linear regression indicated that microbial C and P limitations were significantly correlated with the stoichiometry of soil nutrient, suggesting that the balance of nutrient stoichiometry is an important factor maintaining microbial metabolism and elemental homeostasis. C and P limitations in the microbial communities were the lowest in the natural grassland. This implies that both vegetation and microbial communities under the restoration pattern of natural grassland were more stable under environmental stress, so the restoration of natural grassland should be recommended as the preferred option for ecosystem restoration in these arid and semi-arid regions.

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1. Introduction

Land degradation has become more intense around the world under climate change, especially in arid and semi-arid regions due to

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anthropogenic activities and poor land management (Lal, 2001). Approximately 1.5 billion people are directly affected by land degradation (Godfray et al., 2010). Land degradation not only induces the deterioration of ecological functions and land productivity by soil erosion, desertification, and salinization, but also threatens socio-economic and cultural development at regional and global scales (Zhang et al., 2011; Liu and Shao, 2015). Ecological restructuring and vegetation restoration have recently been carried out globally for preventing land degradation and mitigating climate change (Deng et al., 2014).

Ecological restructuring and vegetation restoration have brought large ecological benefits. Vegetation restoration can prevent soil erosion (Zhang et al., 2011; Deng et al., 2012), increase the sequestration of soil carbon (Lal, 2002; Deng et al., 2014), improve soil nutrient status (Cao et al., 2008; Wang et al., 2011) and improve microbial properties (Fu et al., 2009; Zhang et al., 2016). Ecological restructuring and vegetation restoration in arid and semi-arid regions, however, are confronted with serious challenges due to the constraints of various environmental factors. Soil water, one of the most limiting factors in arid and semi-arid regions, is the key factor affecting vegetation survival and ecosystem stability in arid and semi-arid ecosystems (Wang et al., 2008). For example, continuous water consumption by vegetation leads to a deficiency of soil water and the emergence of dried soil layers, which can cause the degeneration of vegetation communities on the Loess Plateau in China (Wang et al., 2011; Liu and Shao, 2015). Nutrient contents and plant-microbe interactions also play key roles in plant growth and ecosystem stability (Bloom et al., 1985; Fowler, 1986; Forde and Lorenzo, 2001), a deficiency of soil water is not the sole cause of vegetation degradation. The roles of nutrients regulated by microorganisms in vegetation degradation in arid and semi-arid ecosystem have nevertheless rarely been studied.

Microorganisms provide plants with available nutrients but also compete nutrients with roots for nutrients when deficient (Inselsbacher et al., 2010; Sinsabaugh and Shah, 2011). Nutrient availability is generally low in arid and semi-arid regions such as the Loess Plateau because of the low primary productivity, high nutrient immobilization by calcium and magnesium, and high nutrient loss by soil erosion (Feng et al., 2013). The low nutrient availability may thus also be an important factor limiting plant growth. Soil water deficiency can also limit the transport and availability of soil nutrients, which would intensify the competition for nutrients between plants and microbes in micro-environments (Ouyang et al., 2016). A deficiency of available nutrients may greatly aggravate vegetation degeneration in arid and semi-arid regions due to the strong competition for nutrients between plants and microbes. The characteristics of microbial metabolic limitations are therefore important indicators for determining the relationship between vegetation degeneration and soil nutrient limitation in arid and oligotrophic ecosystems.

Microorganisms acquire soil nutrients by secreting extracellular enzymes to degrade complex organic compounds. The secretion of extracellular enzymes is also the rate-limiting step for microorganic metabolism (Jones et al., 2009). Extracellular enzymatic activities (EEA) can thus be used to identify the relationships between microbial metabolism and nutrient cycling (Sinsabaugh et al., 2008; Burns et al., 2013; Duan et al., 2018). Ecoenzymatic stoichiometry is an indicator of the ability of microorganisms to use nutrients, which can be used to assess the flow of energy in an ecological system (Jones et al., 2009; Sinsabaugh et al., 2008, 2009) and to estimate the balance between microbial nutrient demand and soil nutrient supply (Sterner and Elser, 2002; Sinsabaugh et al., 2009). For example, ecoenzymatic stoichiometry analysis has been used to identify phosphorus (P) limitation in tropical forest eocsystems (Waring et al., 2014) and nitrogen (N) and P colimitation in arid and oligotrophic ecosystems (Tapia-Torres et al., 2015). Studying ecoenzymatic stoichiometry for identifying microbial metabolic limitations in anthropogenic arid and semi-arid ecosystems is thus crucial.

To illustrate the microbial metabolic limitations through ecoenzymatic stoichiometry, Sinsabaugh et al. (2008) developed a method to visualize the relative C, N and P controls on soil microbial communities by plotting ratios of enzymatic activities associated with C, N and P acquisition. Moorhead et al. (2016) proposed using the 'length' and 'angle' of vectors in plots of proportional C:N vs. C:P activities of enzyme to quantify the relative investments in C vs. nutrient acquisition (vector length) or P vs. N acquisition (vector angle). Translating these ratios into vector lengths and directions (angles) indicated the simultaneous, relative resource demands of the microbial community independent of variations in total enzyme activity and provided clear metrics of relative C limitation (length) and relative P vs. N limitation (angles) (Moorhead et al., 2016). Fanin et al. (2016), for example, used vector analysis of ecoenzyme activities to determine that the overall N requirement of microbial communities increased relative to P during litter decomposition, but that the C requirement increased more rapidly than either N or P in most of ecosystems. The application of this method can help to identify microbial metabolic limitation, which may provide key evidence of plant death during vegetation restoration.

Vegetation degradation is particularly serious on the Loess Plateau (Lal, 2002; Wu et al., 2003). The 'Grain-for-Green' programme, one of the world's most ambitious conservation set-aside programmes, was launched in China in 1999 (Feng et al., 2013; Deng et al., 2017). Much of the reconstructed vegetation on the Loess Plateau has died and aged, as in many other places in recent years (Chen et al., 2007; Zhao et al., 2014). The Loess Plateau, as a typical area with degraded vegetation, therefore provides an opportunity to identify the factors that play important roles in limiting survival of vegetation in arid and semi-arid regions and to determine the best patterns of vegetation restoration.

Considering the water-limited and oligotrophic environmental conditions in arid and semi-arid regions, we hypothesized that: (1) soil microbial communities would suffer from strongly nutrient limitation because soil water limits nutrient availability and microbial activity, (2) microbial nutrient limitations would vary greatly under different patterns of vegetation restoration due to the distinct plant-microbe feedbacks and strategies of nutrient acquisition, and (3) microbial nutrient limitations would be strongly correlated with the stoichiometries of soil nutrients and the activities of extracellular enzymes due to the elemental stoichiometric balance of microbial biomass. We therefore investigated the characteristics of extracellular enzymes involved in C, N, and P cycling, and identified microbial nutrient limitation in various patterns of vegetation restoration on the central Loess Plateau (a typical fragile ecosystem). This study provides fundamental information and novel ideas for assessing vegetation degradation in arid and semi-arid systems.

2. Materials and methods

2.1. Study site and sampling

The study site was in the Zhifanggou Watershed, Shaanxi Province, China (36°46′N, 109°16′E, Fig. 1). The watershed contains the typical types of vegetation in this hilly-and-gully region of the Loess Plateau. There has a semi-arid climate, with a mean annual temperature of 8.8 °C and a mean annual precipitation of 510 mm, most of which falls from July to September. The soil is mainly composed of a Huangmian soil (Calcaric Cambisol, FAO classification), developed on wind-deposited loessial parental material and characterized by yellow particles, absence of bedding, silty texture, looseness, macroporosity, and wetness-induced collapsibility. The study area is subjected to both wind and water erosion.

Soil and water conservation, integrated management, and vegetation restoration have been carried out in this area since the mid-1980s as a pilot project (Ping and Liu, 2009). The conversion has caused a significant change in vegetation type. The region currently consists of grassland, shrubland, forest, and sloped cropland. It has also been used to monitor vegetation restoration as a field experimental site of the
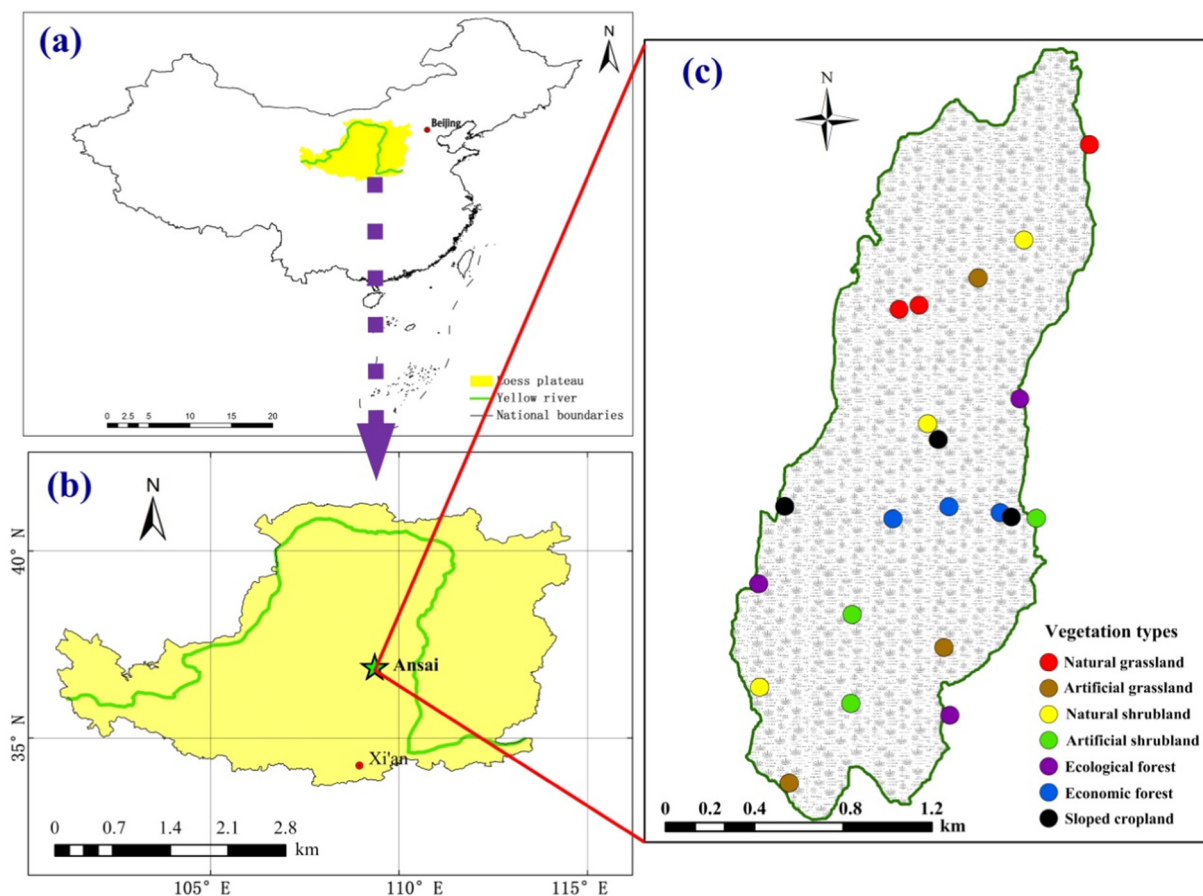
Fig. 1. Locations of the Loess Plateau in China and of the experiment sites in the northern region of the Loess Plateau, and map of the sampling sites.

Institute of Soil and Water Conservation, Chinese Academy of Sciences. The restoration of the vegetation has improved the ecological environment and the living standard of the local villagers. The grassland especially provides feed for livestock, and the economic forests have greatly increased the incomes of farmers (Zhang et al., 2013).

Six types of vegetation were selected as study lands at subjects in June 2016: natural grassland, artificial grassland, natural shrubland, artificial shrubland, ecological forest, and economic forest. Natural vegetation was restored after farmland was abandoned, whereas the artificial land uses were planted with vegetation but not fertilized or subsequently managed. All soils had developed from the same parental materials. The sites had similar elevations and had been previously subjected to similar farming practices. The main crops grown at these sites prior to the conversion of sloped cropland were millet (*Setaria italic*) and soybean (*Glycine* max) in rotation. Sloped cropland currently growing millet and soybean was used as a control site. The crops were manually harvested, and the plots at this site were manually ploughed to a depth of 20 cm each year after harvesting. The sloped cropland was fertilized each year with 6.0 t ha^{-1} goat manure, 60 kg ha^{-1} N, and 45 kg ha^{-1} P$_2$O$_5$. Properties of the experimental sites are shown in Table 1.

Three 100×100 m sampling sites with similar slopes, aspects, and altitudes were selected for each vegetation type, which were considered as true replicates. Three plots (10×10 m) were established at each sampling site with >15 m between the plots. Soil samples (0–20 cm) were collected using soil auger (diameter, 4 cm) from five random locations at each plot. Each sample was passed through a 2-mm sieve and then divided into two subsamples. One subsample was immediately stored at 4 °C for assays of enzymatic activity, and the other subsample was air-dried for physicochemical analysis. Soil bulk density and soil water content were measured at each plot.

2.2. Measurement of soil physical and nutrient properties

About 120 g of fresh soil for each sample were oven-dried at 105 °C to constant weight for the determination of soil water content using the

Table 1
Geographical features and floristic composition of the sampling sites.

Vegetation types	Restoration years (a)	Altitude (m)	Slope aspect	Slope gradient	Main vegetation or crop species
Natural grassland	30 ± 5	1166	E45°S	14°	*Agropyron cristatum*; *Stipa bungeana*; *Incarvillea sinensis*
Artificial grassland	30 ± 5	1132	E55°S	18°	*Medicago sativa*; *Artemisia argyi*; *Agropyron cristatumda*
Natural shrubland	30 ± 5	1123	E55°N	20°	*Robinia pseudoacacia*
Artificial shrubland	30 ± 5	1076	E45°S	19°	*Caragana microphylla* L.
Ecological forest	30 ± 5	1148	E15°N	22°	*Pinus tabuliformis*; *Salix*; *Armeniaca sibirica*
Economic forest	30 ± 5	1220	E12°S	10°	*Malus pumila* M.
Sloped cropland	30 ± 5	1194	E35°N	15°	*Zea may*; *Broom corn millet*; *Glycine max* (L.)

gravimetric method. Soil bulk density was determined using ring sampler weighing. Soil pH was estimated for a 1:2.5 soil:water (w/v) mixture using a glass electrode meter (InsMark™ IS126, Shanghai, China). Soil organic matter was analyzed using dichromate oxidation; about 0.60 g air-dried soil with 5 ml of 0.8 M $K_2Cr_2O_7$ and 5 ml of H_2SO_4 was digested for 5 min at 170–180 °C and was then titrated with 0.2 M $FeSO_4$. Dissolved organic C was extracted with deionized water after shaking for 45 min and then filtered through a Millipore 0.45-μm filter (Jones and Willett, 2006). Total N (TN) was measured by the Kjeldahl method (Bremner and Mulvaney, 1982). In brief, about 0.700 g of air-dried soil with 1.85 g of mixed catalyst (K_2SO_4:$CuSO_4$: Se = 100:10:1) and 5 ml H_2SO_4 was digested for 45 min at 385 °C and was then titrated with 0.02 M HCl. NO_3^--N and NH_4^+-N content were measured using a Seal Auto Analyzer. Total P (TP) content was determined by melt molybdenum, antimony, and scandium colorimetry; 0.25 g of air-dried soil was digested with 2 ml of $HClO_4$ and 3 ml of H_2SO_4 for 60 min at 120–130 °C and then diluted with water to 50 ml. After the overnight stratification of digestion liquid, 5 ml of the supernatant liquid was added to 5 ml the molybdenum antimony reagent, and water was then added to 50 ml. The amount of TP in the solution was measured by an ultraviolet spectrophotometer (Hitachi UV2300) at 700 nm.

2.3. Assays of enzymatic activity

The potential activities of C-acquiring enzymes (β-1,4-glucosidase (BG), β-D-cellobiosidase (CBH)), N-acquiring enzyme (β-1,4-*N*-acetylglucosaminidase (NAG), L-leucine aminopeptidase (LAP)), and organic P-acquiring enzyme (alkaline phosphatase (AP)) were determined by the method of Saiya-Cork et al. (2002). The activities of the extracellular enzymes were measured fluorometrically using a 200 μM solution of substrates labeled with 4-methylumbelliferone (MUB) or 7-amino-4-methylcoumarin (AMC). Using a pipette, 50 μl of 50 mM buffer were dispensed into the wells of black 96-well microplates to serve as the blanks (buffer + slurry); 200 μl of 50 mM buffer were dispensed into wells as the reference standard (buffer + standard) and negative control ((buffer + substrate) (eight analytical replicates per soil per assay each). One gram of previously defrosted soil was homogenized for 2 h with a constant temperature shaker in 125 ml of 50 mM buffer. The soil suspension (soil slurry) was continuously stirred as 200 μl aliquots were dispensed into the microplate wells that served as the sample assay (16 analytical replicate soil suspensions for each sample per assay), blank, and quench standard (slurry + standard) (eight analytical replicates each). Twenty-five microliters of a fluorescence standard solution (10 μM 4-methylumbelliferone-MUB, or 7-amino-4-methylcoumarin-AMC for the LAP assay) was dispensed into microplate wells that served as a reference standard (buffer + standard) and as a quench standard. Finally, sample assays (slurry + substrate) and negative controls (buffer + substrate) also received 25 μl of a 200 μM substrate solution in a final reaction volume of 125 μl. Prepared plates were incubated in the dark at 25 °C for up to 4 h following substrate addition.

Fluorescence was measured without addition of NaOH using a microplate reader with 365 nm excitation and 450 nm emission filters (German et al., 2011). The measurements of well fluorescence for the negative controls, blanks, and quench standard wells were corrected, and enzymatic activity was expressed as nanomoles of substrate released per hour per gram of soil organic matter (nmol g SOM^{-1} h^{-1}).

2.4. Vector analysis of ecoenzymatic activities

We calculated the lengths and angles of the vectors for enzymatic activity for all data based on untransformed proportional activities (*e.g.* [BG + CBH]/[BG + CBH + NAG + LAP]). Vector length was calculated as the square root of the sum of the squared values of *x* and *y*, where *x* represents the relative activities of C *vs.* P acquiring enzymes and *y* represents the relative activities of C *vs.* N acquiring enzymes (Moorhead et al., 2013; Moorhead et al., 2016):

$$Length = SQRT\ (x^2 + y^2) \tag{1}$$

Vector angle was calculated as the arctangent of the line extending from the plot origin to point (*x*, *y*):

$$Angle\ \left(°\right) = Degrees\ (Atan2\ (x, y)) \tag{2}$$

2.5. Data analysis

A one-way ANOVA was used to determine the effects of type of vegetation restoration on soil physicochemical properties, enzymatic activities, and vector characteristics, and means were then compared with a Tukey's multiple comparisons test ($P < 0.05$) using the R software package v.3.3.2. A canonical correspondence analysis (CCA) was used after Hellinger transformation of the data for enzymatic activity and standardization of the data for the environmental factors to identify the most important factors that shaped soil ecoenzymatic activities using the Vegan package in R. Linear regressions were carried out between vector lengths and angles *vs.* components of soil nutrient stoichiometry (soil C:N, soil C:P, and soil N:P) and components of enzymatic stoichiometry (C:N, C:P, and N:P acquisition activities) using OriginPro 9.0. All bar graphs and box plots were drawn using OriginPro 9.0.

3. Results

3.1. Soil physiochemical properties and nutrient stoichiometry

The amounts of soil nutrients differed significantly amongst the patterns of vegetation restoration ($P < 0.05$) (Table 2). Soil organic C (SOC) and TN contents were higher in the soils from natural grassland, natural shrubland, artificial shrubland, and sloped cropland than in the artificial grassland and forest. SOC (8.32 ± 1.32 g kg^{-1}) and TN (1.05

Table 2
Selected soil physicochemical properties from the different vegetation types.

Vegetation types	Soil moisture (%)	Bulk density (g cm^{-1})	pH	SOC (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	C:N ratio	C:P ratio	N:P ratio
Natural grassland	20.20 ± 1.28 a	1.21 ± 0.04 a	8.33 ± 0.05 a	6.86 ± 0.18 ab	0.922 ± 0.02 ab	0.571 ± 0.02 ab	7.42 ± 0.08 ab	12.02 ± 0.30 ab	1.61 ± 0.03 ab
Artificial grassland	15.20 ± 1.48 ab	1.23 ± 0.04 a	8.39 ± 0.04 a	3.37 ± 0.18 c	0.605 ± 0.02 c	0.547 ± 0.03 cd	5.52 ± 0.12 c	6.20 ± 0.35 c	1.11 ± 0.05 c
Natural shrubland	16.86 ± 1.15 ab	1.20 ± 0.04 a	8.32 ± 0.04 a	6.57 ± 0.34 ab	0.858 ± 0.03 ab	0.582 ± 0.03 a	7.57 ± 0.18 a	11.24 ± 0.56 b	1.47 ± 0.05 bc
Artificial shrubland	12.43 ± 1.34 b	1.13 ± 0.04 a	8.36 ± 0.02 a	6.39 ± 0.35 ab	0.862 ± 0.04 ab	0.545 ± 0.05 de	7.37 ± 0.19 ab	11.70 ± 0.62 ab	1.58 ± 0.07 ab
Ecological forest	14.11 ± 1.11 ab	1.14 ± 0.02 a	8.45 ± 0.03 a	4.88 ± 0.21 bc	0.705 ± 0.02 bc	0.532 ± 0.04 de	6.96 ± 0.22 ab	9.15 ± 0.35 bc	1.33 ± 0.04 bc
Economic forest	15.27 ± 2.13 ab	1.29 ± 0.03 a	8.27 ± 0.07 a	4.71 ± 0.05 bc	0.706 ± 0.01 bc	0.562 ± 0.03 bc	6.72 ± 0.12 b	8.38 ± 0.06 bc	1.25 ± 0.02 bc
Sloped cropland	13.00 ± 0.89 b	1.17 ± 0.05 a	8.31 ± 0.06 a	8.32 ± 1.32 a	1.052 ± 0.14 a	0.530 ± 0.02 e	7.27 ± 0.24 ab	15.67 ± 2.47 a	1.98 ± 0.26 a

Note: Values are means ± standard error (n = 27). Different letters in the same column indicate significant differences ($P < 0.05$) amongst the vegetation types based on one-way ANOVA followed by Tukey's test.

± 0.14 g kg^{-1}) contents were the highest in sloped cropland. TP content was higher in the natural grassland and natural shrubland than the other types of vegetation restoration. TP (0.58 ± 0.03 g kg^{-1}) content was the highest in the natural shrubland. Nutrient stoichiometry also differed significantly amongst the types of vegetation restoration ($P < 0.05$). The C:N ratios (7.57 and 5.52) were the highest and lowest in the natural shrubland and artificial grassland, respectively. The C:P ratios (15.67 and 6.20) and the N:P ratios (1.98 and 1.11) were the highest and lowest in the sloped cropland and artificial grassland, respectively.

3.2. Ecoenzymatic activities and enzymatic stoichiometry

The five enzymatic activities and their stoichiometries varied significantly amongst the types of vegetation restoration ($P < 0.05$) (Table 3). BG and CBH activities were higher in the ecological forest than the natural grassland and sloped cropland. NAG activity did not differ significantly amongst the types of vegetation restoration but was the highest in the natural grassland (1.97 × 10^3 ± 208.1 nmol g SOM^{-1} h^{-1}). LAP (1.13 × 10^3 ± 79.2 nmol g SOM^{-1} h^{-1}) and AP (10.52 × 10^3 ± 159 nmol g SOM^{-1} h^{-1}) activities were the highest in the artificial grassland and the lowest (0.4 × 10^3 ± 27.6 and 5.53 × 10^3 ± 82.4 nmol g SOM^{-1} h^{-1}, respectively) in the natural grassland. The (BG + CBH):(NAG + LAP) and (BG + CBH):AP ratios were significantly higher in the artificial shrubland than the other types of vegetation restoration ($P < 0.05$). The (BG + CBH):(NAG + LAP) (0.96 ± 0.2) and (BG + CBH):AP (0.18 ± 0.01) ratios were the lowest in the natural grassland and natural shrubland, respectively. The (NAG + LAP):AP ratio was also the highest (0.42 ± 0.04) and lowest (0.1 ± 0.01) in the natural grassland and natural shrubland, respectively.

3.3. Effect of soil properties on enzymatic activities

The CCA indicated that the variations in soil enzymatic activities were well accounted for (59.5%) by soil properties and nutrient ratios (Fig. 2A). BG activity was strongly negatively correlated with TP content, and NAG activity was strongly positively correlated with most soil nutrients and nutrient stoichiometries. CBH and LAP activities were negatively correlated with most soil nutrients and nutrient stoichiometries ($P < 0.01$ and $P < 0.05$, respectively). The soil properties and nutrient ratios, though, were well accounted for by the ecoenzymatic stoichiometries (65.3%) (Fig. 2B). Enzymatic C:N was strongly negatively correlated with most soil nutrients and nutrient stoichiometries, whereas enzymatic N:P was positively correlated with most soil nutrients and nutrient stoichiometries.

3.4. Characteristics of the vector analysis for microorganism nutrient limitation

The characteristics of ecoenzymatic stoichiometry differed amongst the types of vegetation restoration (Fig. 3), suggesting that the vegetation restoration strongly affected microbial nutrient supply. All datapoints were above the line (1:1 line), which suggesting strong P limitation in the microbial community. Microbial relative C limitation had a wider range in the natural grassland than the other vegetation types due to clusters of higher distribution along the diagonal. We quantified microbial C, N, and P limitation by calculating the vector lengths and angles using relative proportional enzymatic activities (*e.g.* [BG + CBH] / [BG + CBH + AP]) (Fig. 4). The vector lengths (ranging from 0.415 to 0.786) and angles (ranging from 62.1 to 76.6°) differed significantly amongst the types ($P < 0.01$). Vector length and angle (0.415 ± 0.05 and 62.1 ± 5.0°) were the lowest for the natural grassland, indicating the lowest relative C and P limitations of this type of vegetation restoration. Vector length (0.786 ± 0.02) was highest for the artificial shrubland, suggesting the highest relative C limitation. Similarly, the

Table 3
Soil extracellular enzymes activities and enzyme activity ratios from the different vegetation types.

Vegetation types	BG	CBH	NAG	LAP	AP	C:N-enzymes	C:P-enzymes	N:P-enzymes
Natural grassland	0.99 × 10^3 ± 73.7 c	0.23 × 10^3 ± 34.8 d	1.97 × 10^3 ± 208.1 a	0.40 × 10^3 ± 27.6 d	5.53 × 10^3 ± 82.4 d	0.96 ± 0.20 d	0.23 ± 0.02 c	0.42 ± 0.04 a
Artificial grassland	1.90 × 10^3 ± 180.2 b	0.48 × 10^3 ± 29.2 ab	0.43 × 10^3 ± 18.9 b	1.13 × 10^3 ± 79.2 a	10.52 × 10^3 ± 159 a	1.49 ± 0.07 cd	0.23 ± 0.02 c	0.15 ± 0.01 cd
Natural shrubland	1.30 × 10^3 ± 121.2 bc	0.29 × 10^3 ± 25.5 cd	0.35 × 10^3 ± 24.3 b	0.53 × 10^3 ± 54.5 cd	8.58 × 10^3 ± 607 b	1.82 ± 0.06 bc	0.18 ± 0.01 c	0.10 ± 0.01 d
Artificial shrubland	2.75 × 10^3 ± 217.8 a	0.30 × 10^3 ± 10.3 cd	0.41 × 10^3 ± 22.8 b	0.69 × 10^3 ± 34.0 bc	6.75 × 10^3 ± 348 cd	2.91 ± 0.27 a	0.51 ± 0.06 a	0.17 ± 0.01 c
Ecological forest	2.71 × 10^3 ± 168.3 a	0.49 × 10^3 ± 51.1 a	0.71 × 10^3 ± 64.8 b	0.87 × 10^3 ± 104.7 ab	8.94 × 10^3 ± 425 b	2.12 ± 0.10 b	0.35 ± 0.01 b	0.18 ± 0.01 c
Economic forest	2.92 × 10^3 ± 232.0 a	0.37 × 10^3 ± 7.0 bc	0.67 × 10^3 ± 19.5 b	0.89 × 10^3 ± 66.4 ab	7.94 × 10^3 ± 187 bc	2.14 ± 0.13 b	0.40 ± 0.02 ab	0.20 ± 0.01 bc
Sloped cropland	1.03 × 10^3 ± 63.6 c	0.20 × 10^3 ± 15.4 d	0.70 × 10^3 ± 51.9 b	0.71 × 10^3 ± 85.8 bc	5.72 × 10^3 ± 410 d	0.98 ± 0.05 d	0.22 ± 0.01 c	0.24 ± 0.02 b

Note: Values are means ± standard error (n = 27). Different letters in the same column indicate significant differences ($P < 0.001$) amongst the vegetation types based on one-way ANOVA followed by Tukey's test. BG: β-1,4-glucosidase (nmol g SOM^{-1} h^{-1}); CBH: β-D-cellobiosidase (nmol g SOM^{-1} h^{-1}), NAG: β-1,4-N-acetylglucosaminidase (nmol g SOM^{-1} h^{-1}), LAP: L-leucine aminopeptidase (nmol g SOM^{-1} h^{-1}), AP: alkaline phosphatase (nmol g SOM^{-1} h^{-1}), C:N-enzymes: (BG + CBH) / (NAG + LAP), C:P-enzymes: (BG + CBH)/AP, N:P-enzymes: (NAG + LAP) / AP.
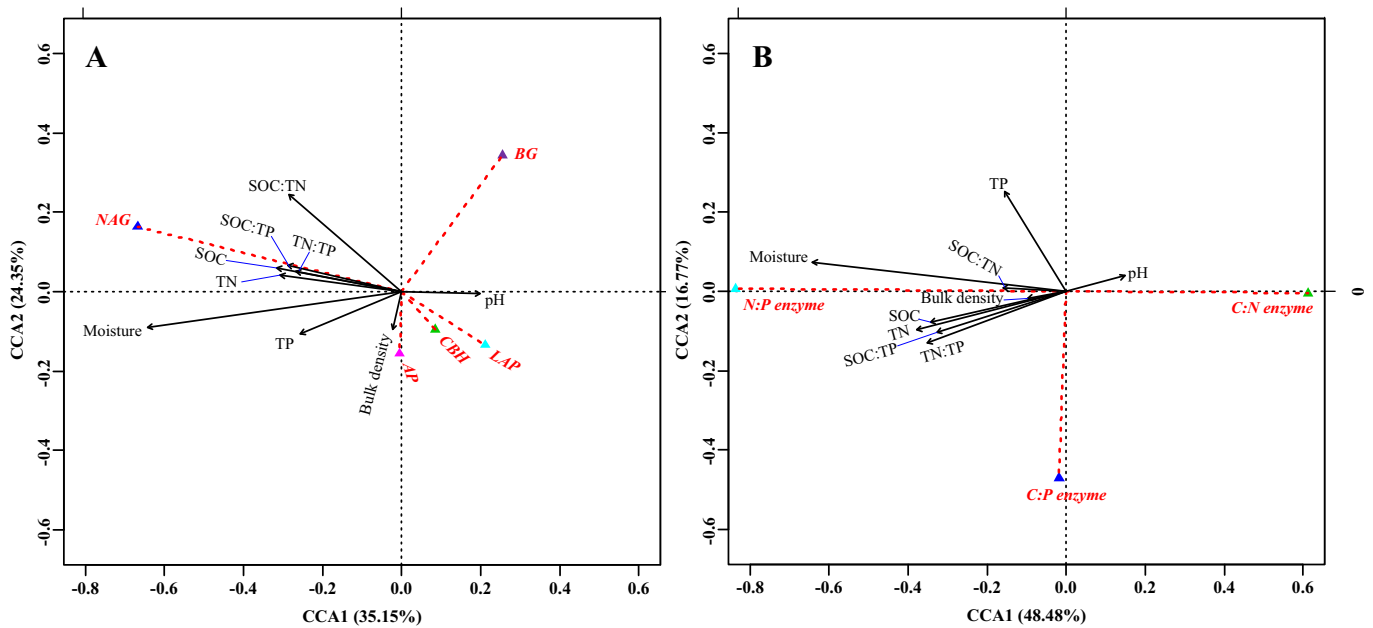
Fig. 2. The canonical correspondence analysis (CCA) used to identify the relationships between soil enzyme activities and soil properties (A), between soil enzyme stoichiometry and soil properties (B). Moisture: soil moisture content, SOC: soil organic carbon, TN: total nitrogen, TP: total phosphorus, BG: β-1,4-glucosidase, CBH: β-ᴅ-cellobiosidase, NAG: β-1,4-N-acetylglucosaminidase, LAP: ʟ-leucine aminopeptidase, AP: alkaline phosphatase. C:N enzyme, C:P enzyme, and N:P enzyme represent (BG + CBH):(NAG + LAP), (BG + CBH):AP and (NAG + LAP):AP, respectively.

vector angle (76.6 ± 1.4°) was highest for the natural shrubland, suggesting the strongest P limitation.

The linear regression analysis identified highly significant correlations between vector lengths/angles and soil nutrient stoichiometry ($P < 0.001$) (Fig. 5). Soil nutrient stoichiometry (soil C:N, soil C:P, and soil N:P was

Fig. 3. Eco-enzyme stoichiometry of the relative proportions of C to N acquisition *versus* C to P acquisition. Vector length was calculated as the square root of the squared sum of the values of x and y [Length = Sqrt($x^2 + y^2$)], and the angle of the vector was calculated as the arctangent of the point (x, y) [Angle (degrees) = Degrees (Atan2(x, y))], where x represents the relative proportion of C *versus* P acquiring enzyme activities and y represents the relative proportion of C *versus* N acquiring enzyme activities. C acquisition is represented by the sum of β-1,4-glucosidase (BG) and β-ᴅ-cellobiosidase (CBH), N acquisition is represented by the sum of β-1,4-N-acetylglucosaminidase (NAG) and ʟ-leucine aminopeptidase (LAP); and P acquisition is represented by alkaline phosphatase (AP).

negatively correlated with vector length and vector angle for both the natural and artificial grasslands (Fig. 5). The vector characteristics, however, were not significantly correlated with soil nutrient stoichiometry for the shrublands, forests or croplands.

4. Discussion

4.1. Effect of vegetation restoration on microbial metabolic activities

The potential activities of the C-, N-, and P-acquiring enzymes were the highest in the ecological forest, natural grassland, and artificial grassland, respectively (Table 3), suggesting that the metabolism of microbial C, N and P was the highest in these three types of vegetation restoration. The influence of vegetation on microbial metabolic activity can be due to 1) the effect on soil physicochemical and nutrient status by the return of litter to the soil (Wang et al., 2012; Yin et al., 2014), and 2) the effect of roots on the microbial community and soil microenvironment by the exchange of matter at the root-soil interface (Okubo et al., 2016; Cui et al., 2018).

Litter from dead leaves, roots, and woody tissue returned to the soil can greatly affect soil properties and the availability of nutrients to microbes (Aerts et al., 1992; Spohn et al., 2013), thus affecting soil microbial biomass and community structure. These effects depend on vegetation type because of the variation of litter quality and quantity for different plant species (Bell et al., 2013; Yin et al., 2014). The effects of the patterns of vegetation restoration on EEA and ecoenzymatic stoichiometry were also attributed to the regulation of soil physicochemical and nutrient status by the vegetation (Cui et al., 2018). SOC, TN, and TP contents and nutrient stoichiometry accounted for most of the variation in EEA and ecoenzymatic stoichiometry (Fig. 2 and Table S2). EEA and ecoenzymatic stoichiometry are normally affected by the status of soil nutrients (Sinsabaugh et al., 2008, 2009). Microbes can secrete more enzymes to meet the demand for nutrients when nutrient availability is low (Sinsabaugh et al., 2008), and soil nutrient contents can have a large impact on EEA and ecoenzymatic stoichiometry by altering the concentrations of available substrates and C, N, and P stoichiometry (Kivlin and Treseder, 2014). The species of vegetation in our study strongly affected SOC, TN, and TP contents and nutrient stoichiometry
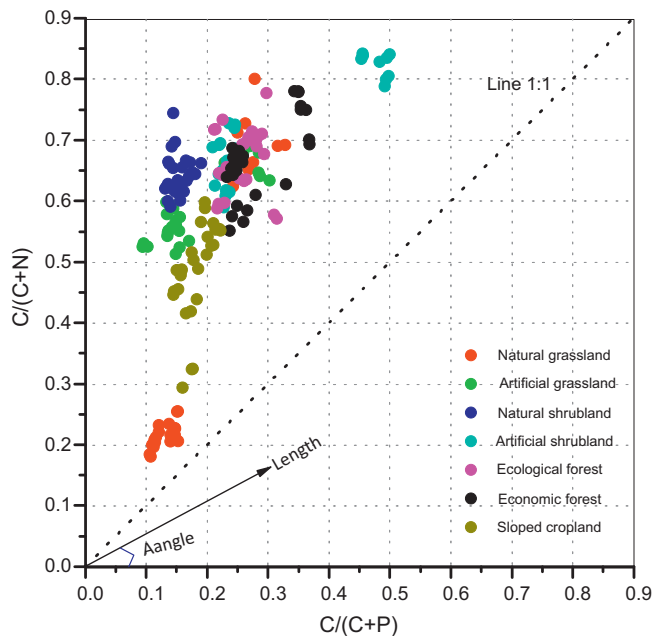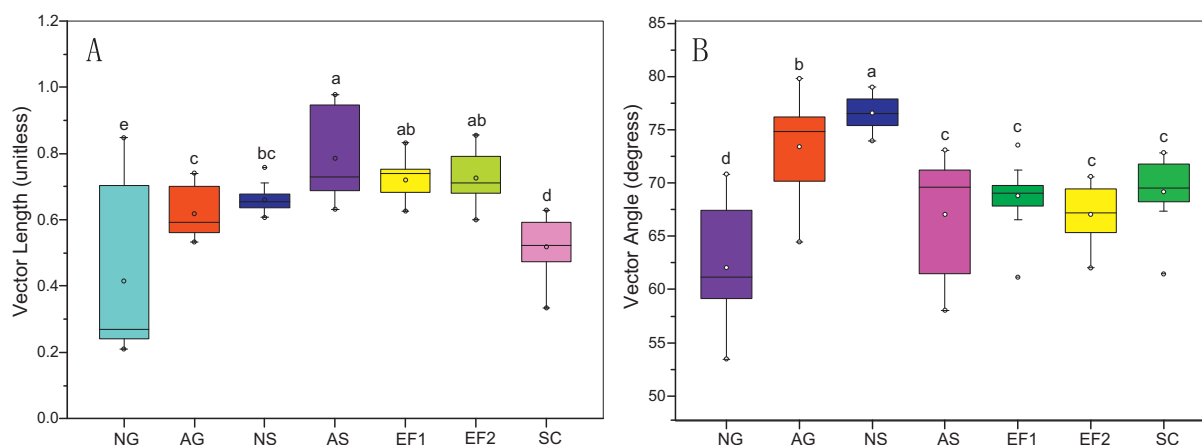
Fig. 4. Comparison of vector lengths and angles amongst different vegetation restoration types. NG: natural grassland, AG: artificial grassland, NS: natural brushland, AS: artificial brushland, EF1: ecological forest, EF2: economic forest, SC: sloped cropland. Vector length and angles defined by plot loci of proportional enzyme activities (BG + CBH) / (BG + CBH + NAG + LAP) and (BG + CBH) / (BG + CBH + AP); BG: β-1,4-glucosidase, CBH: β-D-cellobiosidase, NAG: β-1,4-N-acetylglucosaminidase, LAP: L-leucine aminopeptidase, AP: alkaline phosphatase.

(Table 2). The changes of EEA and ecoenzymatic stoichiometry were thus affected by the pattern of vegetation restoration. We therefore conclude that soil microbial metabolic activity and nutrient demand can be dramatically affected by anthropogenic interventions such as patterns of vegetation restoration, which can potentially influence the stability of above and belowground ecosystems.

4.2. C and P limitations of microorganisms under the various types of vegetation restoration

The vector analysis of EEA indicated that relative C limitation in the microbial communities was the lowest and highest in the natural grassland and artificial shrubland, respectively (Figs. 3 and 4A). Plant species and litter quality have strong effects on soil enzymatic activities and microbial community structures (Weand et al., 2010; Yin et al., 2014), implying that vegetation types have an indirect impact on microbial nutrient limitation by affecting microbial community structure and soil nutrient supply. Litter from herbs is more easily decomposed by microorganisms than litter from shrubs and trees, because herbaceous plants have higher carbohydrates and cellulose and lower lignin (Domenach et al., 1994), C resources from herbaceous plants are thus more easily exploited by microbes. The variation of SOC contents under various types of vegetation also strongly affects the supply of C sources for microbes (Owen et al., 2007).

The SOC content (6.86 g kg^{-1}) in our study was higher in the natural grassland than the types of other vegetation (Table 2). The natural grassland therefore had more C sources to feed the microorganisms, and the microbial communities were less restricted by C. In contrast, high activities of the C-acquiring enzymes and high SOC content in the artificial shrubland indicated the recalcitrance of the litter from *Caragana microphylla* L. (the main species in the artificial shrubland) (Tables 2 and 3). In addition, Deng et al. (2014) found that cropland converted to forest or shrubland had a more persistent capacity of C sequestration than cropland converted to grassland. These suggested the effects of vegetation type on C source used by microorganisms. Tree species can also play an important role in determining soil nutrient stoichiometry such as C:N ratios due to the differences of litter lignin:N ratios and decomposition rates (Cools et al., 2014), which can greatly affect microbial nutrient metabolism. Soil water content is also an important factor affecting litter decomposition and microbial activity (Bell et al., 2008), so the low moisture content in the artificial shrubland may have affected microbial metabolism and nutrient acquisition. Relative microbial C limitation was therefore higher for the artificial shrubland than the natural grassland.

P limitation in the microbial communities had a similar pattern with C limitation. The microbial communities were also limited by P under the six types of vegetation restoration (all vector angles > 45°) (Figs. 3 and 4B). P limitation was the lowest and highest in the natural grassland and natural shrubland, respectively (Fig. 4B). Long-term ecological observations and experiments have indicated that P is the primary limiting element in many ecosystems that have been naturally restored for >30 years (Wardle et al., 2004). Soil N is also generally limited in early soil development. The amount of P released by weathering decreases as soil development proceeds, because primary minerals become depleted (Vitousek and Farrington, 1997). Soil erosion also accelerates the loss of soil nutrients (especially P) on the Loess Plateau (Feng et al., 2013). Insufficient available P in the regions of loessial soil is thus an important factor leading to microbial P limitation, which can cause strong competition between soil microbial and vegetation communities during vegetation development. Our previous study indicated that communities of gramineous plants had a larger effect than other vegetation communities on ecoenzymatic stoichiometry (Cui et al., 2018), indicating that gramineous plants (*e.g. Agropyron cristatum* in the natural grassland) can potentially have a greater ability to acquire soil nutrients in arid and oligotrophic ecosystems. In particular, the gramineous plants usually have abundant fibrous root systems, which may secrete more organic acids to activate nutrients (especially N and P) and provide available nutrients for the concomitant microbes to mitigate the P limitation in microbial communities (Bell et al., 2013). P limitation in the microbial communities was thus the lowest in the natural grassland.

The C and P limitations of the microbial communities were more strongly correlated with soil nutrient stoichiometry in the grasslands than in the other vegetation types, which also suggested that vegetation type can have a strong effect on microbial C and P limitations. Previous studies have suggested that microbial and vegetation communities strongly compete for soil nutrients (Dunn et al., 2006; Cui et al., 2018). The litter from herbs is more easily decomposed by microorganisms than litter from trees (Domenach et al., 1994). The soil in natural grassland can thus supply more nutrients, which can relieve the competition between the vegetation and microbial communities. These results also supported the low P limitation in soil microbial communities in the natural grassland.

A deficiency of soil water and the emergence of dried soil layers are important causes of the degeneration of vegetation communities (Wang et al., 2011; Liu and Shao, 2015). Soil fertility in natural grassland can greatly improve during vegetation restoration (Wang et al., 2012). Zhang et al. (2011) also suggested that the conversion of abandoned cropland to natural grassland may best improve soil quality on the Loess Plateau, so natural recovery is recommended as the best pattern
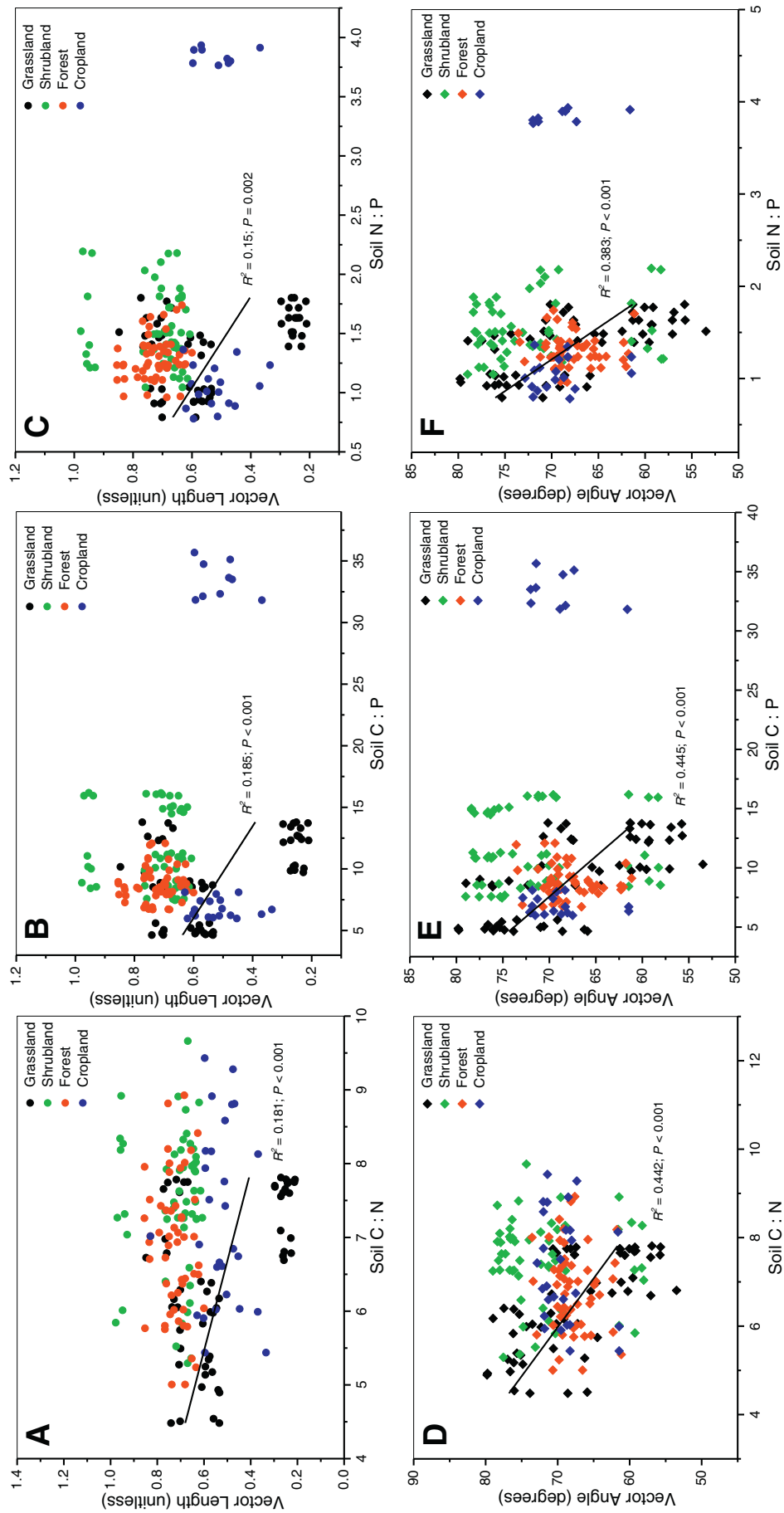
Fig. 5. The relationships of vector lengths and angles with components of soil nutrient stoichiometry (soil C:N, soil C:P, and soil N:P). *Colors* represent each vegetation restoration types (grassland (*black*) = natural grassland + artificial grassland, shrubland (*green*) = natural shrubland + artificial shrubland, forest (*red*) = ecological forest + economic forest, cropland (*blue*) = sloped cropland according to vector lengths (*circle*) or angles (*diamond*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of vegetation restoration. These studies, though, have unfortunately not provided direct evidence of the causes of vegetation degradation in arid and semi-arid regions. Our study found that microbial communities in natural grassland had the lowest relative C and P limitations from the perspective of microbial metabolism and nutrient competition between microbes and plants. Our analysis of ecoenzymatic vectors suggested that microbial-plant nutrient competition in arid and semi-arid regions may be the important cause of vegetation degradation. We therefore suggest that natural grassland is likely the most stable pattern of ecological restoration for arid and semi-arid regions.

4.3. Ecological stoichiometry associated with C and P limitations of microorganisms

The ecological stoichiometric ratios of ecosystem components can highlight the factors that limit biogeochemical cycling and have been increasingly used to predict ecosystem stability and C cycling (Ollinger et al., 2002; Xu et al., 2017). The roles of ecological stoichiometry in microbial metabolic limitations, however, have rarely been reported. Our results indicated that microbial C and P limitations in the grasslands were strongly affected by soil nutrient stoichiometry (soil C:N, soil C:P, and soil N:P) (Fig. 5 and Table S3). The reasons could attribute to microorganisms holding elemental stoichiometric balance and homeostasis as well as unbalance supply of soil nutrients in the various types of vegetation. Nutrient stoichiometry can strongly affect the structures and activities of microbial communities (Taylor and Townsend, 2010; Cui et al., 2018). Delgado-Baquerizo et al. (2017) also reported that bacterial diversity and composition were primarily driven by variation in soil resource stoichiometry (total C:N:P ratios). Soil nutrient stoichiometry can also control the competitive abilities of arbuscular mycorrhizal vs. root-borne non-mycorrhizal fungi (Chagnon and Bradley, 2013). These findings indicate that soil nutrient stoichiometry strongly regulates microbial community metabolism by affecting elemental balance and microbial community structure. Nutrient stoichiometry might therefore be the vital threshold leading to microbial metabolic limitation and shifts in community structures.

5. Conclusions

Our study found that soil microbial communities were co-limited by C and P during vegetation restoration in arid and semi-arid areas from the perspective of microbial metabolism and plants-microbes nutrient competition. The C and P limitations of microbial communities were strongly regulated by the stoichiometry of soil nutrients, implying that nutrient stoichiometric disequilibrium could be a crucial factor limiting microbial metabolism and ecological functions in terrestrial ecosystems. The microbial communities in the natural grassland particularly had the lowest relative C and P limitations, perhaps due to the lower nutrient competition between plants and soil microbes in the natural grassland compared to the other vegetation types. The restoration pattern of natural grassland could therefore potentially create a unique habitat for plant and microbial survival and maintenance of ecological function in arid and semi-arid regions. Our study suggests that the natural restoration of grassland should be the priority for restoring vegetation in arid and semi-arid regions.

Acknowledgements

Appendix A. Supplementary data

The supplementary information provides additional tables of the correlations amongst the soil physicochemical properties and soil enzymatic activities and the results of the regression analysis between vector lengths and angles and the stoichiometries of soil nutrients and enzymatic activities for the various vegetation types. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.08.173.

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