



## Changes of soil microbial and enzyme activities are linked to soil C, N and P stoichiometry in afforested ecosystems



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### ABSTRACT

Afforestation has been shown to strongly affect substrate stoichiometry and profoundly influence the microbial community. However, the degree to which microbial and activity are linked to soil carbon (C), nitrogen (N) and phosphorus (P) stoichiometry in afforested ecosystems remains unclear. In this study, soil samples were collected from *Robinia pseudoacacia* L. (RP42, RP27, and RP17) and farmland (FL) sites with a chronosequence of 42, 27, and 17 years. We determined the microbial biomass C ( $C_{mic}$ ), N ( $N_{mic}$ ),  $C_{mic}$  to organic C ( $C_{soil}$ ) ratio ( $C_{mic}:C_{soil}$ ),  $N_{mic}$  to total nitrogen ( $N_{soil}$ ) ratio ( $N_{mic}:N_{soil}$ ), and metabolic quotient ( $R_{mic}:C_{mic}$ ) to investigate microbial. We also measured soil b-glucosidase (BG), N-acetylglucosaminidase (NAG), acid phosphatase (AP), as well as  $C_{soil}$ ,  $N_{soil}$ , and total phosphorus ( $P_{soil}$ ). The results showed that, compared with FL,  $C_{soil}:P_{soil}$  and  $N_{soil}:P_{soil}$  were increased during aggradation, whereas  $C_{soil}:N_{soil}$  increased in RP27 and RP17 but decreased in RP42.  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$  were 94% and 182% higher in RP42 than FL, respectively. However,  $R_{mic}:C_{mic}$  was lower in RP42, RP27, and RP17 than FL by 64%, 36% and 25%, respectively. Moreover, we found that BG, NAG, AP, BG:NAG, and BG:AP in RP42, RP27, and RP17 were higher than in FL. Compared with global soil (0.62 and 0.13), the lower BG:AP (0.42) and BG:NAG (0.03) ratios in present study indicated that  $P_{soil}$  may be limited. Additionally, redundancy analysis (RDA) revealed that the  $C_{soil}:N_{soil}$  was positively correlated with  $R_{mic}:C_{mic}$  but negatively with  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$ , while  $C_{soil}:P_{soil}$  and  $N_{soil}:P_{soil}$  were also significantly and negatively correlated with BG:AP, BG:NAG, and NAG. Therefore, our results indicated that afforested ecosystem are highly susceptible to changes in soil microbial and enzyme stoichiometry during aggradation and may become P-limited. Such changes were modulated by soil nutrient stoichiometry.

### 1. Introduction

Through ecological succession, afforestation can cause shifts in plant community structure and species composition (Tian et al., 2015), and lead to changes in the microbial community structure and function (Schimel, 2016). As a result, these changes inevitably alter soil microbial metabolism and energy flow (Bardgett and van der Putten, 2014; Xu et al., 2014). However, due to the differences of in substrate availability, enzyme concentration, and resource imbalance after land use change, there has not been a consensus on how soil microbial characteristics, such as activity, functions, and soil microbial hold controversial response to change (Li et al., 2013; Deng et al., 2016). Ultimately, findings have been diverse and substantial uncertainty about

soil microbial in afforested ecosystems exists (Li et al., 2013; Naveed et al., 2016).

Nutrient stoichiometry reflects the interactions between plants, soil, and microbes (Spohn and Chodak, 2015; Pabst et al., 2016; Tian et al., 2017; Zhou et al., 2017b). Particularly, the soil C:N:P ratio is one important factor that drives microbial composition play a vital role in soil-plant interaction and terrestrial nutrient limitations (Zhou et al., 2017b). For example, Fierer et al. (2007) reported that soil microbial communities can shift from r-strategists (fast-growing copiotrophs) to K-strategists (slow-growing oligotrophs) as the C:N ratio increases. Chen et al. (2016) illustrated that variations in the microbial C:N ratio were primarily related to abiotic variables, while variations in microbial C:P and N:P were primarily associated with shifts in the community

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structure of soil microbes. Thus, soil microbial communities respond differently to the imbalances nutrients in the soil (Garcia-Franco et al., 2015; Zhang et al., 2016a; Deng and Shangguan, 2017). Indeed, microbes fed with lower C:N ratio resources would have higher growth efficiency and lower release of C through respiration than microbes fed with higher C:N ratio resources, which allows for more C to be converted to biomass (Zhou et al., 2017b). Consequently, these variations of  $C_{soil}$ ,  $C_{mic}$ , and respiration lead to changes in ratios of  $R_{mic}:C_{mic}$ ,  $C_{mic}:C_{soil}$ , and  $N_{mic}:N_{soil}$ , and according to stoichiometry theory, the  $R_{mic}:C_{mic}$  ratio increases while the  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$  ratios decrease (Insam and Haselwandter, 1989; Pabst et al., 2016). Therefore, it is imperative to investigate the relationship between nutrient stoichiometry and microbial composition, especially the C:N:P ratio, after afforestation and during aggradation.

The extracellular enzyme that is involved in C, N and P cycling, reflects the biogeochemical equilibrium between nutrient requirements of microbial assemblages and nutrient availability of the environment (Nannipieri et al., 2012; Burns et al., 2013; Burns et al., 2013; Sinsabaugh et al., 2009a, 2009b; Kivlin and Treseder, 2014; Peng and Wang, 2016). Previous studies indicated that soil enzyme activity and stoichiometry were affected by soil pH (Snajdr et al., 2008), soil texture (Alvarez and Lavado, 1998), and root biomass (Edwards and Jefferies, 2013). However, large uncertainties remain due to the differential responses of soil extracellular enzymes activity and stoichiometry to nutrient stoichiometry (Burns et al., 2013; Cleveland and Liptzin, 2007). In fact, soil stoichiometry (especially C, N and P) influence soil extracellular enzyme activities by influencing the biochemical properties of soil enzymes and regulating enzyme secretion (Sinsabaugh et al., 2009a, 2009b; Bailey et al., 2011; Nannipieri et al., 2012; Wild et al., 2014). And in turn, soil extracellular enzymes stoichiometry reflects how the microbial community invests in energy and nutrient acquisition under in situ conditions as it copes with nutrient limitation (Tapia-Torres et al., 2015). For example, Chapin et al. (2002) illustrate soil microbes will secrete more acid phosphatase (AP) enzymes to meet the P demand in soils with low pH and P availability. Bowles et al. (2014) found microbes regulate extracellular enzyme production to acquire limiting nutrients, so changes in enzyme activities may reflect patterns of microbial nutrient limitations and hence nutrient availability Xu et al. (2017b) reported that lower b-glucosidase (BG, C-acquiring enzyme) and N-acetylglucosaminidase ratio (BG:NAG) in tropical and subtropical forests indicate greater microbial demand for N versus C. Therefore, understanding the relationships between soil enzymes stoichiometry and the nutrient stoichiometry especially for C, N, P stoichiometry would, therefore, constitute important steps in evaluating the stoichiometric and energy limitations on the metabolism of soil microbes after afforestation and during aggradation.

The Loess Plateau in China covers approximately  $62.4 \times 104 \text{ km}^2$  and is known for its long agricultural history and severe soil erosion (Chen et al., 2007). In recent years, numerous studies have been conducted to study the effects of afforestation on soil physicochemical properties (Chen et al., 2014; Deng and Shangguan, 2017), microbial dynamics and soil enzymes activity (Ren et al., 2016c; Zhang et al., 2016b). However, information on the relationship between soil microbial and enzyme activity linked to nutrient stoichiometry is scarce. Thus, Our objectives in this study were to investigate afforested farmlands to (i) evaluate changes in the soil C, N, and P stoichiometry, (ii) characterize the variations of  $C_{mic}:C_{soil}$ ,  $N_{mic}:N_{soil}$  and  $R_{mic}:C_{mic}$ , and (iii) demonstrate the relationships between soil microbial, soil enzyme activity and stoichiometry and soil C, N, and P stoichiometry after afforestation. We hypothesized that  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$  increased, whereas  $R_{mic}:C_{mic}$  decreased with afforestation aggradation. We also predicted that the soil C, N, and P stoichiometry was one important driver of the trends in microbial and soil enzyme activity and stoichiometry.

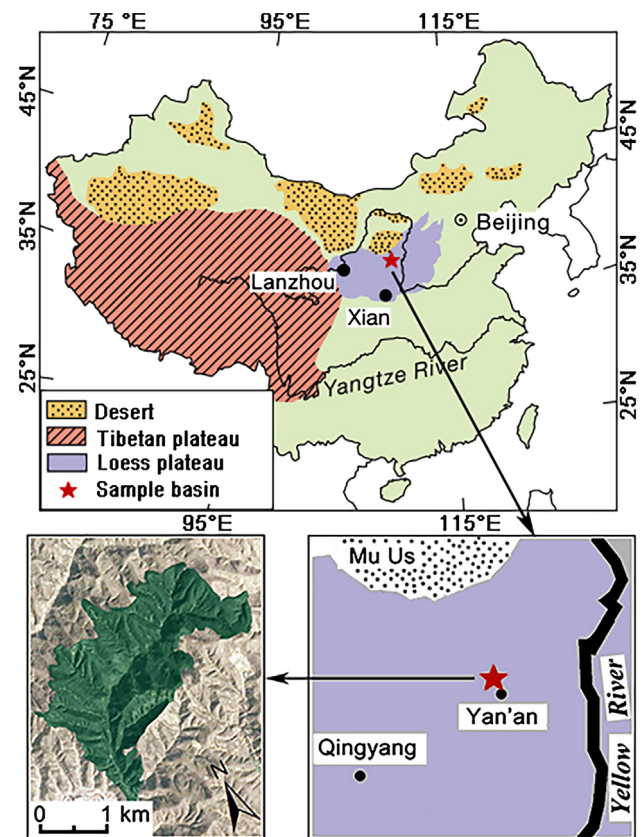


Fig. 1. Location of the Loess Plateau and the study basin.

## 2. Materials and methods

### 2.1. Study area

The study area was in the Wuliwan Watershed, Ansai County, Shaanxi Province, Northern China ( $36^{\circ}46'42'' - 36^{\circ}46'28''\text{N}$ ,  $109^{\circ}13'46'' - 109^{\circ}16'03''\text{E}$ ) (Fig. 1). The study site is a temperate semiarid area and the average monthly temperature ranges from  $-6.2^{\circ}\text{C}$  in January to  $37.2^{\circ}\text{C}$  in July, with a mean annual temperature and precipitation of  $8.8^{\circ}\text{C}$  and 505 mm, respectively (Ren et al., 2016b). The soil is highly erodible and classified as loessial (Calcaric Cambisols, WRB classification, 2014). Details of the soil properties are provided in Table 1. Wuliwan catchment is an experimental site of the Chinese Academy of Science (CAS), and vegetation restoration has been implemented due to the serious soil degradation since 1970s (Zhao et al., 2014). The main species used for restoration on farmland (FL) is *Robinia pseudoacacia* L. (RP), and farmland in this region is mainly planted millet. Water resources for crop growth have been dependent entirely on rainfall since no provisions for irrigation during the growing season were made. After 30 years of afforestation, forest area has increased significantly from 5% to 40% (Zhao et al., 2016).

### 2.2. Experimental design

Experiments were carried out in June 2014. Study plots were established in farmland (FL) and three afforested lands with different stand ages (17, 27, and 42 years) of *Robinia pseudoacacia* L. Prior to afforestation, the land-use was essentially farmland which had been subjected to 20 years of millet (*Setaria italica*) and soybean (*Glycine max*) rotations (Ren et al., 2016c). Within each of the four study locations, three independent replicate plots ( $25 \times 50 \text{ m}$ ) were designed with similar slopes, gradients, and altitudes (Zhao et al., 2016; Ren et al., 2016c). In total, 12 plots (four land cover types  $\times$  three replicate

**Table 1**  
The geographical information and soil properties of four *R. pseudoacacia* sites.

Sites	Farmland	<i>R. pseudoacacia</i> (RP17yr)	<i>R. pseudoacacia</i> (RP27yr)	<i>R. pseudoacacia</i> (RP42yr)
Elevation (m)	1205	1303	1298	1293
Coverage (%)	–	70	75	85
SBD (g cm <sup>-3</sup> ) <sup>a</sup>	1.14 ± 0.02 A	1.17 ± 0.01 A	1.20 ± 0.01 A	1.24 ± 0.01 A
Clay (%)	8.12 ± 0.21 A	8.55 ± 0.14 A	9.54 ± 0.13 A	10.11 ± 0.12 A
pH	8.48 ± 0.02 A	8.65 ± 0.01 A	8.67 ± 0.11 A	9.38 ± 0.01 A
SWC (%) <sup>b</sup>	9.34 ± 0.74 C	14.32 ± 0.98 B	15.38 ± 0.79 B	22.12 ± 1.21 A

<sup>a</sup> SBD is soil bulk density of soil.

<sup>b</sup> SWC is soil water content; Capital letters indicate significant difference among different land use types ( $P < 0.05$ ); the error bars are the standard error.

plots) were established in our study area. In addition, six quadrats (0.5 m × 0.5 m) (three trenched quadrats and three untrenched quadrats) were randomly established in each replicate plot, and the trenches (0.5 m wide and 0.8 m deep) were excavated in October 2013. The above-ground plants were carefully removed without any disturbance. After covering the trenches with a 2-mm thick plastic sheet, we refilled the trenches with soil.

### 2.3. Soil respiration measurement and soil sampling

As described in our recent study (Ren et al., 2018), we used polyvinyl chloride (PVC) collars (16 cm inner diameter, 12 cm deep) to determine soil respiration. A total of six PVC collars were inserted to a depth of 10 cm. Three PVC collars in trenched quadrats were used to determine the soil heterotrophic respiration ( $R_{mic}$ ), and another three PVC collars in untrenched quadrats were used to determine soil respiration (SR). In the trenched plots, aboveground portions of all plants were removed before plot establishment to avoid disturbance to the soil, and aboveground biomass was repeatedly removed during the study period. Analysis revealed that the decomposition of dead and dying roots caused by trenching contributed to only about 3%–5% of cumulative soil respiration, thus three trenched sub-plots were used to measure  $R_h$ . One hundred centimeter deep trenches were dug along the outside edges and lined with double-layer plastic sheets to stop root growth into trenched plots; these trenches were then backfilled, layer by layer, to minimize disturbance. Accordingly, soil autotrophic respiration was the difference between SR and  $R_{mic}$ . In June 2014, soil respiration ratios ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) were measured with a portable infrared analyzer (GXH-3010E1). To reduce the measuring error from weather conditions, the measurement time was selected as 09:00 and 11:00 am on days without rain, and the estimates of respiration were obtained from continuous 2- or 3-day measurements to represent the average monthly soil respiration. Finally, three respiration ratio observations were averaged to obtain the results for a given plot, for both autotrophic respiration and  $R_{mic}$ .

After carefully removing the litter layer by hand from the topsoil, soil samples were obtained at 0–10 cm depth using a soil auger (diameter 5 cm) from ten points within an “S” shape in each subplot and then homogenized to provide one final soil sample per subplot. Overall, 36 samples (four stand age types × three plots × three sub-plots) were collected. The samples were sieved through a 2-mm screen, and roots and other debris were removed (Ren et al., 2016a; 2016c). A portion of each soil sample was air dried and stored at room temperature prior to analysis soil properties including soil organic carbon ( $C_{soil}$ ), total nitrogen ( $N_{soil}$ ), and soil water content (SWC), pH, and soil bulk density (BD). A portion of each soil sample was immediately transported to the laboratory for microbial biomass carbon ( $C_{mic}$ ), nitrogen ( $N_{mic}$ ) and enzyme analysis (on ice, and then stored at  $-80^\circ\text{C}$ ). Our approach was based on the stoichiometry theory, which can evaluate trends of microbial, microbial C to soil C ratio ( $C_{mic}:C_{soil}$ ), microbial N to soil N ratio ( $N_{mic}:N_{soil}$ ), and microbial respiration to biomass ratio ( $R_{mic}:C_{mic}$ ) (Odum, 1969; Zhou et al., 2017b). In addition,  $C_{soil}:N_{soil}$ ,  $C_{soil}:P_{soil}$ ,  $N_{soil}:P_{soil}$  stoichiometry were calculated as mass ratio (Ren et al.,

2016c).

### 2.4. Analysis of soil properties and enzymes activities

SWC was determined by oven drying to constant mass at  $105^\circ\text{C}$ . BD was calculated from the gravimetric weight of the cores before and after oven drying at  $105^\circ\text{C}$  for 24 h from the individual core volume. Soil pH was measured using a pH meter after shaking the soil water (1:5 w/v) suspension for 30 min (Ren et al., 2016c; 2018).  $C_{soil}$  was determined using the  $\text{K}_2\text{Cr}_2\text{O}_7$  oxidation method,  $N_{soil}$  were determined using an elemental analyzer (Vario MACRO cube CN; Germany), and total phosphorus ( $P_{soil}$ ) was determined colorimetrically (UV, spectrophotometer) after wet digestion with  $\text{HClO}_4\text{--H}_2\text{SO}_4$  (Ren et al., 2016c).  $C_{mic}$ , and  $N_{mic}$  was estimated from fresh soil samples using a chloroform fumigation-extraction method (Ren et al., 2016c).

The activities of  $\beta$ -glucosidase (BG, C-acquiring enzyme), N-acetylglucosaminidase (NAG, N-acquiring enzymes), and acid phosphatase (AP, organic P acquiring enzyme) were measured by the method of (Saiya-Cork et al., 2002; Peng and Wang, 2016). The reasons for selecting the three enzymes are mainly due to the potential activities of these three enzymes are frequently linked to rates of microbial metabolism and biogeochemical processes, and are generally used as indicators of microbial nutrient demand (Peng and Wang, 2016). These enzymes were determined with the absorbance of *p*-nitrophenol (rNP) at 410 nm on an Evolution 201 Thermo Scientific Inc, spectrophotometer. For ecoenzyme extraction, we used 2 g of fresh soil and 30 ml of modified universal buffer (MUB) pH 9. Then, three replicates and one control (sample without substrate) per sample were prepared including three substrate controls (substrate without sample), and centrifuged the tubes after the incubation period and then 750 ml of supernatant was diluted in 2 ml of deionized water. Ecoenzyme activities were expressed as nanomoles of rNP formed per gram of soil dry weight per hour (Tapia-Torres et al., 2015). The soil C:N acquisition ratios were calculated using BG and NAG, BG:AP, NAG: AP for the soil C:P and N:P acquisition ratios, respectively (Sinsabaugh et al., 2009a, 2009b).

### 2.5. Statistical analyses

Changes in soil properties (SOC, TN, TP,  $C_{soil}:N_{soil}$ ,  $C_{soil}:P_{soil}$ ,  $N_{soil}:P_{soil}$ , SWC, pH, and BD), microbial ( $R_{mic}:C_{mic}$ ,  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$ ), and soil enzyme activity (BG, NAG, AP, BG:AP, BG:AP and NAG:AP) were tested through one-way ANOVA using the R v.3.1.3 software. Redundancy analysis (RDA) in the CANOCO 4.5 software package was used to identify the relations between the carbon fractions and soil microbial community (ter Braak and Smilauer, 2002). We used RDA because the RDA plots provide a representation of relations between dependent and independent variables. The RDA plots were interpreted in terms of the Euclidean distances between centroids, and between centroids and individual objects. The angles of the vectors plotted in the plane of the first two RDA axes, which explain the largest proportion of the variation represent, show the strength of correlation between response and explanatory variables (a narrow angle indicates a

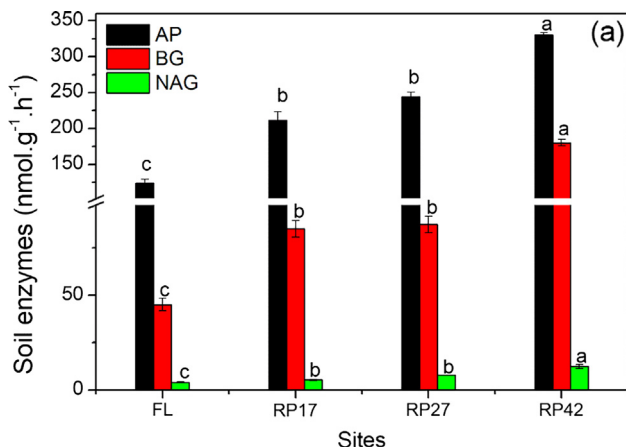


Fig. 2. Soil C, N and P content during afforestation aggradation; Lower case letters indicate significant difference among different sites ( $P < 0.05$ ). The error bars are the standard errors.

strong correlation).

### 3. Results

#### 3.1. Changes in $C_{soil}$ , $N_{soil}$ and $P_{soil}$ content and its stoichiometry

Soil  $C_{soil}$ ,  $N_{soil}$ , and  $P_{soil}$  content showed remarkable changes during aggradation in our study (Fig. 2a). We found  $C_{soil}$ ,  $N_{soil}$ , and  $P_{soil}$  content significantly increased in afforested sites compared with FL ( $p < 0.05$ ). Particularly,  $C_{soil}$ ,  $N_{soil}$ , and  $P_{soil}$  content in RP42 were more than 440%, 444%, and 41% higher than FL, respectively. Among afforested sites,  $C_{soil}$ ,  $N_{soil}$  and  $P_{soil}$  content in RP42 also higher than in RP27 and RP17 by 14%–89% and 29%–156%, respectively.

#### 3.2. Changes in $C_{soil}$ , $N_{soil}$ and $P_{soil}$ stoichiometry

We found  $C_{soil}:P_{soil}$  and  $N_{soil}:P_{soil}$  increased during aggradation, which in RP42 was higher than in FL by 3, respectively (Fig. 3). However,  $C_{soil}:N_{soil}$  was higher in RP27 and RP17, but lower in RP42 when compared to FL.

#### 3.3. Changes in microbial activities

Significant differences were found in the values of  $C_{mic}:C_{soil}$ ,  $N_{mic}:N_{soil}$  and  $R_{mic}:C_{mic}$  during forest aggradation in our study (Fig. 4)

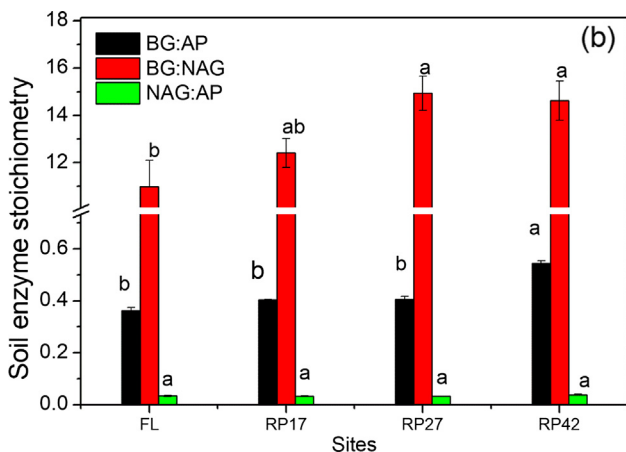


Fig. 3. Soil stoichiometry during afforestation aggradation; Lower case letters indicate significant difference among different sites ( $P < 0.05$ ). The error bars are the standard errors.

( $P < 0.05$ ). Our result showed that  $C_{mic}:C_{soil}$ ,  $N_{mic}:N_{soil}$  and  $P_{mic}:P_{soil}$  increased during aggradation, whereas  $R_{mic}:C_{mic}$  decreased after afforestation. Particularly,  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$  were higher in RP42 than FL by more than 94%, 182% and 148%, respectively. However,  $R_{mic}:C_{mic}$  was lower in RP42, RP27, and RP17 than FL by more than 64.%, 36%, and 25%, respectively.

#### 3.4. Changes in soil enzyme activity

Soil enzyme activity and its stoichiometry was different among sites (Fig. 5). We found BG, NAG, and AP content increased significantly during forest aggradation (Fig. 4a, b and c). More specifically, BG, NAG, and AP content in RP42, RP27, and RP17 were higher than in FL by 89%–300%, 30%–201%, and 70%–167%, respectively. Among afforested sites, BG, NAG, and AP content was higher in RP42 than in RP27 (57%–112%) and RP17 (35%–133%). Meanwhile, BG:NAG and BG:AP had similar trends with BG, NAG, and AP content (Fig. 4d, e and f). BG:NAG and BG:AP in RP42, RP27, and RP17 were higher than in FL by 13%–36% and 11%–51%, respectively. However, NAG:AP slightly increased, but was not significant.

#### 3.5. Relationship between soil microbial, enzyme activity and $C_{soil}$ , $N_{soil}$ and $P_{soil}$ stoichiometry

The relations between soil microbial ( $R_{mic}:C_{mic}$ ,  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$ ), enzyme activity (BG, NAG, AP, BG:NAG, BG:AP and NAG:AP) and the  $C_{soil}$ ,  $N_{soil}$  and  $P_{soil}$  stoichiometry ( $C_{soil}:N_{soil}$ ,  $C_{soil}:P_{soil}$ ,  $N_{soil}:P_{soil}$ ) were examined with redundancy analysis (Fig. 6). In detail, the  $C_{soil}:N_{soil}$  ratio was correlated positively with the  $R_{mic}:C_{mic}$  ratio, but negatively with the  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$  ratios. In particular, soil enzyme activity and stoichiometry (BG:AP, BG:NAG, and NAG:AP) were significantly and negatively correlated with soil  $C_{soil}:P_{soil}$  and  $N_{soil}:P_{soil}$ , but there was no significant difference between  $C_{soil}:N_{soil}$  and BG:AP, AP, BG, and NAG (Table 2).

### 4. Discussion

#### 4.1. Trends in microbial linked to C, N and P stoichiometry

Changes in soil microbial abundance, community composition, and activity can alter microbial and nutrient flows after afforestation and during aggradation (Zhou et al., 2017c). According to Zhou et al. (2017b)  $R_{mic}:C_{mic}$ ,  $C_{mic}:C_{soil}$ ,  $N_{mic}:N_{soil}$  and  $P_{mic}:P_{soil}$  are used evaluate and activity in microbial ecology. In general, as available substrate ( $C_{mic}:C_{soil}$ ,  $N_{mic}:N_{soil}$  and  $P_{mic}:P_{soil}$ ) decreases so does the microbial substrate use efficiency, resulting in higher  $R_{mic}:C_{mic}$  (Manzoni et al., 2012; Spohn, 2015; Zhou et al., 2017b). This relationship was also observed in our result, which showed that  $C_{mic}:C_{soil}$ ,  $N_{mic}:N_{soil}$  and  $P_{mic}:P_{soil}$  increased whereas  $R_{mic}:C_{mic}$  decreased after afforestation. In other words, early stages of afforestation tended to be dominated by r-strategists (bacteria) that had higher  $R_{mic}:C_{mic}$  and lower  $C_{mic}:C_{soil}$ ,  $N_{mic}:N_{soil}$  and  $P_{mic}:P_{soil}$ , whereas late stages tended to be dominated by K-strategists (fungi) that behaved oppositely. This could indicate that the community shifted from r- to K-strategists following afforestation (Anderson, 2003) and that microbial stability increased during aggradation (Blagodatskaya et al., 2014). Moreover, a previous study reported non-forest systems were characterized by low  $C_{mic}:C_{soil}$  ratios with higher  $R_{mic}:C_{mic}$ , while forest ecosystems had higher  $C_{mic}:C_{soil}$  with lower  $R_{mic}:C_{mic}$  (Pabst et al., 2016). Particularly, recent global synthesis by Xu et al. (2017a) based on a total of 2444 observations illustrate that the  $R_{mic}:C_{mic}$  is about two times higher in cropland than forest ecosystems. Therefore, it may be concluded that the lower  $R_{mic}:C_{mic}$  in the forest ecosystems indicated a higher efficiency of the substrate utilization by the soil microbial community.

Changes in microbial are associated with changes in soil C, N, and P stoichiometry during aggradation (Hartman and Richardson, 2013;

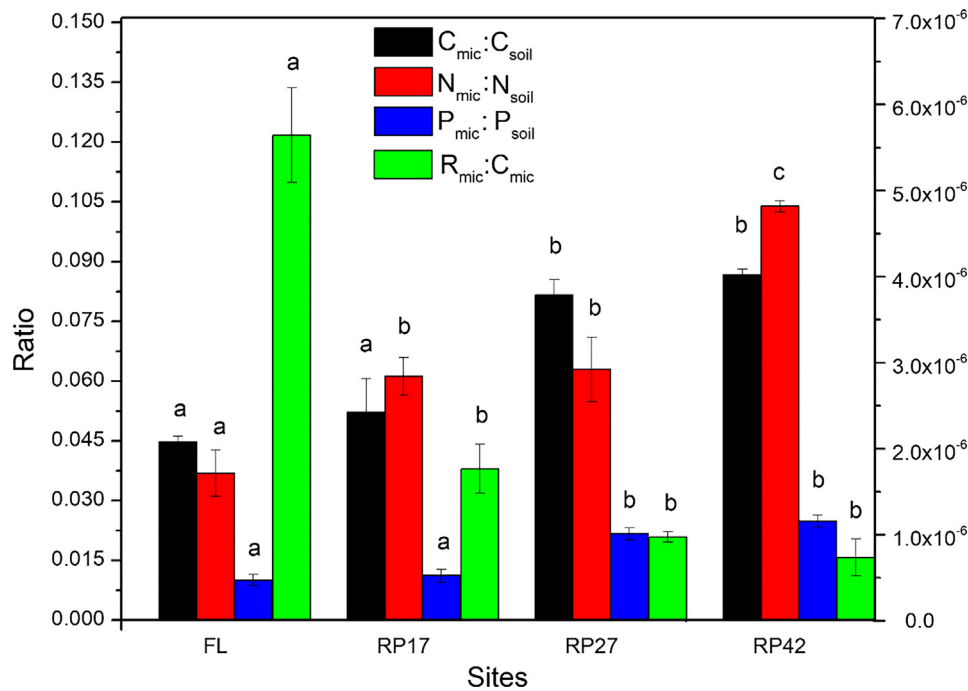


Fig. 4. The value of  $C_{mic}:C_{soil}$ ,  $N_{mic}:N_{soil}$  and  $R_{mic}:C_{mic}$  during afforestation aggradation ( $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$  for Y-axis on the left, while  $R_{mic}:C_{mic}$  value for Y-axis on the right); Lower case letters indicate significant difference among different sites ( $P < 0.05$ ). The error bars are the standard errors.

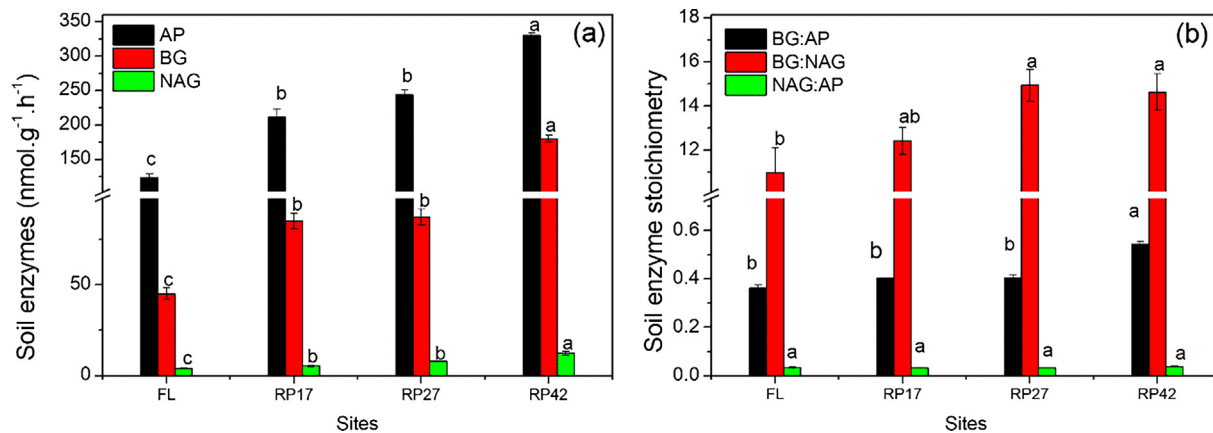


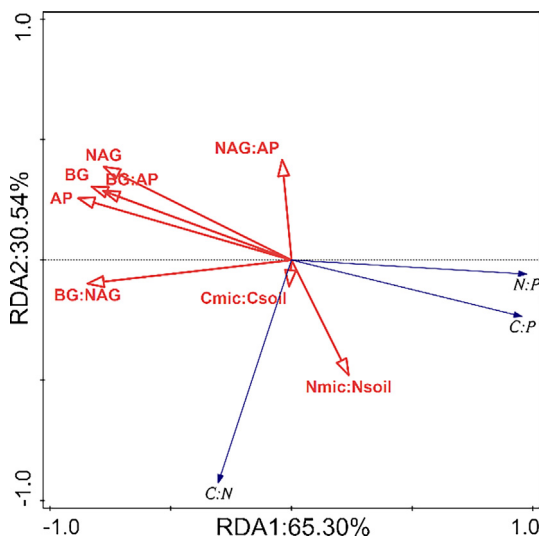
Fig. 5. Soil enzyme activity and its stoichiometry during afforestation aggradation; Lower case letters indicate significant difference among different sites ( $P < 0.05$ ). The error bars are the standard errors.

Zhou et al., 2017b). We found the  $C_{soil}:N_{soil}$  ratio was correlated positively with the  $R_{mic}:C_{mic}$  ratio, but negatively with the  $C_{mic}:C_{soil}$  and  $N_{mic}:N_{soil}$  ratios (Fig. 6), which suggested soil resource quality had a positive effect on microbial assimilation of soil organic C (Zhou et al., 2017b). It is probably that microbes in soils with a lower  $C:N_{soil}$  ratio would have higher growth efficiency and lower release of C through respiration. Conversely, microbes in soils with a higher  $C:N_{soil}$  ratio have more C available to be converted to biomass (Schimel, 2016; Shoemaker et al., 2017; Zhou et al., 2017a; 2017b). Additionally, a weak but significant negative relationship was found between  $N_{soil}:P_{soil}$  and  $R_{mic}:C_{mic}$  (Fig. 6). This result might have occurred because higher  $R_{mic}:C_{mic}$  is associated with bacteria-dominated microbial communities (Fierer et al., 2007), and lower  $R_{mic}:C_{mic}$  is associated with a fungi-dominated microbial communities, which normally results in higher N mineralization and low N availability, respectively (Schimel and Bennett, 2004). This result is also consistent with recent field research (Spohn, 2015), meta-analyses (Xu et al., 2017a), modeling, (Xu et al., 2014) and biochar addition experiments (Zhou et al., 2017a). Therefore, the soil C, N, and P stoichiometry was an important determinate of

the trends in microbial composition and.

#### 4.2. Trends in microbial enzyme activity linked to C, N and P stoichiometry

The microbial decomposition of SOC causes soil respiration by producing soil C-degrading extracellular enzymes (EEs) (Waring et al., 2014; Ren et al., 2016a). As a result, soil extracellular enzymes are the proximate agents of nutrient decomposition (Peng and Wang, 2016). We found soil enzymes (BG, AP, BG, NAG, and AP) increased after afforestation (Fig. 5). It is possible that the above- and below-ground plant residues in afforested ecosystems contain more substrates that stimulate the synthesis of soil enzymes and thus had higher concentration (Zhang et al., 2011; Ren et al., 2016a). We also found that the BG:AP and BG:NAG ratios in our dataset averaged 0.42 and 0.03, respectively (Fig. 5), which was lower the global value (0.62 and 0.13) (Sinsabaugh et al., 2008; 2009a, 2009b). It is implied that microbial growth and C uptake in the soils of our study area may be limited by P availability. This limitation has been observed by previous studies in tropical and subtropical forests, which may be subject to much more



**Fig. 6.** Ordination plots of the results from the redundancy analysis to identify the relationships between microbial bioenergetics, soil enzyme activity (red arrows) and soil C, N and P stoichiometry (blue arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**  
Principal component analysis between “soil microbial, enzyme activity and  $C_{soil}$ ,  $N_{soil}$  and  $P_{soil}$  stoichiometry.

Variable	Components		
	PC1	PC2	PC3
CP	-0.761	0.573	0.1
CN	0.485	0.02	0.784
NP	-0.786	0.541	-0.076
qMBC	0.205	-0.419	-0.535
qMBN	-0.193	-0.636	0.715
Qco2	0.444	0.408	-0.503
AP	0.981	-0.003	-0.124
BG	0.972	0.17	-0.114
NAG	0.953	0.231	0.103
BG:AP	0.921	0.32	-0.109
BG:NAG	0.492	-0.679	-0.359
NA:GAP	0.624	0.562	0.416

serious P limitations (Xu et al., 2017b). Ren et al. (2016a) used the response of bacterial communities in imbalanced soil to show changes in soil P following afforestation in Loess plateau. Therefore, the enzyme ratio trends indicated that high microbial demand for P relative to C and N persists across diverse soils, in which multiple interacting climatic and edaphic factors may drive low P bioavailability.

Additionally, soil C, N, and P contents might largely impact soil extracellular enzyme stoichiometry by affecting the concentration of soil available substrate and soil C, N, and P stoichiometry, implying that soil extracellular enzyme stoichiometry was largely controlled by soil C, N, and P stoichiometry (Peng and Wang, 2016). We found fewer studies conducted in our study area one the relationship between soil extracellular enzyme stoichiometry and soil C, N, and P stoichiometry. Although factors such as temperature (Stone et al., 2012), pH (Kivlin and Treseder, 2014), and soil nutrients (Cleveland and Liptzin, 2007) influence soil enzyme activity and stoichiometry, the soil C, N, P stoichiometry also were important driving factors (Peng and Wang, 2016). We found soil enzyme activity and stoichiometry (BG:AP, BG:NAG and NAG:AP) were significantly and negatively correlated with soil  $C_{soil}:P_{soil}$  and  $N_{soil}:P_{soil}$  (Fig. 5). These results might have been observed because aggradation plant litter may modify soil nutrient availability, thereby regulating the enzyme production soil microbial organisms. Variations

in soil enzyme activity are frequently linked to rates of microbial metabolism and biogeochemical processes, and largely influenced by nutrient balance. In our result, changes of NAG and AP led to variations of C, N, and P concentration, and consequently, influence C:P and N:P after afforestation. Our result was also consistent with a recent study that reported BG:AP and NAG:AP ratios were negatively correlated with soil  $C_{soil}:P_{soil}$  and  $N_{soil}:P_{soil}$  due to soil N and P availability (Xu et al., 2017b). Therefore, the results of this study emphasized the importance of soil nutrient stoichiometry in determining soil enzyme activity and stoichiometry.

In summary, soil C, N, P stoichiometry largely impacted soil microbial and enzyme stoichiometry by affecting the concentration of soil available substrate during aggradation of afforested farmlands. However, soil microbial activities and enzymes, as biological catalysts, mainly come from soil microbes, root secretion, and the decomposition of plant and animal residues. Therefore, soil microbial activities and enzyme stoichiometric can be coupled via plant-soil-microbe feedbacks in soil nutrient cycles. Knowledge about variations in soil and plant tissue stoichiometry regulating the enzyme production of the soil microbial organism is topic of future study that is urgently needed.

## 5. Conclusions

In conclusion, changes in soil microbiology after afforestation and during aggradation indicate an increased nutrient demand but higher substrate use efficiency. Soil extracellular enzyme stoichiometry indicated P became increasingly limiting during forest aggradation in the study area. Furthermore, the relationship between soil C, N, and P stoichiometry and soil microbial and enzyme stoichiometry indicated soil microbial and enzyme stoichiometry was largely controlled by soil nutrient stoichiometry. Collectively, our results provided useful insights into the controls on soil C and nutrient cycling in forest ecosystems.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foreco.2018.06.011>.

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