

## Article

# Extracellular Enzyme Activity and Stoichiometry Reveal Nutrient Dynamics during Microbially-Mediated Plant Residue Transformation

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**Abstract:** Extracellular enzymes are the major mediators of plant residue and organic matter decomposition in soil, frequently associated with microbial metabolic processes and the biochemical cycling of nutrients in soil ecosystems. However, the dynamic trends and driving factors of extracellular enzymes and their stoichiometry during plant residue transformation remain to be further studied. Here, we investigated the dynamics of extracellular enzymes and enzymatic stoichiometry in the “litter-soil” transformation interface soil (TIS) layer, an essential occurrence layer for microbially-mediated C transformation. The results indicated an unbalanced relationship between substrate resource supply and microbial metabolic demand. Microbial metabolism was limited by C (C/N-acquiring enzymes > 1) and P (N/P-acquiring enzymes < 1) throughout the observed stages of plant residue transformation. The initially higher extracellular enzyme activity reflected the availability of the active components (dissolved carbon (DC), nitrogen (DN), microbial biomass carbon (MBC), nitrogen (MBN), and phosphorus (MBP)) in the substrate and the higher intensity of microbial metabolism. With the transformation of plant residues, the active fraction ceased to be the predominant microbial C source, forcing the secretion of C-acquiring enzymes and N-acquiring enzymes to obtain C sources and N nutrients from refractory substrates. Moreover, C/N-acquiring enzymes decreased, while C/P-acquiring enzymes and N/P-acquiring enzymes subsequently increased, which suggested that the microbial demand for N gradually increased and for P relatively decreased. Soil microorganisms can be forced into dormancy or intracellular mineralization due to the lack of substrate resources, so microbial biomass and extracellular enzyme activities decreased significantly compared to initial values. In summary, the results indicated that soil nutrients indirectly contribute to extracellular enzymes and their stoichiometry by affecting microbial activities. Furthermore, extracellular enzymes and their stoichiometry were more sensitive to the response of soil microbial biomass carbon.

**Keywords:** transformation interface soil layer; transformation of plant residues; extracellular enzyme activities; extracellular enzymatic stoichiometry; soil microbial biomass



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## 1. Introduction

Recently, the decomposition mechanisms regulated by soil microorganisms during plant residue transformation have attracted considerable attention [1–4]. Plant residue inputs are the primary driver of soil microbial maintenance activity [5,6], significantly affecting organic matter turnover in the topsoil [7,8]. As regulators of global biogeochemical cycles [9], soil microorganisms participate in core ecosystem processes through ex vivo modifications (extracellular enzymes attack and transform plant residues) or in vivo turnovers (cell uptake–biosynthesis–growth–death), such as soil organic matter decomposition, turnover, and sequestration [10]. Meanwhile, most plant residues enter the soil

microbially-mediated in the forms of labile compounds, root exudates, and microbial necromass, which provide energy and nutrients for microbial metabolism [11]. The *ex vivo* modification pathway mainly regulates the production of specific extracellular enzymes that degrade macro-molecular structural compounds of plant residues into low molecules that can be assimilated and used by microorganisms [12,13]. The microbially-mediated transformation of plant residues is closely associated with extracellular enzyme activities (EEAs) [14].

Extracellular enzymes are mainly derived from soil microbial activities, root exudates, and the decomposition of soil, plant, and microbial necromass, which increases microbial acquisition of limiting elements to ensure strict stoichiometric homeostasis in the organism [15,16]. In plant-microbes-soil systems, microbially-mediated decomposition of plant residues and soil nutrient cycling are always dynamic [17], which could result in an imbalance between substrate resource availability and microbial requirements [18]. In addition, soil enzymes can be used to evaluate soil health [19], and some studies have found that increased microbial enzyme activities can promote plant growth and soil fertility [20,21]. Microorganisms mobilize resources by adjusting their elemental utilization efficiency or by producing specific extracellular enzymes to adapt to the insufficient substrate supply to maintain normal life activities [22,23]. Changes in EEAs are driven by complex ecological factors with temporal and spatial heterogeneity. Factors such as soil properties, microbial biomass, and physiological status are considered to be critical in controlling EEAs [24]. Thus, extracellular enzyme activities are frequently used to indicate soil habitat changes and functional characteristics of microbial communities, owing to their sensitivity to the resources [25]. The ratios of extracellular enzymes involved in soil nutrient cycling represent extracellular enzymatic stoichiometry (EES) [26]. EES, as the intersection of ecological metabolic and stoichiometric theories, results from cellular metabolism and is regulated by substrate availability [27–29]. Thereby, EES can reflect the biogeochemical balance between microbial metabolism, nutrient requirement, and substrate availability [30]. On the Loess Plateau in China, revegetation significantly increased aboveground inputs, which can alter soil elemental content and, thus, microbial community composition [31]. Therefore, studying the changes and drivers of soil extracellular enzyme activities and stoichiometry during plant residue transformation is essential to understand the mechanisms of microbially-mediated soil regulation.

The inputs of plant residues are the beginning of the conversion from the plant C pool to the soil C pool, which significantly affects the capacity and stability of the SOC [32,33]. The transformation of plant residues C and the formation of SOC are not isolated but constitute a continuous systemic process that occurs simultaneously [34]. As the decomposition and transformation of aboveground plant residue have been further studied, researchers are increasingly focusing on the “litter-soil” transformation interface. Previously, Kandeler et al. [35] revealed that soil enzyme activities were relatively high in the “litter-soil” transformation interface due to the superior aeration and hydrothermal conditions of the topsoil. Ai et al. [36] also discovered that microorganisms were most active in the organic C-transformed layer. The decomposition products of plant residues mainly accumulate in the topsoil layer (i.e., the interfacial layer), which cannot move to below ground in a short time [37]. Xue et al. [38] defined the narrow organic matter layer between the litter and the surface soil as the “litter-soil” transformation interface soil (TIS) layer, and found strong interactions between vegetation composition, soil properties, and microbial nutrient requirements in the TIS layer. The TIS layer is a continuum of interacting plant fragments with varying degrees of humification, microbial metabolites, and soil (Figure S1) [38]. It is the most active and complicated part of the ecosystem linking above- and below-ground and central to transforming plant residue C to SOC. However, studies focusing on microbially-mediated organic C transformation in the TIS layer are scarce.

To clarify the balance of C, N, and P between microbial requirement and substrate availability in the TIS layer, we investigated the variations of extracellular enzymes and their stoichiometry during the transformation of plant residues. We addressed the following

hypothesis: (1) soil microorganisms can access scarce resources by regulating extracellular enzymes, and as available resources are continuously utilized, microbial metabolic limitations grow, leading to increased activities of the corresponding extracellular enzymes; (2) soil extracellular enzymes and their stoichiometry are influenced by microbial biomass, substrate nutrients, and their stoichiometric characteristics; (3) soil microbial response to substrate limitation could be reflected by the expression of the extracellular enzyme.

## 2. Materials and Methods

### 2.1. Site Description

The sampling site was located in Shanghuang Village, Guyuan City, northwest of the Loess Plateau, China (105°19′–106°57′ E, 35°14′–36°31′ N), which has undergone nearly 40 years of vegetation restoration. This area belongs to the semi-arid mid-temperate to warm temperate transition monsoon climate. The soil type is dominated by Huangmian soil (Calcaric Cambisols, FAO) developed on loess parent material, which is extremely susceptible to erosion. *Stipa bungeana* (*St. B*) is a typical herbaceous plant of the semi-arid region of the Loess Plateau, and belongs to the dominant and constructive species in the sampling area. *St. B* is also called “standing litter” since it will not collapse immediately after death. The standing litter and litter layer accumulated over many years can lead to a thicker TIS layer.

### 2.2. Experimental Design

To eliminate the influence of surface litter on soil properties, we selected abandoned land in the study area with similar years of vegetation restoration and carefully removed surface biological crusts and litter residues before sample sampling. Soil samples were collected at a depth of 0–5 cm using a stainless steel corer with a 2 cm diameter. Soil samples were cleared of debris, mixed, passed through a 2 mm sieve, and air-dried to preserve for later culture. Three sampling sites were selected in the *St. B* community, and three random plant investigations were conducted at each site. The whole *St. B* in the 1 m × 1 m sample square was taken away at the end of the growing season, mixed and dried to constant weight at 70 °C, then crushed and reserved. The crushed litter residue was mixed with the soil sample to simulate the interfacial layer, and the soil and litter residues were mixed in the ratio of 100:1 by mass and laid flat in culture dishes to form the 0.5 cm interfacial layer. Soil samples were pre-cultured for 7 days before mixing to restore the activity of soil microorganisms. The samples were placed in an artificial climate chamber at 28 °C, and the water content was adjusted to 60%–70% of the field holding capacity. Soil moisture was replenished every 3 days to ensure a state of constant soil humidity.

Based on previous indoor incubation experiments fitting the time required for the decomposition of the litter (*St. B*) stages, the time taken to reach 50% and 60% weight loss was 412 and 799 days, respectively (Figure S2). Decomposition was slow, and neared stasis after the weight loss rate reached 63.12 % [39]. Therefore, this experimental cycle was designed for 512 days, and destructive sampling was carried out at certain time intervals. For each sampling time, 4 parallel culture dishes were randomly selected as replications of the decomposition time points. Thus, with 512 days and 42 time points, we have 168 repeats. According to the substrate decomposition, three stages were divided, i.e., early (0–95), middle (95–353), and later (535–512).

### 2.3. Measurement

The soil organic C (SOC) content was determined using the Walkley–Black method [40]. Soil total N (TN) was determined using the micro-Kjeldahl method [41]. Soil total P (TP) was determined with the H<sub>2</sub>SO<sub>4</sub>-HClO<sub>4</sub> digestion-molybdenum blue colorimetric method [42]. Microbial biomass carbon (MBC), nitrogen (MBN), and phosphorus (MBP) were determined by chloroform fumigation extraction [43,44]. Litter–soil mixture samples were fumigated with CHCl<sub>3</sub> at 25 °C for 24 h. The control used unfumigated soil of the same weight and conditions. MBC and MBN were then extracted with 100 mL of 0.5 M

K<sub>2</sub>SO<sub>4</sub> at a ratio of 1:4 (*w/v*), and MBP was extracted with 0.5 M NaHCO<sub>3</sub> at a ratio of 1:20 (*w/v*). MBC, MBN, and MBP contents were calculated based on the differences between fumigated and unfumigated samples and adjusted using experimentally derived conversion factors *E*, where *E<sub>C</sub>*, *E<sub>N</sub>*, and *E<sub>P</sub>* were 0.45, 0.45, and 0.40, respectively [45]. Dissolved carbon (DC), nitrogen (DN), and phosphorus (DP) in the samples were calculated from unfumigated samples [46,47]. Fresh samples stored at 4 °C were used to determine five hydrolytic enzyme activities in the soil. In this study, C-cycle-related enzymes (BG, β-1,4-glucosidase; CBH, β-D-cellobiohydrolase), N-cycle-related enzymes (NAG, β-N-acetyl-glucosaminidase; LAP, leucine-aminopeptidase), and P-cycle related enzymes (AP, alkaline phosphatase) were selected, as these enzymes are mainly involved in catalytic reactions with the ends. One gram of fresh soil was added to 250 mL of 0.5 M acetate buffer in autoclaved jars and dispersed by ultrasonic disaggregation (50 J/s for 120 s). All enzyme activities were determined by fluorometric analysis using 96-well plates and standard fluorometric techniques with appropriate and sufficient substrates (Table S1) [48,49].

#### 2.4. Statistical Analyses

We used the normal probability (Q-Q) graph to test the statistical distribution of various enzyme activities and vector characteristics and calculate the skewness and kurtosis of each measure. A homogeneity test was used to assess all data before analysis, and log transformation was performed for data with uneven variance. Linear regression analysis was performed with SPSS 26.0 statistical software and plotted using Origin Pro 2021. Pearson correlation analysis was performed using SPSS 26.0 for total C, N, P, microbial biomass C, N, P, dissolved C, N, P, soil EEA, and EES. Redundancy analysis (RDA) was performed using Canoco 5.0. All data are the mean of four repeats.

### 3. Results

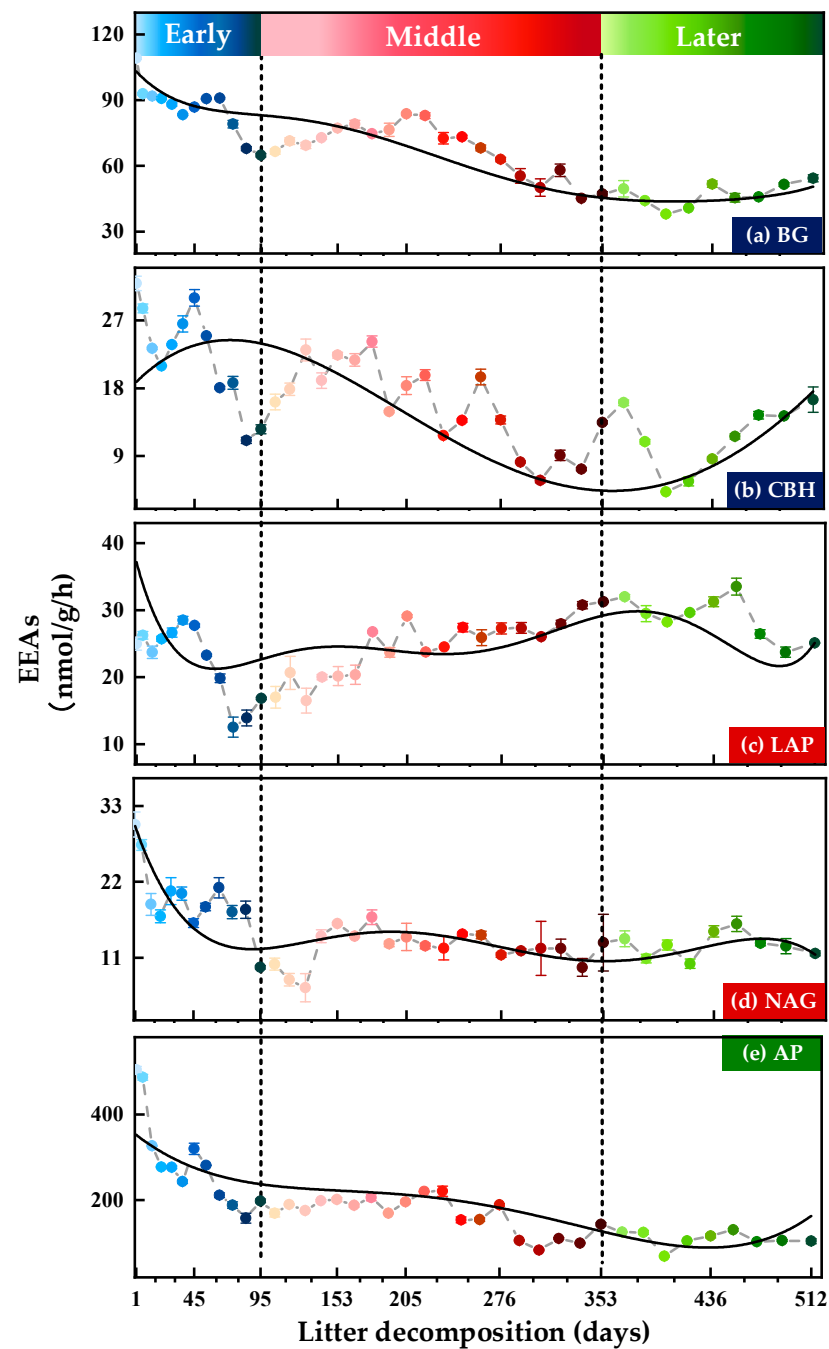
#### 3.1. Variations in Soil Extracellular Enzyme Activities (EEAs) and Extracellular Enzymatic Stoichiometry (EES)

##### 3.1.1. Soil Extracellular Enzyme Activities (EEAs)

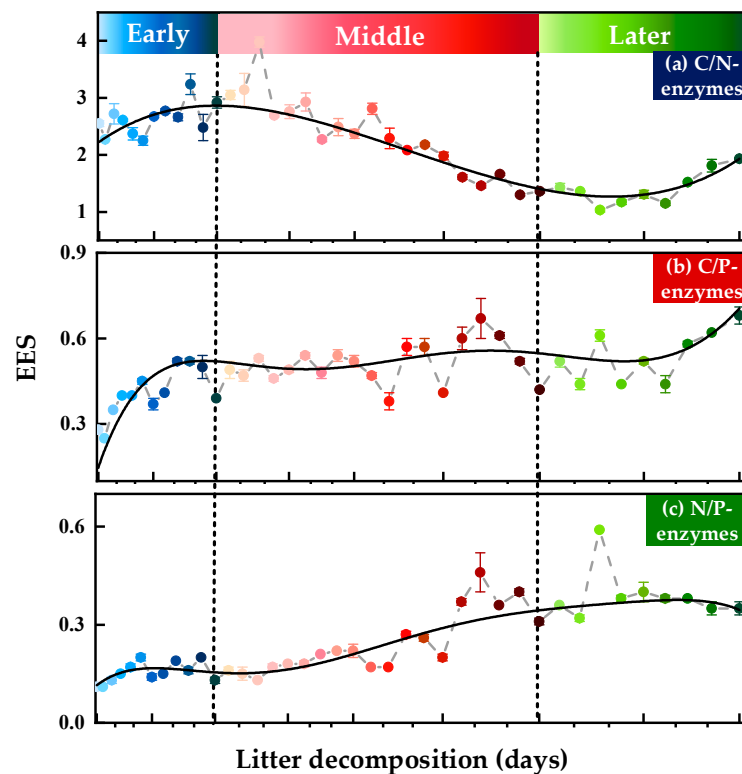
C-enzymes (BG and CBH) fluctuated and decreased in the early and middle stages of plant residue transformation (Figure 1a,b), both reaching the bottom at the 401st day (37.99 nmol/g/h and 4.26 nmol/g/h for BG and CBH, respectively). Subsequently, BG remained in the range of 40.81–54.33 nmol/g/h with slight variations, while CBH rebounded slightly. Eventually, BG and CBH decreased by 50.2% and 48.30%, respectively, compared to the initial stage. N-enzymes (NAG and LAP) activities were both significantly reduced in the early stage (Figure 1c,d). NAG activities remained in a relatively stable range (9.62–14.33 nmol/g/h) in the middle and later stages, with the lowest value of 6.69 nmol/g/h occurring on the 129th day (Figure 1d). The lowest value of LAP appeared on the 74th day (13.46 nmol/g/h) and then slowly recovered to the initial activity, with the final value 1.0 times higher than the initial value (Figure 1c). In addition, the P-enzyme (AP) activities decreased overall during the decomposition of plant residues, with only 20.7% of the initial value on the 512th day (Figure 1e).

##### 3.1.2. Soil Extracellular Enzymatic Stoichiometry (EES)

Soil EES showed different patterns of change at different stages of plant residue transformation in the TIS layer (Figure 2). C/N-enzyme increased significantly in the early stage, peaking on the 129th day (3.98) and then decreasing significantly to the lowest (1.03) on the 401st day (Figure 2a). C/P-enzyme increased significantly in the early stages, remained essentially stable in the middle stages, and increased slightly in the later stages (Figure 2b). C/P-enzyme eventually elevated by 63.24%. N/P-enzyme continued to rise during the transformation of plant residues, with the final value being 3.22 of the initial value (Figure 2c).



**Figure 1.** The variations of soil extracellular enzyme activities (EEAs) during the plant residue transformation. (a) BG,  $\beta$ -1,4-glucosidase; (b) CBH, cellobiohydrolase; (c) NAG,  $\beta$ -N-acetylglucosaminidase; (d) LAP, leucine aminopeptidase; (e) AP, acid phosphatase. The extracellular enzyme activities are expressed as the mean  $\pm$  SE. The dashed lines in the graph represent the actual variation rules. The solid lines are the fitted curves, BG:  $R^2 = 0.97$  \*\*\*; CBH:  $R^2 = 0.95$  \*\*\*; LAP:  $R^2 = 0.59$  \*\*\*; NAG:  $R^2 = 0.58$  \*\*\*; AP:  $R^2 = 0.89$  \*\*\*. \*\*\*  $p < 0.001$ .



**Figure 2.** The variations of soil extracellular enzymatic stoichiometry (EES) during the plant residue transformation. (a) C/N-enzymes; (b) C/P-enzymes; (c) N/P-enzymes. All ratios are expressed as the mean  $\pm$  SE. The dashed lines in the graph represent the actual variation rules. The solid lines are the fitted curves. C/N-enzymes:  $R^2 = 0.94$  \*\*\*; C/P-enzymes:  $R^2 = 0.46$  \*\*; N/P-enzymes:  $R^2 = 0.86$  \*\*\*. \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

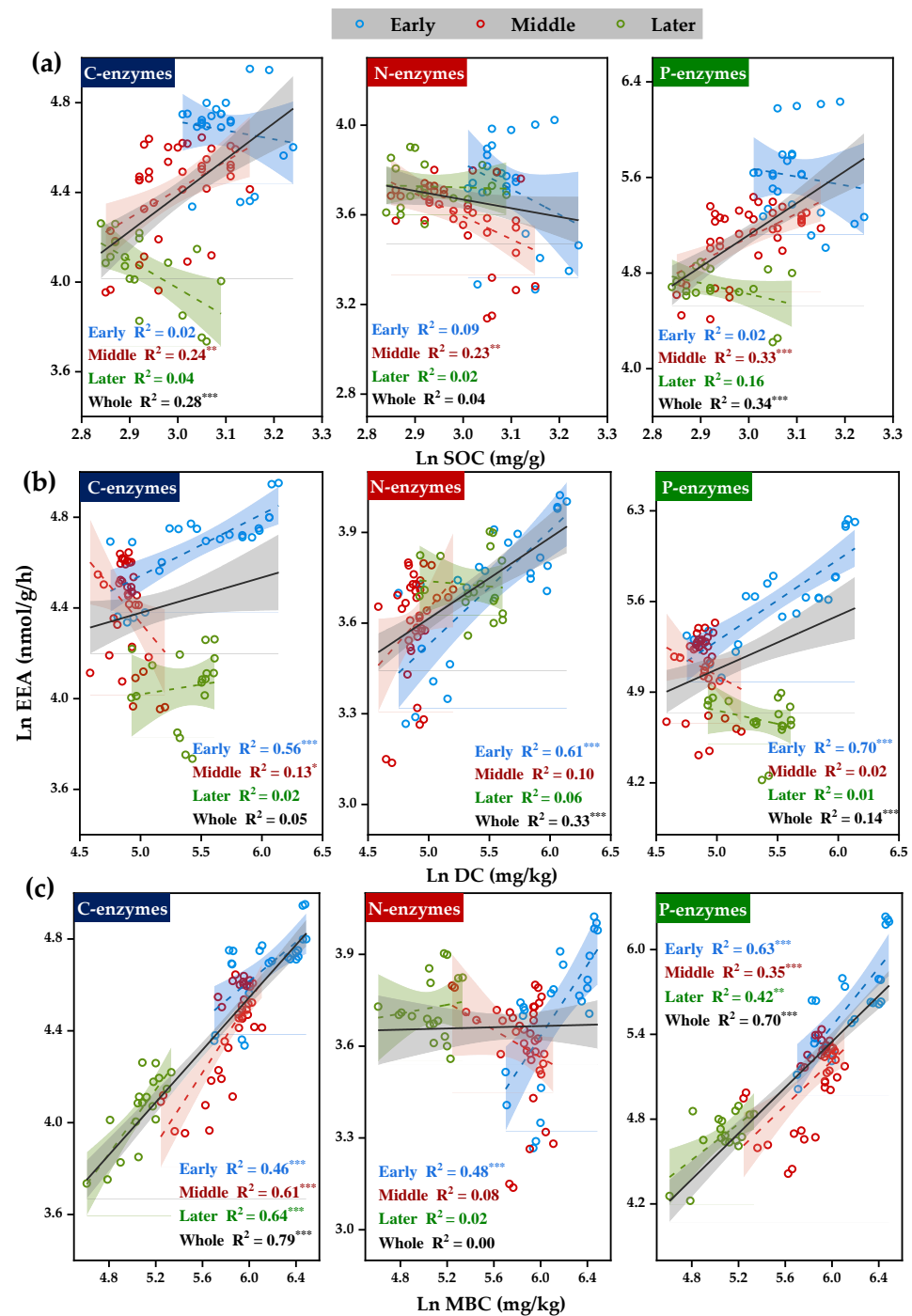
### 3.2. Correlation of Soil Extracellular Enzyme Activities with C, N, and P Transformations

#### 3.2.1. Correlation between Soil Extracellular Enzyme Activities and Substrate C

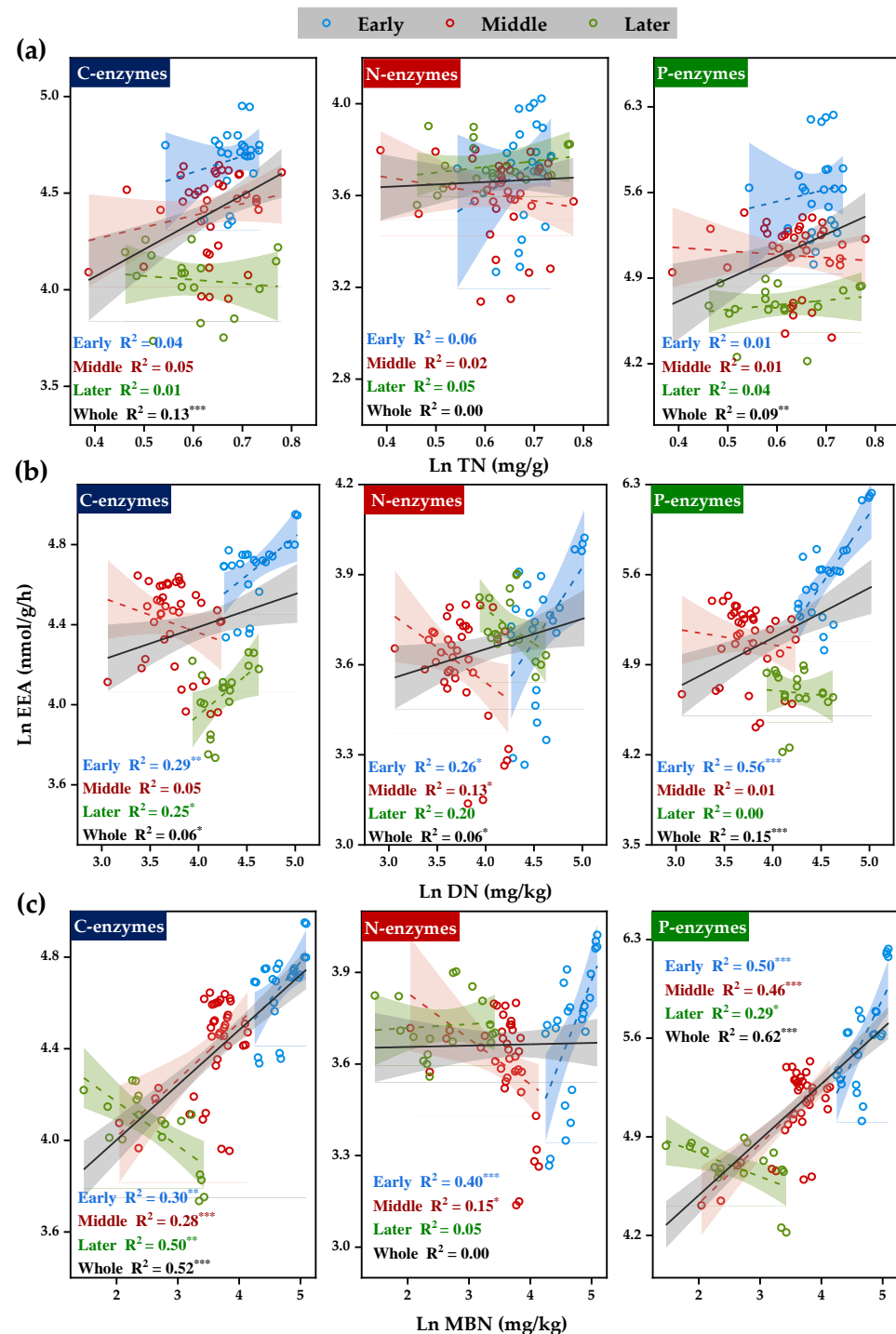
C- and P-enzymes were significantly and positively correlated with SOC throughout the decomposition of plant residues (Figure 3a), especially in the middle stage. C-, N-, and P-acquired EEAs were all significantly and positively correlated with DC in the early stage of plant residue decomposition (Figure 3b). Moreover, C- and P-enzymes showed a significant positive correlation with MBC at all observed stages (Figure 3c). N-enzymes significantly and positively correlated with MBC in the early stages of decomposition but not in other stages.

#### 3.2.2. Correlation between Soil Extracellular Enzyme Activities and Substrate N

C-, N-, and P-acquiring EEAs were insignificantly correlated with TN at early, middle and late stages of plant residue decomposition (Figure 4a). However, both C-, N-, and P-enzymes showed a significant positive correlation with DN in the early stage (Figure 4b). Furthermore, C- and P-enzymes were positively correlated with MBN in the early and middle stages while negatively correlated with MBN in the later stages (Figure 4c).



**Figure 3.** Relationship between soil extracellular enzyme activities (EEAs) and soil C fractions. (a) C-enzymes (BG+CBH) and soil organic C (SOC); N-enzymes (LAP+NAG) and SOC; P-enzymes (AP) and SOC. (b) C-enzymes and dissolved C (DC); N-enzymes and DC; P-enzymes and DC. (c) C-enzymes and microbial biomass C (MBC); N-enzymes and MBC; P-enzymes and MBC. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

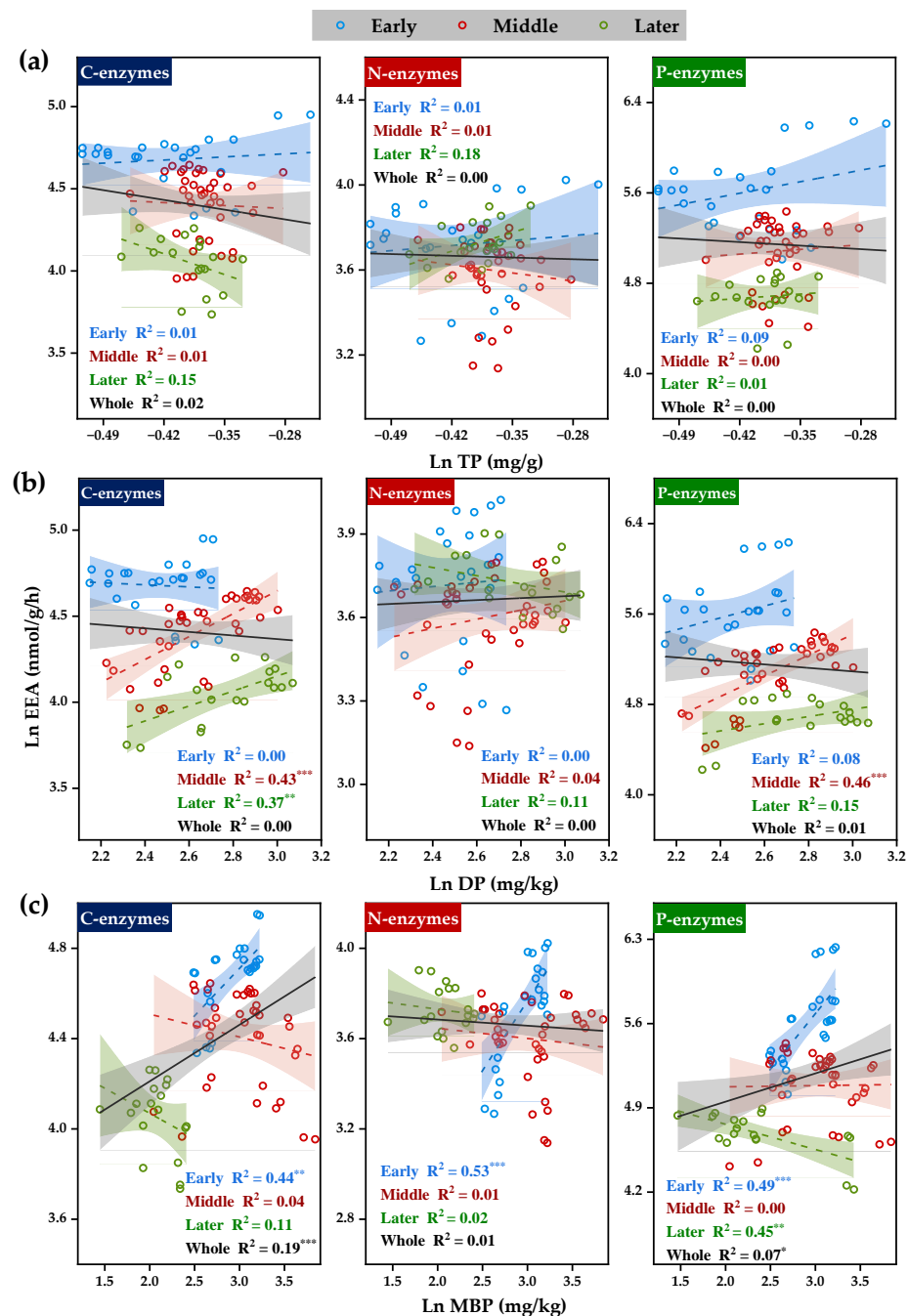


**Figure 4.** Relationship between soil extracellular enzyme activities (EEAs) and soil nitrogen fractions. (a) C-enzymes (BG+CBH) and total nitrogen (TN); N-enzymes (LAP+NAG) and TN; P-enzymes (AP) and TN. (b) C-enzymes and dissolved nitrogen (DN); N-enzymes and DN; P-enzymes and DN. (c) C-enzymes and microbial biomass nitrogen (MBN); N-enzymes and MBN; P-enzymes and MBN. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

### 3.2.3. Correlation between Soil Extracellular Enzyme Activities and Substrate P

There was no significant correlation between EEAs and TP during the transformation of plant residues (Figure 5a). C- and P-enzymes were significantly and positively correlated with DP in the middle stage (Figure 5b). However, N-enzymes exhibited an insignificant correlation with DP. N-enzyme showed a significant positive correlation with MBP only

in the early stage, while C- and P-enzymes showed a significant positive correlation with MBP throughout the observed stages (Figure 5c).



**Figure 5.** Relationship between soil extracellular enzyme activities (EEAs) and soil phosphorus fractions. (a) C-enzymes (BG+CBH) and total phosphorus (TP); N-enzymes (LAP+NAG) and TP; P-enzymes (AP) and TP. (b) C-enzymes and dissolved phosphorus (DP); N-enzymes and DP; P-enzymes and DP. (c) C-enzymes and microbial biomass phosphorus (MBP); N-enzymes and MBP; P-enzymes and MBP. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

### 3.3. Influential Factors of Soil Extracellular Enzyme Activities and Extracellular Enzymatic Stoichiometry

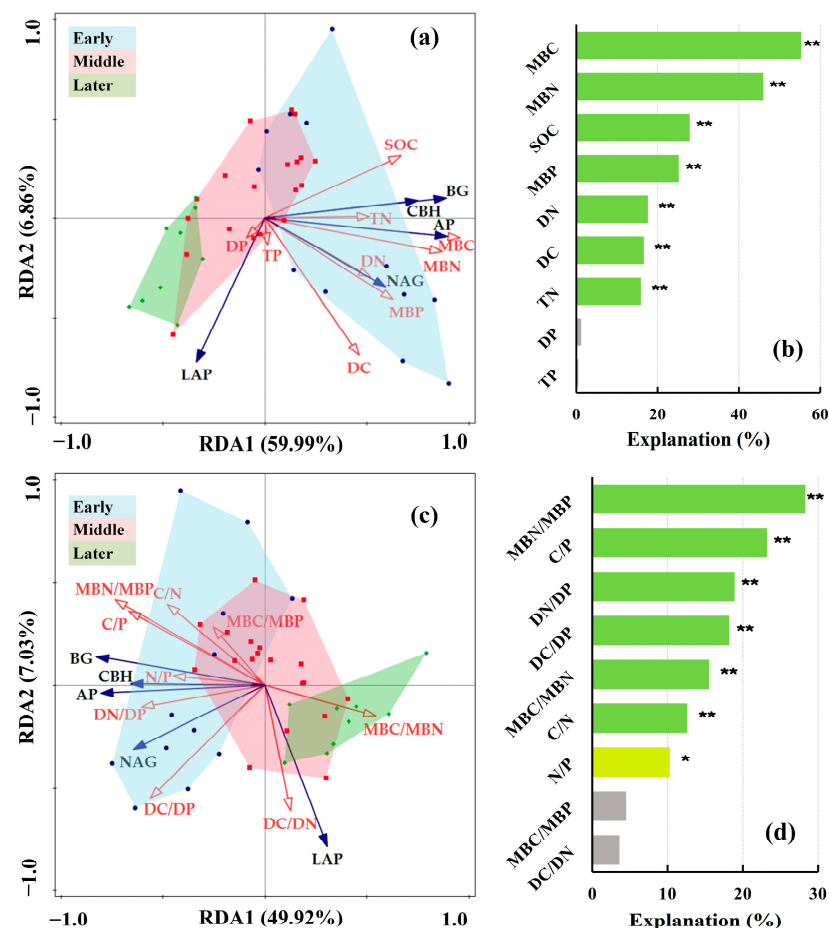
#### 3.3.1. Influential Factors of Soil Extracellular Enzyme Activities

Correlation analysis of EEAs with substrate C, N, and P contents and their stoichiometry during plant residue transformation in the TIS layer was performed based on RDA and

Pearson (Figure 6 and Table 1). The results indicated that LAP showed significant negative correlations with SOC, C/N, C/P, and MBN/MBP ( $p < 0.01$  \*\*) and positive correlations with MBC/MBN and DC/DN ( $p < 0.05$  \*; Table 1). In addition, BG, CBH, NAG, and AP exhibited significantly positive correlations with SOC, TN, DC, DN, MBC, MBN, and MBP, as well as stoichiometric C/P, DC/DP, DN/DP, while negatively correlated with MBC/MBN (Figure 6a,c). MBN/MBP and C/N were also positively correlated with C- and P-enzymes. The highest explanation for extracellular enzymes was MBC (55.3%) (Figure 6b,d).

### 3.3.2. Influential Factors of Extracellular Enzymatic Stoichiometry

The factors influencing EES during plant residue transformation in the TIS layer were analyzed based on RDA and Pearson (Figure 7 and Table 2). C/N-enzyme significantly and positively correlated with SOC, TN, MBC, MBN, C/N, C/P, MBC/MBP, and MBN/MBP, while significantly and negatively correlated with DC/DN and MBC/MBN (Figure 7a,c). C/P-enzyme and N/P-enzyme were negatively correlated with microbial biomass, TN, SOC, C/N, C/P, DN/DP, and MBN/MBP but positively correlated with MBC/MBN. In addition, C/P-enzyme was also significantly and negatively correlated with DC and DN. MBC explained the largest variation in EES (38.1%), followed by DC (15.6%) and DN (10.6%), while the explanation of MBN/MBP in the substrate stoichiometry was the highest (46.8%) (Figure 7b,d).



**Figure 6.** Redundancy analysis (RDA) identifies the relationships between extracellular enzyme activities (EEAs) and (a,b) microbial biomass, total nutrients, dissolved nutrients, and (c,d) their stoichiometry. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

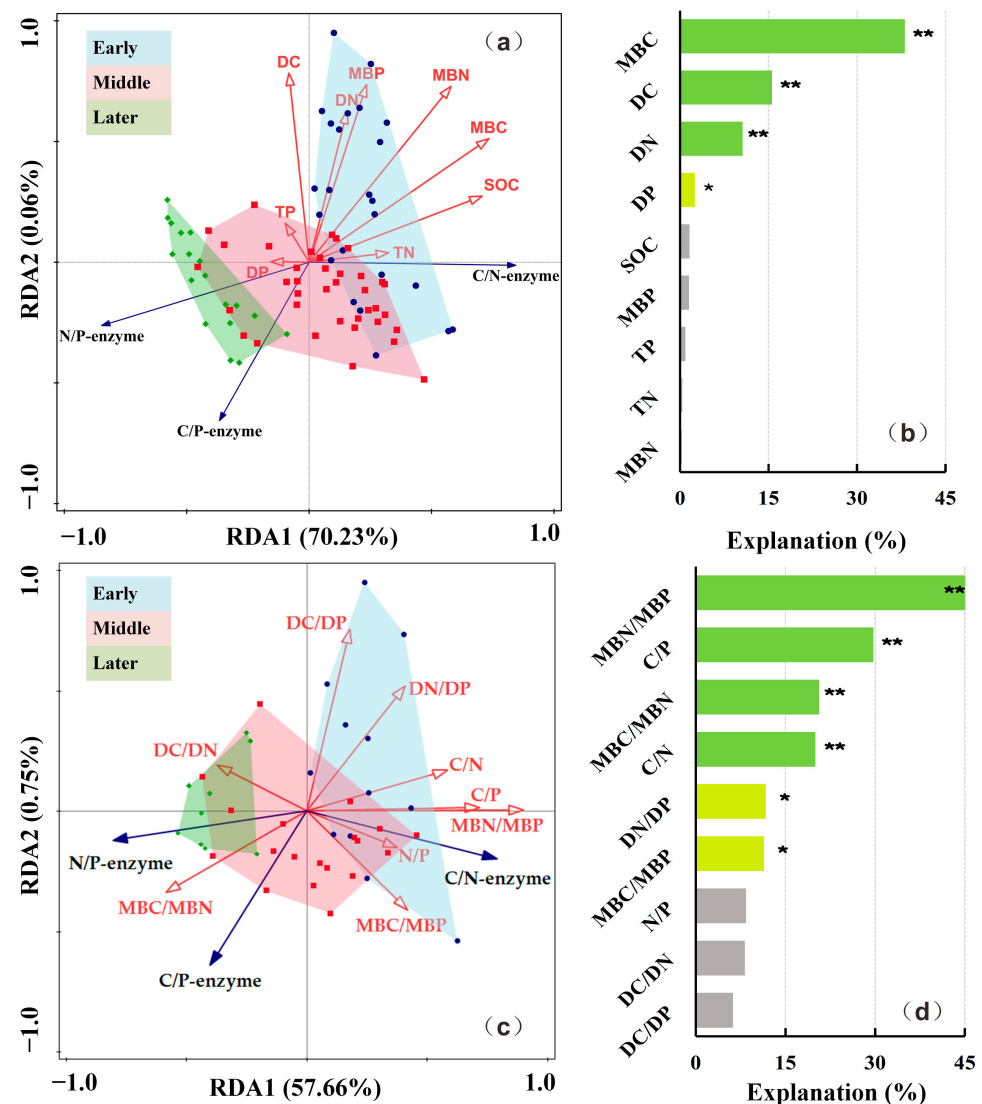
**Table 1.** Pearson correlation coefficients between soil C, N and P fractions variables and enzyme activities.

Correlation Coefficients		BG	CBH	LAP	NAG	AP
Total C, N, P	SOC	0.587 **	0.526 **	−0.543 **	0.438 **	0.548 **
	TN	0.486 **	0.444 **	−0.199	0.389 *	0.402 *
	TP	−0.001	−0.026	0.033	0.125	0.220
	C/N	0.352 *	0.320 *	−0.478 **	0.248	0.364 *
	C/P	0.543 **	0.491 **	−0.499 **	0.359 *	0.422 **
	N/P	0.395 *	0.373 *	−0.162	0.254	0.210
Microbial biomass C, N, P	MBC	0.892 **	0.731 **	−0.277	0.624 **	0.854 **
	MBN	0.790 **	0.650 **	−0.285	0.727 **	0.850 **
	MBP	0.538 **	0.511 **	0.006	0.655 **	0.682 **
	MBC/MBN	−0.501 **	−0.313 *	0.321 *	−0.364 *	−0.464 **
	MBC/MBP	0.295	0.143	−0.229	−0.116	0.043
	MBN/MBP	0.700 **	0.430 **	−0.532 **	0.288	0.511 **
Dissolved C, N, P	DC	0.334 *	0.414 **	0.249	0.688 **	0.624 **
	DN	0.382 *	0.428 **	−0.101	0.662 **	0.617 **
	DP	−0.161	−0.046	0.176	−0.099	−0.134
	DC/DN	−0.072	−0.075	0.536 **	0.013	−0.030
	DC/DP	0.418 **	0.445 **	0.174	0.695 **	0.661 **
	DN/DP	0.459 **	0.438 **	−0.198	0.637 **	0.616 **

Note: \*  $p < 0.05$ , \*\*  $p < 0.01$ .**Table 2.** Pearson correlation coefficients between soil C, N and P fractions variables and soil extracellular enzymatic stoichiometry.

Correlation Coefficients		C/N-Enzyme	C/P-Enzyme	N/P-Enzyme
Total C, N, P	SOC	0.598 ***	−0.493 ***	−0.604 ***
	TN	0.280 *	−0.147	−0.282 *
	TP	−0.090	−0.060	0.062
	C/N	0.466 **	−0.357 *	−0.453 **
	C/P	0.583 **	−0.328 *	−0.540 **
	N/P	0.312	−0.083	−0.276
Microbial biomass C, N, P	MBC	0.695 ***	−0.502 ***	−0.824 ***
	MBN	0.606 ***	−0.647 ***	−0.728 ***
	MBP	0.156	−0.515 ***	−0.300 *
	MBC/MBN	−0.418 **	0.470 **	0.458 **
	MBC/MBP	0.363 *	0.081	−0.339 *
	MBN/MBP	0.685 **	−0.378 *	−0.717 **
Dissolved C, N, P	DC	−0.156	−0.418 *	−0.046
	DN	0.088	−0.409 **	−0.201
	DP	−0.126	0.051	0.039
	DC/DN	−0.378 *	0.042	0.265
	DC/DP	−0.024	−0.505 **	−0.155
	DN/DP	0.227	−0.461 **	−0.317 *

Note: \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .



**Figure 7.** Redundancy analysis (RDA) identifies the relationships between extracellular enzymatic stoichiometry (EES) and (a,b) microbial biomass, total nutrients, dissolved nutrients, and (c,d) their stoichiometry. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

## 4. Discussion

### 4.1. Association of Extracellular Enzyme Activities (EEAs) with Environmental Nutrient Variables

Moorhead et al. [50] indicated that enzyme activities characterize soil nutrient turnover capacity and play a critical role in plant residue transformation. Different kinds of extracellular enzymes not only divide the labor involved in substrate decomposition at different stages but also synergistically maintain microbial homeostasis [14]. The pre-culture phase at constant temperature and humidity is conducive to the activation of dormant microorganisms, which are generally in a “starved” state. The exogenous addition of crushed plant residues first supplements the labile substrate, providing energy and nutrients that can be used directly by microorganisms [51,52]. The results of the present study showed that, with the exception of LAP, the initial values of other extracellular enzyme activities were the highest (Figure 1). EEAs began to decrease as the active ingredient (soil dissolved nutrients and microbial biomass nutrients) decreased. This is contrary to our hypothesis 1 that continued substrate depletion would increase metabolic restriction, leading to more extracellular enzyme production. Zheng et al. [53] indicated that when the active substrate dominates the microbial nutrient source, the higher extracellular enzyme levels reflect

energy availability in the substrate and a higher rate of microbial metabolism. This view is consistent with the results of the correlation analysis between labile substrate and EEAs. The labile components (DC, DN, MBC, MBN, and MBP) were significantly and positively correlated with the extracellular enzymes in the early stages of plant residue transformation (Figures 3–5). And when the labile fraction of plant residue is no longer the main source of C, microorganisms are forced to secrete relevant extracellular enzymes to get energy and nutrients from recalcitrant substrates [54]. In the plant–microbial soil system, the microbial “C pump” has the significant function of regulating the sequestration and allocation of organic C (labile part, recalcitrant part, microbial necromass, and soil “old C”) to maintain stoichiometric homeostasis [10,55].

Guillaume et al. [56] indicated that the higher the C/N ratio, the slower the mineralization rate as the labile substrate is depleted. The decrease in the value of soil C/N ratio, which characterizes the rate of SOC decomposition, indicates a gradual increase in the decomposition of organic C during the plant residue transformation, resulting in a positive priming effect that led to a decrease in SOC (Table S2). During the plant residue transformation, C-enzymes were significantly and positively correlated with SOC (Table 1), especially in the middle stages (Figure 3). Soil microorganisms secrete EEAs through *ex vivo* modification pathways to convert large molecular compounds into small ones, thus providing deficient energy and nutrients [10,57]. As a result, the extracellular enzyme activities of BG, CBH, and LAP all picked up to varying degrees in the middle or late stages to obtain C and N from recalcitrant substrates (Figure 1). Moreover, throughout the plant residue transformation, the EEAs and microbial biomass decreased significantly compared to their initial values (Figure 1 and Table S2), except for LAP. This implied that soil microbial activity gradually decreased as the plant residues decomposed. The microbial “C pump” is mainly regulated by heterotrophic microorganisms, which require continuous inputs of exogenous organic C for the long-term activity to maintain the pump operation [5,58]. In contrast, the decomposition and utilization of recalcitrant compounds or “old C” by soil microorganisms is a lagging and slow process, which is a desperate choice for microbial metabolic limitation.

The results showed that N-enzymes (LAP and NAG) or P-enzymes (AP) correlate with different soil C fractions (SOC, DC, or MBC). This is attributable to the fact that C-related substrates dominate the transformation of plant residues as the major energy source and influence C-, N-, and P-enzyme activities [59]. There was also a significant and positive correlation between P-enzyme with DN and MBN (Figure 6 and Table 1), which means that large amounts of C and N are required to satisfy the conditions for P-enzyme production when P is metabolically limited [18,60]. Soil C-, N-, and P-enzymes were insignificantly correlated with TP while mainly influenced by DP and MBP (Figures 5 and 6, and Table 1). Most of the P in the soil is bound into chemical complexes [61], so high P levels do not necessarily indicate sufficient effective P for microbial use. Consistent with hypothesis 2, soil EEAs were driven by a combination of factors, including substrate nutrient status and microbial demand [15,29].

#### 4.2. Driving Factors of Soil Enzymatic Stoichiometry (EES)

Previous meta-analyses showed that the ratio of different EEAs (BG, NAG, LAP, and AP) to SOC was close to 1.0 [14]. However, the absolute values of the slopes between EEA and SOC in the present study ranged from 0.06 to 2.00 (Figure 3a). This suggests the extreme imbalance between the availability of substrate resources and the microbial metabolic demands. Soil microbial communities are able to access limited resources from complex substrates by secreting specific extracellular enzymes, resulting in a high degree of flexibility in soil enzyme stoichiometry [62]. Soil microbes respond to metabolic C- and P-limitations by secreting C-enzymes (C/N enzyme > 1) and P-enzymes (N/P enzyme < 1; Figure 2), which respond to target resource imbalances through EEAs to maintain microbial homeostasis [15,60]. The decrease in C/N-enzyme and MBN/MBP and the increase in C/P-enzyme and N/P-enzyme (Figure 2 and Table S2) indicated that soil microbial demand

for N gradually increased during the transformation of plant residues, which corresponded to the trend of increasing LAP enzyme activity. In addition, Mori et al. [63] presented new findings on substrate resource allocation, which suggested that the declining C/N-enzymes do not represent the utilization of C and N resources by soil microorganisms, but can be used to determine the source of C acquisition by soil microorganisms (plant-derived or microbial-derived). Soil microbes can acquire C sources by secreting N-enzymes to degrade N-containing compounds, e.g., lignin-embedded protein and N-rich microbial necromass [53,64]. This microbial metabolic process not only provides N sources but also alleviates microbial C-limitation.

According to the correlation between soil EES and substrate stoichiometry, soil microbial biomass and its stoichiometry significantly influenced extracellular enzymes and their stoichiometry (Figure 7 and Table 2). Because microbial biomass is the labile part of organic matter, it is more sensitive than chemical properties to respond to the transformation processes of plant residual C [65]. MBC explained the highest percentages of EEAs and EES among many factors (Figures 6b and 7b). This is attributed to the fact that MBC is the available and preferred energy utilized by soil microorganisms, which is the driving force for organic matter decomposition [66]. Unlike the available part, soil microorganisms first converted recalcitrant C fractions into labile ones via the *ex vivo* modification pathway (EEAs' action), and then further transformed to the microbial biomass via the *in vivo* turnover pathway [10], which is invoked in case microbial metabolic limitation occurs [67,68]. The active substrates decreased with the transformation of plant residues, forcing some microorganisms to dormancy or intracellular element mineralization. As a result, microbial biomass (MBC, MBN, and MBP) decreased throughout decomposition (Table S2). In addition, microbial biomass stoichiometry changes may be related to the shift in microbial community structure [69], which could affect enzymatic stoichiometry as well. In summary, the present results indicated that soil microbial biomass stoichiometry and, in particular, the influence of MBC are most prominent for soil extracellular enzyme activity and its stoichiometric variation than other factors.

## 5. Conclusions

Soil extracellular enzyme activities are essential in material cycling and energy flow. When labile fractions (DC, DN, MBC, and MBN) are the predominant C source for microbes, the high extracellular enzyme activity implies the availability of substrate resources. As dissolved substances were depleted, the microorganism was forced to secrete relevant extracellular enzymes to obtain energy and nutrients from the recalcitrant substrate. The relative decrease in cellulose with plant residue transformation prompted soil microbes to degrade protein-containing compounds by secreting N-enzymes to supplement both N and C sources, leading to a decrease in C/N-enzymes. However, long-term monitoring of plant residue transformation revealed that microbial biomass and enzyme activities decreased significantly. Therefore, the decomposition of recalcitrant compounds for nutrients is a lagging and slow process, which is insufficient to sustain the long-term activity of some microorganisms. The results demonstrated that extracellular enzymes and their stoichiometry are affected by the combination of substrate and microbial factors. Soil microbial biomass factors, especially MBC, are essential indicators of labile C forms and microbial activity, which significantly influenced variations in extracellular enzymes and their stoichiometry in the present study. This study contributes insight into extracellular enzymes and their stoichiometric drivers, which is valuable for further exploration of substrate element dynamics during the microbially-mediated transformation of plant residues.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f14010034/s1>, Figure S1: definition of the transformation interface soil layer (TIS); Figure S2: dynamic fitting of weight loss rates and decomposition processes of *Stipa bungeana* (St. B); Table S1: basic information on the soil extracellular enzymes (EEAs) investigated here; Table S2: variation in substrate properties.

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