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Impact of soil leachate on microbial biomass and diversity affected by plant diversity

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Abstract
Aims High plant diversity is usually linked with high soil microbial diversity, which is hypothesized to be attributed to a high diversity of components in the soil leachate, but experimental evidence is scarce. The aim of this study was to determine if the variation in soil leachate caused by plant diversity could affect the soil microbial community.
Methods A microcosm experiment was conducted to determine the effect of plant diversity on the soil microbial community by measuring soil leachate in a gradient of plant richness from levels 1 (one species) to 3 (three species).
Results Plant richness significantly affected the diversity of soil leachate and microbial communities. The amount and diversity of soil leachate, microbial biomass carbon (C), basal respiration, β-1,4-glucosidase activity, β-1,4-N-acetylglucosaminidase activity, bacterial biomass, fungal biomass, total microorganism biomass, and microbial diversity (Shannon diversity index and evenness) were highest at richness level 3. Changes in the microbial community were best explained by variation in the amount and diversity of leachate. Linear regression and correlation analyses indicated that leachate diversity had a close association with microbial Shannon diversity and evenness, whereas leachate amount had a close association with microbial biomass C, total microbial biomass, bacterial biomass, enzyme activities, and abundance of microbial groups. An ordinary least squares multiple regression and the structural equation model demonstrated that leachate amount had a greater effect on microbial biomass than leachate diversity, which had a greater impact on microbial Shannon diversity and evenness.
Conclusions Our results indicate that plant diversity drives changes in soil microbial communities by altering the amount and diversity of leachate in the soil. The diversity of soil leachate determined the diversity of the microbial community to some extent.

Keywords Soil leachate · Plant richness · Microbial diversity · Biomass · Grass species

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Introduction

Plants are the primary producers in most terrestrial ecosystems and make use of resources in the soil through their root systems. Ecosystem services and functions, including aboveground productivity, soil nutrient levels,

C stocks, and belowground biochemical processes depend on plant diversity (Eisenhauer et al. 2010; Spehn et al. 2000). Plants affect soil microorganisms via the production of aboveground biomass, litter quality, root decomposition, and root exudation (Steinauer et al. 2016; Wardle et al. 2004). Previous studies have demonstrated that an increase in plant diversity can increase the biomass and activities of microbial communities (Eisenhauer et al. 2017; Lange et al. 2015). Plant community composition is therefore a decisive factor in determining the composition of the soil microbial community (Kuzyakov and Blagodatskaya 2015).

In the belowground ecosystem, root biomass increases as plants grow, and increased root biomass can provide many available resources for soil microorganisms via root turnover and exudation (Mueller et al. 2013). Microorganisms use these resources as food substrates to maintain their daily activities and, in doing so, produce active or inactive metabolic compounds (Badri and Vivanco 2009). Soil leachate is a complex solution containing abundant root exudates, some rhizodeposition, and low microbial metabolite content. Root turnover and exudates tend to decrease with plant diversity (Mommer et al. 2015; Philippot et al. 2013), but the impact of plant diversity on soil leachate has rarely been investigated. Thus, little information is available on the interaction between soil leachate and microbial communities, even though the amount and variety of compounds from leachate may be responsible for changes in soil microbes (Steinauer et al. 2015, 2016).

The quantity and quality of soil leachate (mainly root exudates) depend on plant characteristics such as the species, abundance, and growth and on soil variables such as type (Murugan et al. 2014), moisture, and nutrient conditions (Lorenzo et al. 2013). It is accepted that plant diversity determines the quality and quantity of root exudates released into the soil. Different plant species release various chemicals into the soil that change soil conditions and consequently change the composition, abundance, and activity of the microbial community (Dennis et al. 2010; Read et al. 2003). The biochemical activity of root exudates in leachate depends on the structure of soil compounds and is usually changeable and neighboring or other plants can have synergistic effects on root exudates that can affect exudate activity and subsequently influence soil microorganisms (Haichar et al. 2014). A more diverse plant community is therefore assumed to have a more diverse composition of soil leachate and thereby a higher soil microbial diversity (Philippot et al. 2013; Thakur et al. 2015).

Changes in the components of soil leachate can lead to large variations in the activities of extracellular enzymes (Steinauer et al. 2016). These enzymes are generated predominantly by soil microbes and partly by plant roots for decomposing polymeric substances, such as cellulose and lignin, into bioavailable compounds (e.g., sugars and amino acids) for microbial metabolism and plant growth (Janusz et al. 2017). For example, glucosidase plays an essential role in the degradation of cellulose and usually illustrates the increased activity of specialized soil bacterial and fungal groups (Zhao et al. 2015), and phosphatase activities responded uniformly to an increasing dominance of heterotrophic bacteria (Spohn and Kuzyakov 2013). Knowledge of specific extracellular enzymes could thus increase our understanding of soil microbial activities.

Interactions between soil leachate and microorganisms have usually been determined by indirect methods, including the amount of variance explained in linear regression models and the contribution of variables in structural equation models (Eisenhauer et al. 2017). Mellado-Vazquez et al. (2016) recently reported the essential role of root exudates in profiling microbial distribution using continuous ^{13}C labeling in a controlled environment. These authors reported that increased plant diversity facilitated the accessibility of plant-derived C to bacteria and arbuscular mycorrhizal fungi. Sauheitl et al. (2010) found that the composition of amino acids varied along a gradient of plant diversity. How plant diversity regulates the dependence of changes in microbial communities on the variations in the quantity and diversity, or their combinations, of soil leachate, however, remains uncertain.

In this study, we aimed to determine whether the diversity of soil leachate affected by plant diversity affects the soil microbial community. To this end, we established a microcosm experiment with a gradient of increasing plant richness ranging from monocultures to mixtures of two and three species. Three functionally and physiologically dissimilar grass species dominating the natural grassland of the Loess Plateau, a semiarid area in China, were used. Leachate components in the soil were measured by gas chromatography-mass spectrometry (GC-MS), and microbial community structure was determined by measuring the quantities of phospholipid fatty acids (PLFAs). We hypothesized that (i)

changes in plant richness lead to variations in microbial properties due to changes in soil leachate (amount and diversity); and (ii) the amount of leachate in the soil has a greater effect on microbial biomass and enzyme activity than leachate diversity, which has a larger impact on microbial diversity.

Materials and methods

Experimental set-up

Three dominant plant species *Artemisia sacrorum*, *Lespedeza davurica*, and *Bothriochloa ischaemum* from the grassland community on the Loess Plateau, a semi-arid area in China, were selected for a pot experiment. *A. sacrorum*, *L. davurica*, and *B. ischaemum* belong to the families Compositae, Leguminosae, and Gramineae, respectively. These species were chosen according to their physiological dissimilarity to ensure differences in their root exudates. Plant monocultures and mixtures of two and three species were established to create a gradient of plant diversity representing richness levels 1, 2, and 3, respectively. A control treatment (pots without plants) was conducted to rule out the microbial effects from the original soils and artifacts from the pots. Soil leachate compounds and microbial properties at different levels of plant richness were calculated by subtracting the values obtained in the control treatment from those obtained in the richness treatments (Table 1).

The microcosm experiment was conducted using flower pots (diameter 28 cm, height 42 cm) with a 6.0-mm mesh covering the bottom for draining water. The pots were filled with 1500 g of 2-mm mesh-sieved and homogenized soil (pH 8.4, 4.6% C, 0.3% N, C:N ratio 15.7, 16% water content) collected from the natural grassland of the Ansai grassland restoration experiment at the Chinese Academy of Sciences. The soil was defaunated by three freeze-thaw cycles at −20 °C and 25 °C before the experiment to eliminate the possible effects of competition for nutrients at higher trophic levels, such as Collembola, on soil microbial biomass and diversity (Steinauer et al. 2016). We planted the same total densities (12 plants pot^{-1}) for the various monocultures and species mixtures, and pre-grown seedlings 5 cm in height were transplanted into the pots. Each richness level had twelve replicates. The plants were grown in a climate-controlled chamber (day/night cycles of 16/8 h and 25/16 °C ± 2.0 °C, photosynthetically active radiation 400 μE) for 180 d. Irrigation was conducted using distilled water and the amount was increased from 100 ml every two days during the initial 60 days to 200 ml every day after that. The same amount of water was added to each pot to exclude the effects of plant diversity on soil microorganisms caused by soil water content. No nutrients were added throughout the experiment.

Harvest and laboratory analyses

The experiment was terminated after six months. The entire soil content in each pot was sieved using a 2-mm mesh to remove the roots, which were washed and weighed to determine the total root biomass after drying at 65 °C for 48 h. The sieved soil was divided into two subsamples; one was kept at 4 °C for measuring microbial biomass C and enzymatic activities, and the other was immediately kept at −40 °C for determining microbial biomass and composition based on PLFA quantities.

Collection of soil leachate for GC-MS analysis

Soil leachate were collected based on the method described by Badri et al. (2013) and modified by Zhu et al. (2016). The procedures included the preparation of purified extracts containing chemical components of the soil, component identification by GC-MS, and data processing. First, 300 ml of deionized water was added to each pot, and approximately 210 ml soil extract was collected. The extracts were filtered through a 0.45-μm film, and 150 ml of the liquid was collected and subsequently freeze-dried and powdered. The extract powder was dissolved in 80% methanol, and the solution was centrifuged at 6000 rpm for 30 min. The supernatant was transferred into a Teflon tube and dried using a termovap sample concentrator under N_2 (Hengao technology Co., LTD, Tianjin, China). Two milliliters of 75% methanol was then added to the tube, and the solution was transferred to GC-MS glass vials for centrifugation and then dried under N_2. The compounds in the leachate were identified using GC-MS at the Chinese Academy of Sciences. A mixture of internal retention index markers was prepared using fatty acid methyl esters from C8–C30 dissolved in chloroform at concentrations of 0.8 mg ml^{-1} (C8–C16) and 0.4 mg ml^{-1} (C18–C30) (Sana et al. 2010). A mixture of 1 μl was added to the dried extracts, and 10 μl of a

Table 1 Design of the experiment

Richness levels	Plant species	Plant number/pot	Number of pot
Control	*No plant*	0	12
1	*A. sacrorum*	12	4
	L. davurica	12	4
	B. ischaemum	12	4
2	*A. sacrorum: L. davurica*	6: 6	4
	A. sacrorum: B. ischaemum	6: 6	4
	L. davurica: B. ischaemum	6: 6	4
3	*A. sacrorum: L. davurica: B. ischaemum*	4: 4: 4	12

A. sacrorum: *Artemisia sacrorum, L. davurica: Lespedeza davurica; B. ischaemum: Bothriochloa ischaemum*

solution of 20 mg ml^{-1} 98% methoxyamine hydrochloride (Sigma-Aldrich Inc., St. Louis, USA) in pyridine was added and shaken at 35 °C for 100 min, and 100 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide was also added and shaken at 37 °C for 30 min. Aliquots of 1 μl were analyzed by an Agilent 7890A gas chromatograph (Agilent Technologies Inc., Santa Clara, USA) equipped with Leco ChromaTOF Version 2.3. The compounds were separated using a Rxi-5Sil MS Integra column (Restek, Bellefonte, USA) 30 m in length, an inner diameter of 0.25 mm, and a film thickness of 0.25 μm, with a 10-m guard column. Injection temperature was set at 240 °C for full gasification. The separation temperature was established as follows: 3 min at 85 °C and subsequently increased from 5 °C min^{-1} to 300 °C for 10 min. The exported data containing the spectral intensities were filtered in the metabolomics BinBase database. The compounds in the leachate were identified by the BinBase identifier according to the retention index of the peak and mass spectra, and the compounds were quantified based on peak height. The data in the BinBase were matched to the Fiehn mass spectral library (http://fiehnlab.ucdavis.edu/Metabolite-Library) and were normalized as described by Fiehn et al. (2008). The amount of soil leachate was treated as the total dry weight, with units of g kg $soil^{-1}$.

Diversity of soil leachate

The diversity of soil leachate was estimated using the Shannon index H (H_{leachate}) to investigate the overall change in leachate at different plant richness levels:

$$H = -\sum_{i=1}^{n} pi \ln pi$$

Where p_i is the relative proportion of each compound, and n is the number of detected compounds.

Microbial community activities

Microbial biomass C, basal respiration, and enzymatic activities were used as indicators of microbial community activity. Microbial biomass C was measured via chloroform fumigation (Vance et al. 1987). Soil basal respiration was measured via alkali absorption (Jenkinson and Powlson 1976). The activities of β-1, 4-glucosidase (BG), β-1,4-N-acetylglucosaminidase (NAG), and alkaline phosphatase (AP) involved in C, N, and P cycles were measured via a fluorescent microplate assay using substrates labeled with 4-methylumbelliferone (4-MUB). The determination of microbial biomass C, basal respiration, and enzymatic activities were conducted as previously reported (Zhang et al. 2011, 2018).

PLFA-based microbial biomass

The biomass of a microbial community was represented by the specific PLFA signatures, including total microorganisms, bacteria, fungi, and actinomycetes. The extraction of PLFAs was performed using the Bligh-Dyer method (Frostegard et al. 1993) and modified by Zhang et al. (2015). Briefly, 2.5 g of soil sample was mixed with a solution of chloroform:methanol:citrate (1:2:0.8 *v*/v/v) and subsequently shaken for 40 min. Two phases, liquid and solid, were separated after 12 h. Three components of lipids, including neutral lipids, glycolipids, and phospholipids, were fractionated using silicic-acid columns through a successive elution of chloroform, acetone, and methanol. Alkaline methanolysis was subsequently performed and the fatty-acid methyl esters were determined

via the Agilent 7890A gas chromatograph (Agilent Technologies Inc., Santa Clara, USA) installed with MIDI Sherlock peak-identification software (Version 4.5; MIDI Inc., Newark, USA). The total amounts of all PLFAs were used to represent the biomass of microorganisms. Bacteria were characterized by iso-branched, anteiso-branched, and monounsaturated fatty acids, including 13:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:0 anteiso, 17:0 iso, 19:0 anteiso, 22:0 iso, i12:1 w4c, 14:1 w5c, 15:1 iso w9c, 16:1 w7c, 17:1 iso w9c, 17:1 w8c, 17:0 cyclo w7c, 17:1 w5c, 18:1 w9c, 18:1 w7c, 20:1 w9c, 22:1 w9c, 22:1w6c, and 22:1w3c (Zhang et al. 2017). The polyenoic, unsaturated 18:2w6 was indicative of the fungi, and 10-methyl fatty acids indicated actinomycetes. The bacterial:fungal PLFA ratios represented the proportion of bacterial and fungal biomasses.

Microbial community diversity

The Shannon diversity index (H_{microbe}) and evenness index (E_{microbe}) was used to evaluate the diversities of the fatty acids:

$$H = -\sum_{i=1}^{n} pi\ln pi$$

where p_i is the relative abundance of each fatty acid in the total PLFAs, and n is the number of fatty acids detected, and:

$$E = H/ln(\mathrm{S})$$

where S is the total number of fatty acids tested in the community.

Statistical analyses

A one-way analysis of variation (ANOVA) was used to assess the effect of plant richness on soil leachate (amount and diversity), microbial activities (biomass C, respiration, and enzymes), and microbial communities (biomass and diversity). Significance was set at $P < 0.05$. Multiple comparisons were performed using a least significant difference (LSD) test ($P < 0.05$) when the variables differed significantly among richness levels, and a general linear model was further used to identify linear trends in the microbial variables along the gradient of plant diversity. In addition, a one-way ANOVA was also used to assess the effects of plant identity on soil leachate and microbial properties, followed by LSD multiple comparisons ($P < 0.05$). Pearson's correlation coefficient and linear regression were also used to evaluate the relationships between leachate and microbial properties. The ANOVA, correlation, and linear regression were performed via SPSS 24.0 (IBM SPSS for Windows, Chicago, USA). The optimum ordinary least squares (OLS) multiple regression models were selected to estimate the effect of leachate amount and diversity on the microbial community, and the model was confirmed based on the Akaike's information criterion and the variance inflation factor by the R package MASS 7.3–45 and package CAR 2.1–2, respectively. Results of OLS were verified by structural equation modeling (SEM), which evaluated the multivariate effect of soil leachate on microbial biomass, enzyme activities, and microbial diversity.

The first step in SEM requires establishing an a priori model based on the known effects and hypothetical relationships among the drivers of microbial biomass and diversity (Fig. S1). In this study, we assumed that plant richness alters the amount and diversity of soil leachate, which affects microbial biomass, activities, and diversity. Data treatment transformation was initially required before modeling (Grace 2006). Basal respiration and total PLFA was standardized (average = 0 and SD = 1) to achieve normality. In addition, the activities of BG, NAG, and AP were included as a composite variable (enzyme activities) and the first principal components (PC1) of these enzymes' activities obtained from a principal component analysis were used in the SEM analysis (Fig. S2). The use of composite variables does not alter the underlying SEM model but collapses the effects of multiple conceptually related variables into a single composite effect, aiding interpretation of model results (Grace 2006).

When data treatment was completed, we established the model using our data set and tested its overall goodness of fit. There is no single, universally accepted test for overall goodness of fit for SEM that is applicable to all situations regardless of sample size or data distribution. Here, we used (1) the χ^2-test (χ^2; the model has a good fit when χ^2 is low (< 2) and P is high (> 0.05) and (2) the root MSE of approximation (RMSEA; the model has a good fit when RMSEA is low (< 0.05) and P is high (> 0.05)) (Schermelleh-Engel et al. 2003). To improve our interpretation of SEM results, we also calculated the standardized total effects of the amount and diversity of soil leachate on the total PLFAs, microbial biomass C, basal respiration, enzyme activities, and microbial diversity.

Results

Soil leachate

A total of 105 compounds were detected in the leachate of all treatments. Of those, 51 compounds were identified and classified according to their attributes: 10 sugars, 5 sugar alcohols, 4 amines, 9 carboxylic acids, 4 phenolics, 13 lipids, and 6 others. Sugars increased by 34.3% (29.7 to 39.9 μg g soil^{-1}), and lipids increased by about 264% (2.8 to 10.2 μg g soil^{-1}) from richness levels 1 to 3 (Table 2). Sugar alcohols, amines, and carboxylic acids did not differ significantly among richness levels. A total of 9 sugars, 3 sugar alcohols, 2 carboxylic acids, 2 phenolics, and 3 lipids were significantly affected by plant richness (Table 2) and were highest for richness level 3. The amount of leachate did not differ significantly between richness levels 2 and 3 and was significantly higher than that in level 1 (Fig. 1). $H_{leachate}$ differed significantly among the three levels and increased with increasing plant richness (Fig. 1).

Compared with the monocultures, the 3-species and 2-species mixtures showed the highest $H_{leachate}$ and leachate amount. For example, the $H_{leachate}$ and leachate amount in *A. sacrorum-B. ischaemum-L. davurica* and *A. sacrorum-L. davurica* treatments were significantly higher than those in the monoculture treatments (Table S1a,b); this was particularly obvious in the concentrations of phenolics and lipids. Similarly, *B. ischaemum-L. davurica* and *A. sacrorum-B. ischaemum* showed the highest $H_{leachate}$ and amount of leachate than those of the monocultures (Table S1c,d).

Microbial activity

Plant richness had a significant effect ($P < 0.05$) on microbial biomass and enzymatic activities (Table 3). Microbial biomass C and the activities of BG and NAG did not differ significantly between richness levels 1 and 2; however, the corresponding values in both levels were significantly lower than those in level 3. Basal respiration tended to increase with plant richness, while AP activity showed the opposite trend. The 3-species mixtures and all 2-species mixtures showed higher microbial biomass C and respiration than the monocultures (Table S1 a-d). Activities of BG, NAG, and AP showed different trends at different mixture treatments compared to the monocultures.

Microbial community biomass and diversity

Microbial biomass, as indicated by the PLFAs, differed significantly among the three richness levels, including bacterial, fungal, and total microbial biomass ($P < 0.05$) (Table 3). Bacterial PLFAs and fungal PLFAs increased along the gradient of increasing plant richness, but actinomycete PLFA did not differ significantly among the different levels. Total PLFAs were significantly higher for level 2 than for level 1 but did not differ significantly between levels 2 and 3. The bacterial:fungal PLFA ratio was the highest for level 1. Microbial-community diversity was significantly affected by plant richness ($P < 0.05$). $H_{microbe}$ tended to increase along the gradient of increasing plant richness, but $E_{microbe}$ did not differ significantly between levels 2 and 3 ($P = 0.31$), which were both significantly higher than that for level 1.

Bacterial PLFAs and total PLFAs between 3-species and 2-species mixtures did not differ significantly and were significantly higher than those for the monocultures of the three species (Table S1a-d). The highest $H_{leachate}$ and $E_{leachate}$ were found in the 3-species mixture. $H_{leachate}$ of *A. sacrorum-L. davurica* mixture was significantly higher than that of the monocultures (Table S1a,b); this was similar to the mixtures of *B. ischaemum-L. davurica,* and *A. sacrorum-B. ischaemum* (Table S1c,d).

Association between soil leachate and microbial community

The amount of soil leachate was significantly linearly correlated with microbial biomass C, basal respiration, the activities of BG and NAG, bacterial PLFAs, fungal PLFAs, total PLFAs, $H_{microbe}$, and $E_{microbe}$ (Figs. 2 and 3). $H_{leachate}$ was significantly linearly correlated with the change in basal respiration, AP activity, fungal PLFAs, total PLFAs, $H_{microbe}$, and $E_{microbe}$ (Figs. 4 and 5). These results indicate that changes in plant richness led to the variation in the soil microbial community due to changes in soil leachate, and that the amount and diversity of leachate jointly contributed to the change in microbial biomass, activities, and diversity.

The OLS regression indicated that the amount and diversity of leachate were mainly responsible for the change in the microbial community, together accounting for 76.9, 69.6, and 46.6% of the variation in total microbial PLFAs, $H_{leachate}$, and $E_{leachate}$, respectively (Table 4). In addition, the fitted models of SEM

Table 2 Effect of plant diversity on the compounds in soil leachate

ug g soil^{-1}	Compounds	Level 1	Level 2	Level 3	*F*
Sugars	All sugars	29.7 ± 1.0 b	36.7 ± 1.5 ab	39.9 ± 2.2 a	10.32*
	Lactose	2.3 ± 0.2 b	3.5 ± 0.3 a	3.7 ± 0.2 a	22.31***
	Xylose	4.0 ± 0.4 b	5.3 ± 0.5 a	5.9 ± 0.6 a	16.24**
	Fructose	2.1 ± 0.1 b	2.7 ± 0.2 b	3.9 ± 0.3 a	31.17***
	Galactose	6.0 ± 0.8 b	6.7 ± 0.6 ab	7.9 ± 0.5 a	11.30*
	Glucose	1.4 ± 0.2 b	2.7 ± 0.3 a	2.9 ± 0.3 a	10.39*
	Galactinol	0.9 ± 0.1 b	1.8 ± 0.2 a	1.5 ± 0.2 ab	7.61*
	Sucrose	2.2 ± 0.2 b	2.4 ± 0.2 b	3.7 ± 0.4 a	8.82*
	Fucose	0.6 ± 0.1 a	0.6 ± 0.1 a	0.3 ± 0.0 a	0.64 NS
	Mannose	1.9 ± 0.2 b	3.0 ± 0.1 a	3.2 ± 0.2 a	7.79*
	Threose	1.4 ± 0.1 b	1.5 ± 0.2 b	2.7 ± 0.2 a	15.34**
Sugar alcohols	All sugar Alcohols	5.7 ± 0.2 a	6.4 ± 0.8 a	7.0 ± 1.1 a	0.52NS
	Myo-inositol	0.7 ± 0.1 b	0.4 ± 0.0 b	1.7 ± 0.2 a	8.10*
	Erythritol	0.4 ± 0.1 b	1.4 ± 0.2 a	1.1 ± 0.2 ab	7.28*
	Lyxitol	1.5 ± 0.2 b	2.1 ± 0.2 a	1.8 ± 0.1 ab	0.95 NS
	Xylitol	1.8 ± 0.2 a	0.9 ± 0.1 b	1.0 ± 0.3 b	8.02*
Amines	Amines	1.4 ± 1.0 a	1.5 ± 0.9 a	1.5 ± 1.0 a	0.34NS
	Cyclohexamine	0.7 ± 0.2 b	1.3 ± 0.1 ab	1.6 ± 0.1 a	1.03 NS
	Glycine	0.2 ± 0.2 b	0.5 ± 0.1 ab	0.8 ± 0.2 a	1.15 NS
Carboxylic acids	All carboxylic acids	4.0 ± 0.3 a	4.5 ± 0.2 a	5.1 ± 0.8 a	1.42 NS
	azelaic acid	1.0 ± 0.2 ab	0.6 ± 0.1 b	1.5 ± 0.1 a	10.03*
	2-hydroxyvaleric acid	0.7 ± 0.1 a	0.5 ± 0.2 a	0.9 ± 0.2 a	0.52 NS
	4-hydroxybutyric acid	0.6 ± 0.0 a	0.4 ± 0.1 a	0.4 ± 0.1 a	0.56 NS
	Dihydroxymalonic acid	0.5 ± 0.1 ab	0.3 ± 0.1 b	0.8 ± 0.2 a	0.81 NS
	Quinic acid	0.2 ± 0.1 b	0.5 ± 0.1 ab	0.9 ± 0.2 a	7.26*
Phenolics	All phenolics	3.2 ± 0.2 a	3.9 ± 0.4 a	3.7 ± 0.4 a	0.38NS
	Hydroquinone	1.5 ± 0.3 b	1.7 ± 0.2 b	2.9 ± 0.2 a	15.37**
	p-cresol	0.3 ± 0.1 c	1.0 ± 0.1 b	2.1 ± 0.2 a	17.20**
Lipids	All lipids	2.8 ± 0.3 c	7.5 ± 0.5 b	10.2 ± 0.6 a	124.27***
	Palmitoleic acid	1.0 ± 0.2 c	3.3 ± 0.4 b	4.6 ± 0.4 a	39.41***
	2-monopalmitin	0.5 ± 0.2 a	1.0 ± 0.3 a	0.6 ± 0.1 a	0.25 NS
	2-deoxyerythritol	0.1 ± 0.0 b	0.6 ± 0.1 a	1.1 ± 0.2 a	9.62*
	1-monopalmitin	0.2 ± 0.1 b	0.3 ± 0.2 b	1.0 ± 0.2 a	9.03*
	Caprylic acid	0.5 ± 0.1 a	0.4 ± 0.1 a	1.0 ± 0.3 a	0.62 NS

*, **, and ** * indicated the difference at the $p < 0.05$, 0.01, 0.001, respectively by the ANOVA. Different letters indicated the significant difference among the treatment by the LSD multiple test. n = 12 for each level. Only specific compounds with the content >0.1 μg g soil^{-1} are shown

explained 82.4% of the variance in microbial diversity (Fig. 6). Clearly, changes in plant richness had a direct effect on the amount and diversity of soil leachate, which resulted in significant changes in microbial biomass C, total PLFAs, respiration, enzyme activities, and microbial diversity. Soil leachate diversity had a direct and considerable impact on H_{microbe}, and leachate amount exerted a direct effect on total PLFAs, microbial biomass C, and enzyme activity. The compounds of the leachate were variably correlated with microbial properties (Table S2). The content of sugars and lipids were significantly correlated ($P < 0.05$) with microbial biomass C, basal respiration, the activities of BG and NAG, bacterial PLFA, total PLFA, and H_{microbe}.

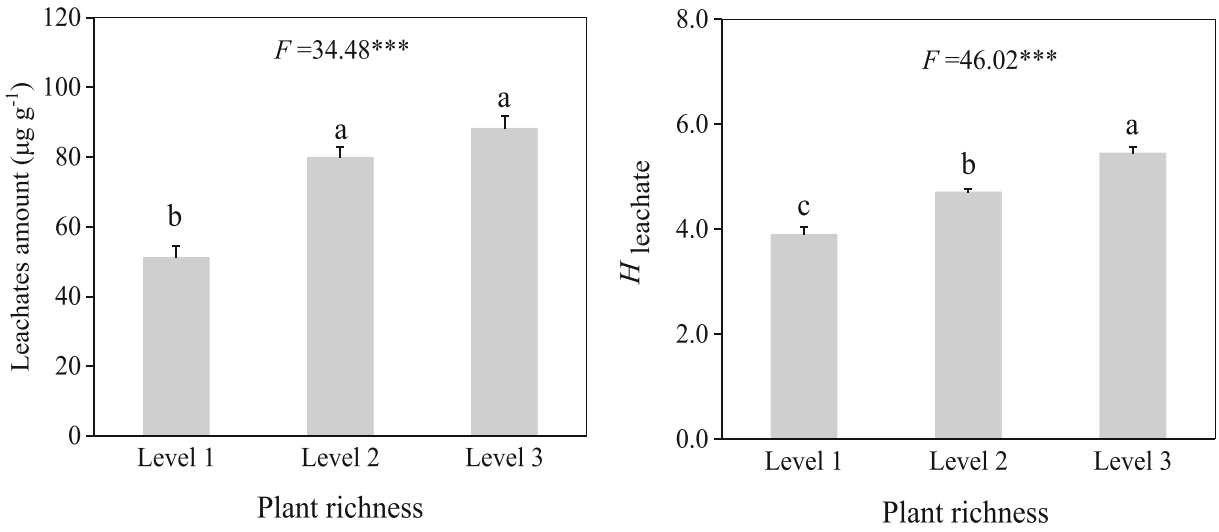


Fig. 1 Effect of plant diversity on the amount and diversity of soil leachate. $H_{leachate}$: Shannon diversity of leachate. *** indicated the difference at the $p < 0.001$by the ANOVA. Different letters indicated the significant difference among the treatment by the LSD multiple test. $n = 12$ for each level

Discussion

Effect of plant diversity on soil leachate

Our aim was to investigate the influence of plant diversity on leachatesoil leachate. Previous studies have illustrated that a change in plant diversity can lead to a variation in the quantity and composition of root exudates (Eisenhauer et al. 2017; Steinauer et al. 2016). Eisenhauer et al. (2017) found that the amount of root exudates increased significantly with increasing diversity (richness levels 1, 3, and 6) but exudate diversity did not increase. We also found an increase in leachate diversity with increasing plant richness in this study (Fig. 1), suggesting that an increase in plant diversity not only increases the quantity of soil leachate, but also increases their variety. This may be due to the increase in the number and variety of plants with different physiological features, which increases the quantity and type of exudate compounds.

Table 3 Effect of plant richness on soil microbial activities, biomass and diversity

Variables	Level 1	Level 2	Level 3	*F*
Root biomass (g kg^{-1})	27.07 ± 0.68	26.84 ± 1.09	26.16 ± 1.39	0.13 NS
Microbial biomass C (mg kg^{-1})	222.3 ± 11.3 b	217.3 ± 9.1 b	265.5 ± 8.4 a	5.07 *
Basal respiration (mg kg $soil^{-1}$)	15.82 ± 4.81 c	27.78 ± 3.37 b	45.45 ± 6.19 a	47.32 **
β-1,4-glucosidase (nmol g^{-1} h^{-1})	108.8 ± 9.2 b	117.6 ± 4.8 b	167.9 ± 8.5 a	10.62**
β-1,4-N-acetylglucosaminidase (nmol g^{-1} h^{-1})	17.03 ± 1.88 b	20.73 ± 2.98 b	26.34 ± 1.31 a	7.86*
Alkaline phosphatase (nmol g^{-1} h^{-1})	221.9 ± 11.5 a	191.1 ± 9.2 b	186.7 ± 5.4 b	5.22 *
Bacterial PLFA (nmol g^{-1})	3.99 ± 0.28 b	4.29 ± 0.16 ab	4.95 ± 0.15 a	4.07 *
Fungal PLFA (nmol g^{-1})	0.30 ± 0.04 c	0.38 ± 0.06 b	0.43 ± 0.05 a	18.68 **
Actinomycete PLFA (nmol g^{-1})	0.14 ± 0.02 a	0.14 ± 0.02 a	0.15 ± 0.01 a	0.07 NS
Total PLFA (nmol g^{-1})	5.53 ± 0.15 b	6.45 ± 0.25 a	6.73 ± 0.18 a	38.60 **
Bacterial: Fungal PLFA ratio	13.48 ± 1.87 a	11.88 ± 0.04 a	11.47 ± 0.87 a	1.27 NS
$H_{microbe}$	2.16 ± 0.09 c	2.69 ± 0.08 b	3.24 ± 0.08 a	18.96 **
$E_{microbe}$	0.71 ± 0.04 b	0.78 ± 0.07 a	0.82 ± 0.05 a	14.04 **

$H_{microbe}$: microbial Shannon diversity, $E_{microbe}$: microbial evenness. *, and ** indicated the difference at the $p < 0.05$, 0.01, respectively by the ANOVA. Different letters indicated the significant difference among the treatment by the LSD multiple test. n = 12 for each level

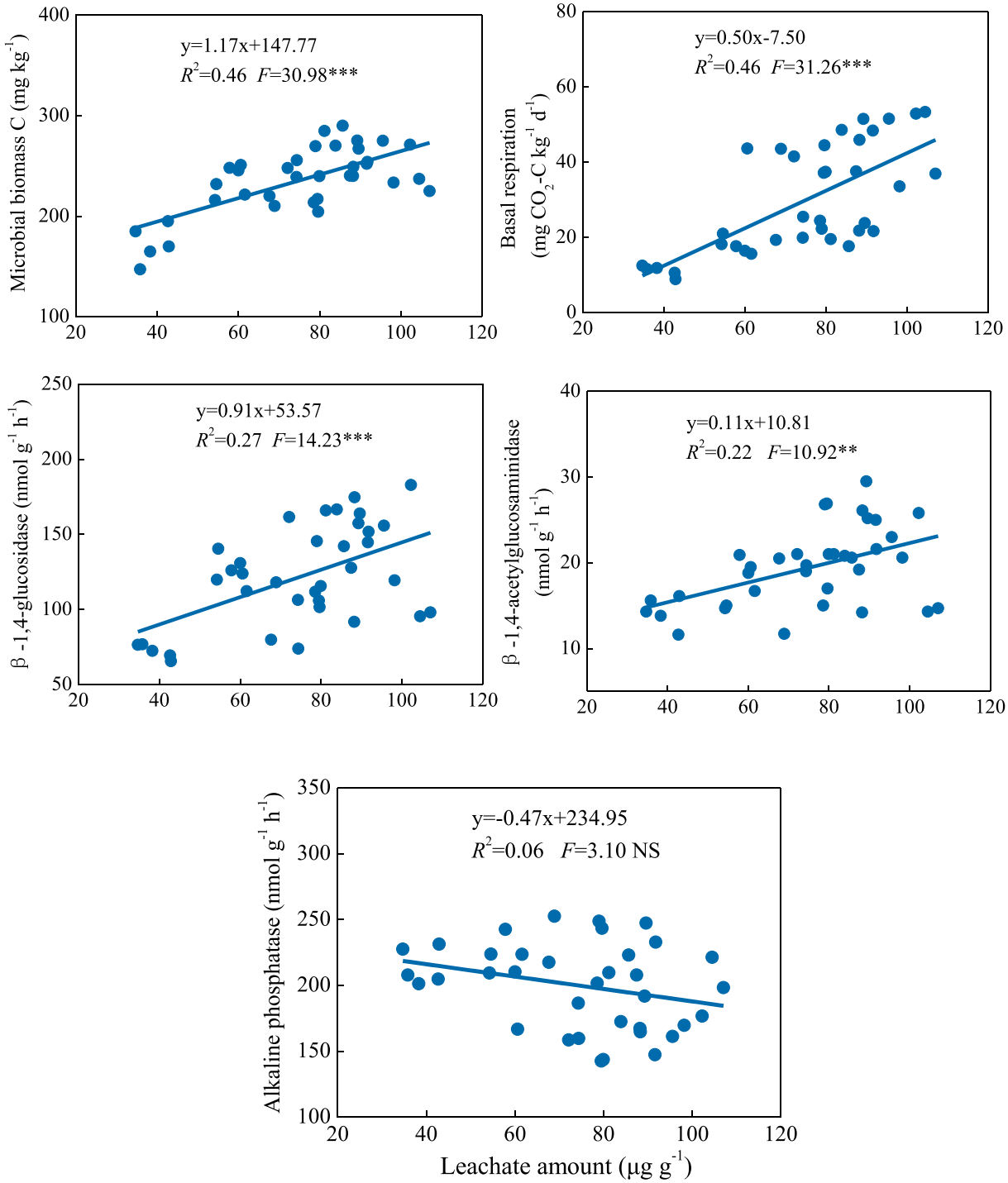


Fig. 2 Linear regression between soil leachate amount and microbial activities. ** and *** indicated the difference at the $p < 0.01$ and 0.001, respectively. NS: not significant

Increases in plant diversity significantly increased the secretion of sugars and lipids and concomitantly increased the abundance of soil microorganisms (Table 2). This was in accordance with previous reports, which found that C-containing metabolites in soil leachate played an important role in shaping soil microbial

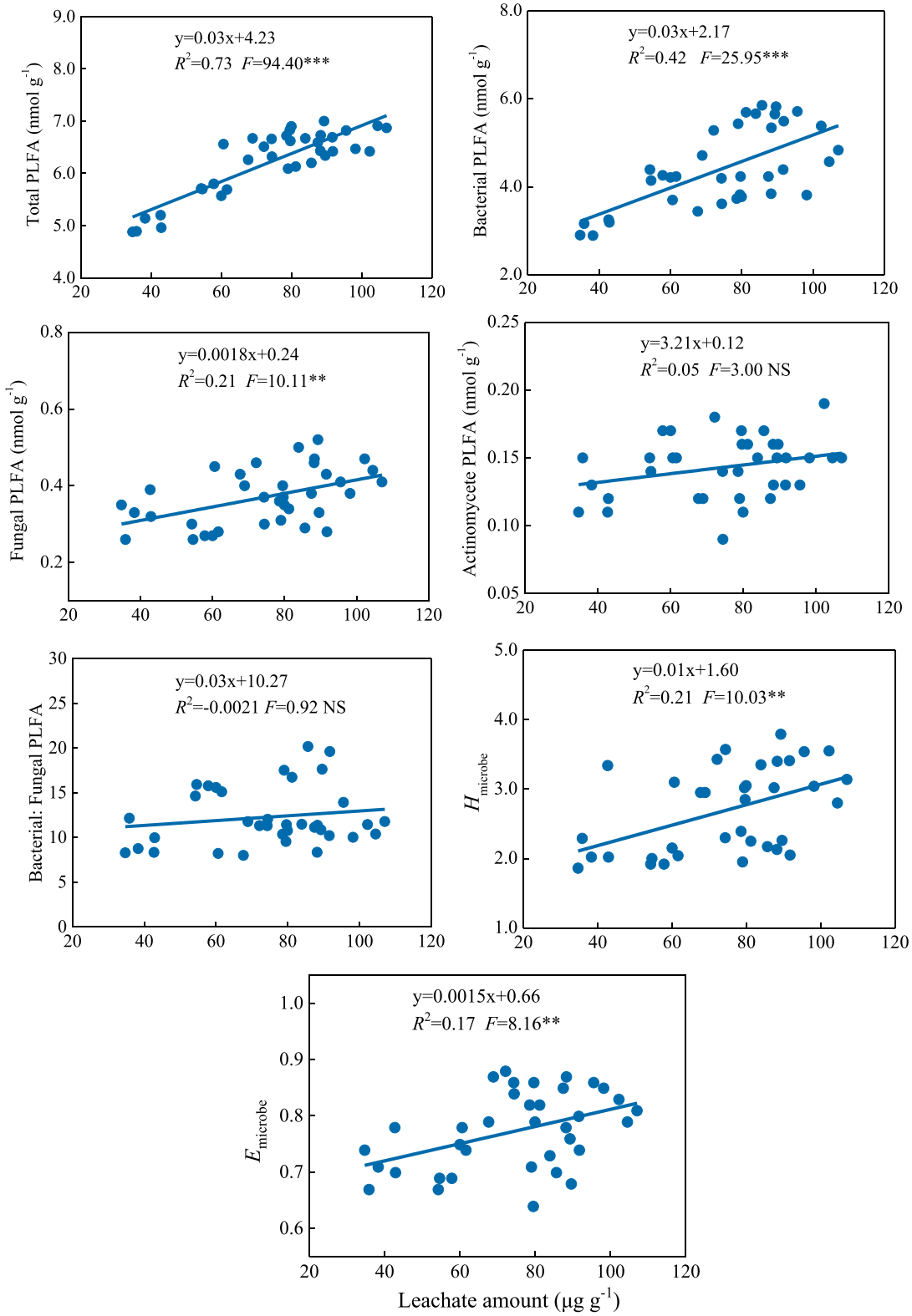


Fig. 3 Linear regression between soil leachate amount and microbial community. $H_{microbe}$: microbial Shannon diversity; $E_{microbe}$: microbial evenness. ** and *** indicated the difference at the $p < 0.01$ and 0.001, respectively. NS: not significant

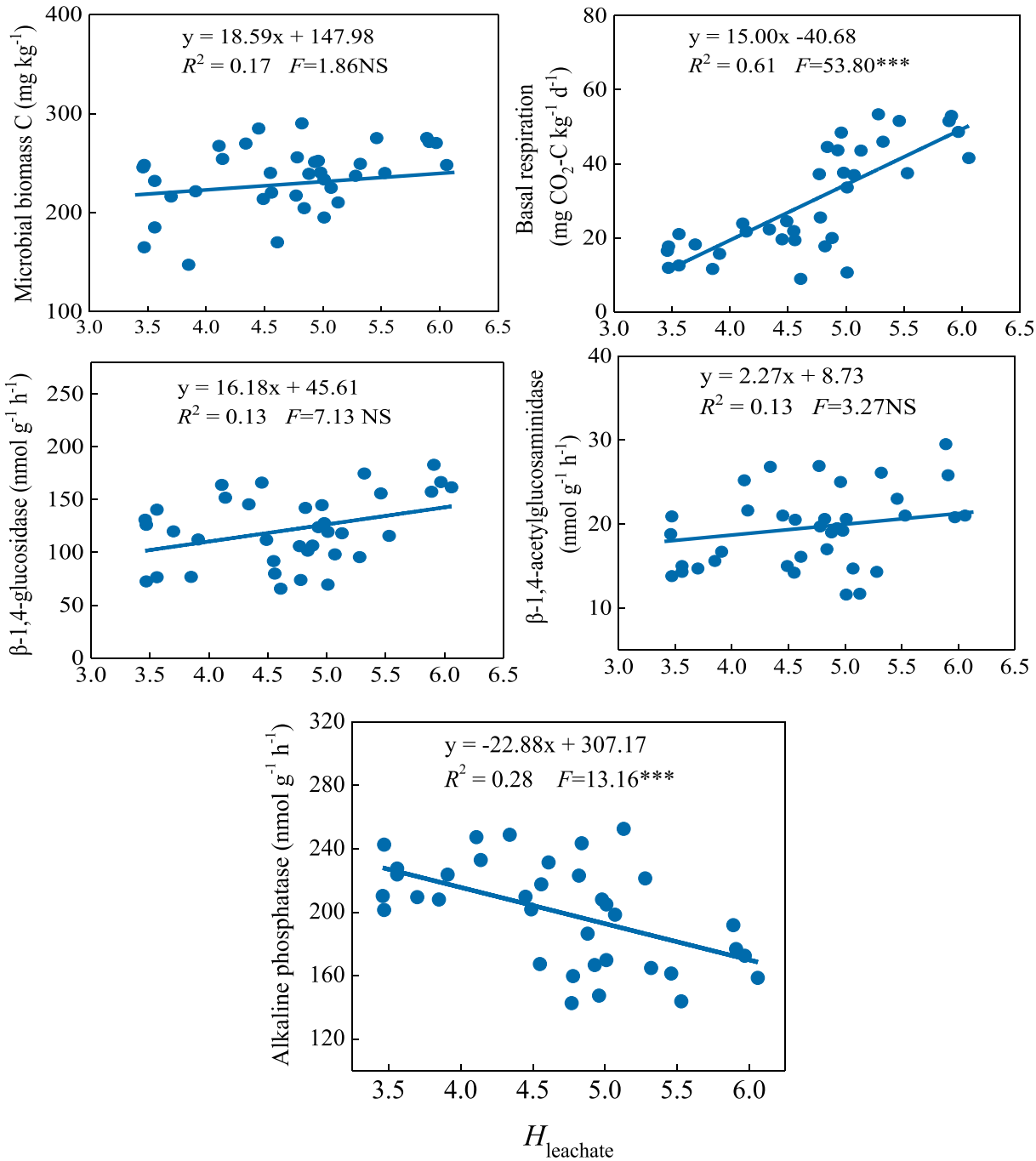


Fig. 4 Linear regression between soil leachate diversity and microbial activities. $H_{leachate}$: Shannon diversity of leachate. *** indicated the difference at the $p < 0.001$. NS: not significant

communities (Haichar et al. 2014; Neumann et al. 2014; Zhu et al. 2016). Sugars serve as sources of energy for a broad range of microbes (Fusconi 2014) and act as general chemotactic substances (Eilers et al. 2010; Kamilova et al. 2006). The abundance of bacterial, fungal, and total microorganisms in our study indicated by microbial PLFAs was correlated with sugar content (Table S2). This is consistent with the observations by Zhu et al. (2016) where the abundance of bacterial groups such as Bacillales, Nitrosomonadales, and Rhodocyclales tended

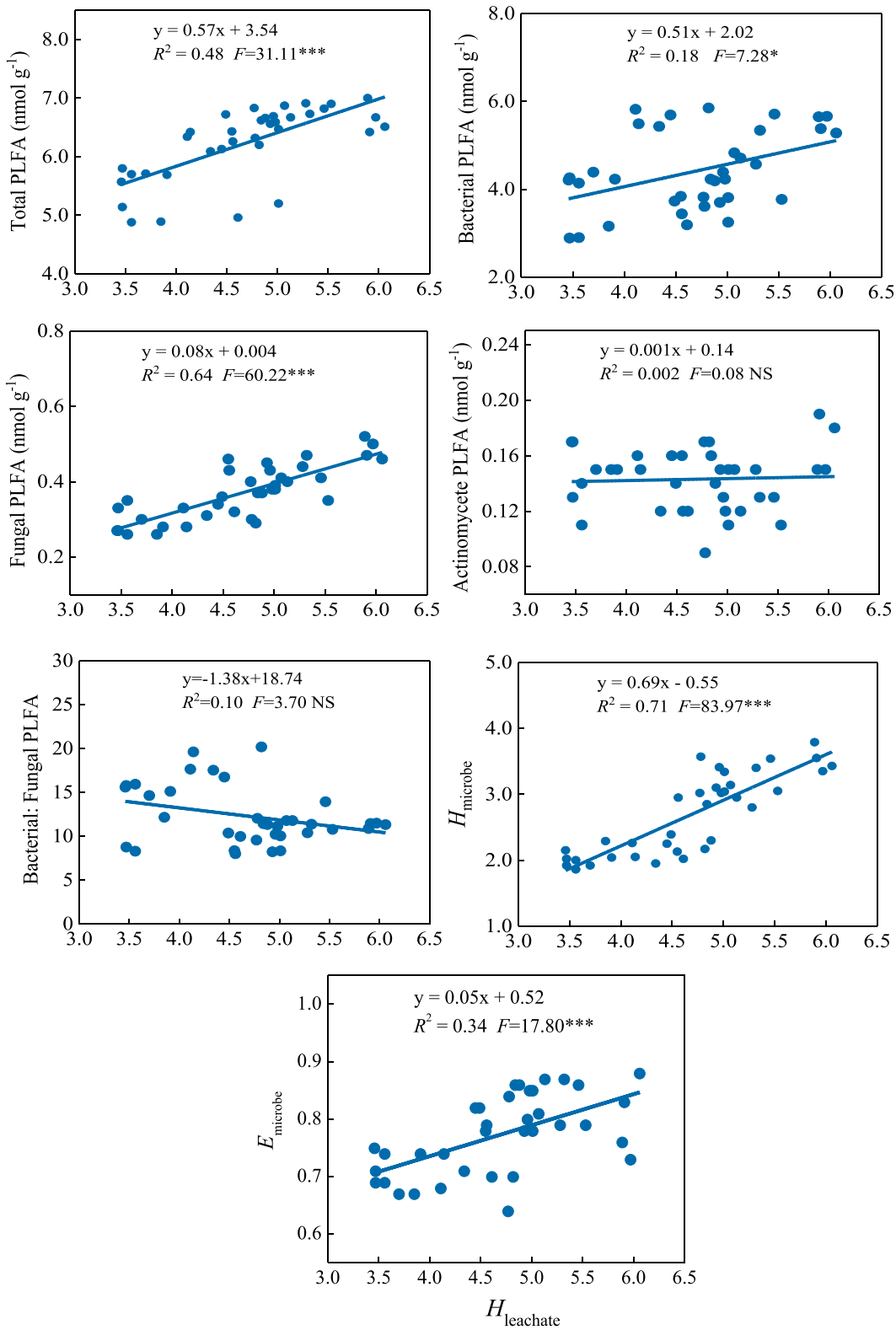


Fig. 5 Linear regression between soil leachate diversity and microbial community. $H_{microbe}$: microbial Shannon diversity. $E_{microbe}$: microbial evenness. $H_{leachate}$: Shannon diversity of leachate. * and *** indicated the difference at the $p < 0.05$ and $p < 0.001$, respectively. NS: not significant

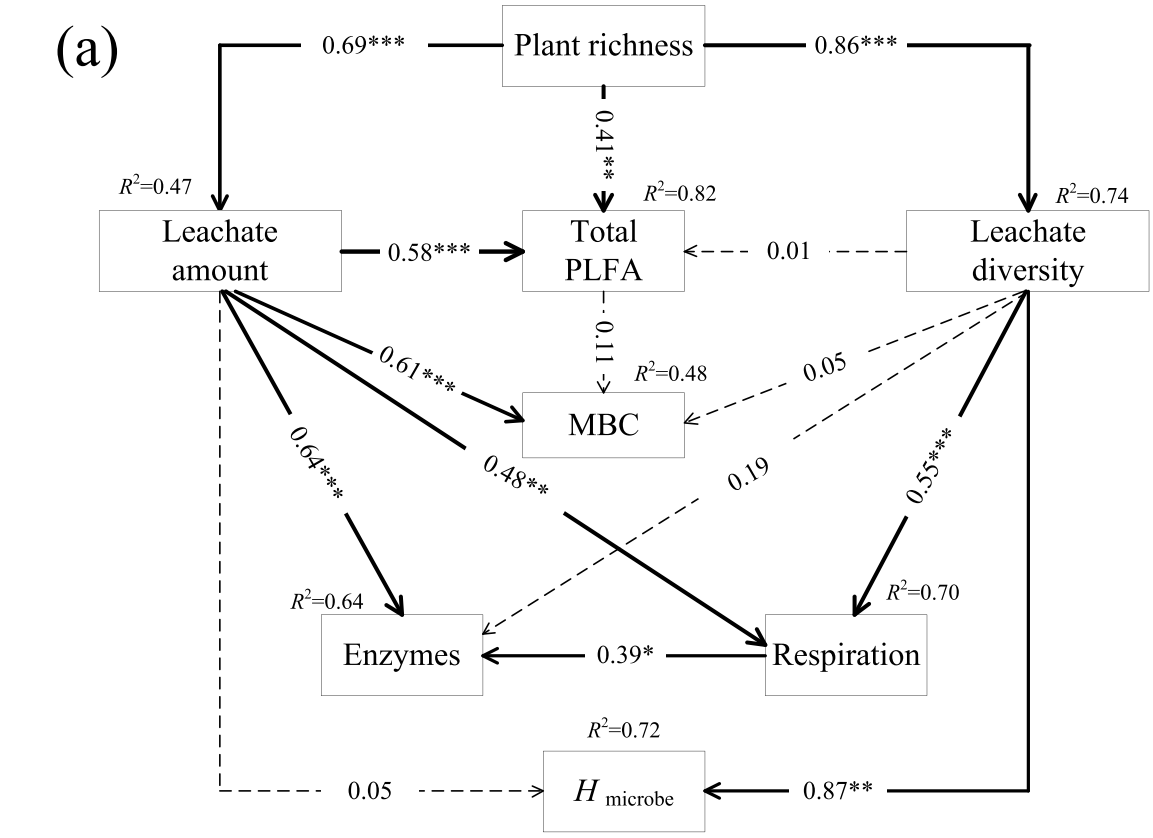
Table 4 Summary of the best Ordinary Least Square (OLS) multiple regression model for the effects of soil leachate amount and diversity on microbial community. $df = 32$

Regression model	Estimate		SE		t-value		Significance Pr (>|t|)		R^2_{adj}	$SE_{residue}$	AIC
	SLA	SLD	SLA	SLD	SLA	SLD	SLA	SLD			
Total PLFA = 3.554 + 0.227 × SLA + 0.022 × SLD	0.022	0.227	0.003	0.085	6.802	2.683	<0.001	0.011	0.769	0.299	19.992
$H_{microbe}$ = −0.552–0.002 × SLA + 0.717 × SLD	−0.002	0.717	0.004	0.096	−0.432	7.498	0.669	<0.001	0.696	0.338	28.978
$E_{microbe}$ = 0.520 + 0.001 × SLA + 0.046 × SLD	0.001	0.046	0.001	0.016	0.772	2.877	0.446	<0.001	0.466	0.057	−99.329

SLA soil leachate amount; *SLD* soil leachate diversity; $H_{microbe}$ microbial Shannon diversity; $E_{microbe}$ microbial evenness; *SE* standard errors; *df* degree of freedom; *AIC* Akaike information criterion

to increase when sugars were abundant. Phenolics are a dominant class of plant secondary metabolites (Zwetsloot et al. 2018) and may be general antimicrobial compounds (Makarova et al. 2016; Yang et al. 2015). The content of phenolics did not differ significantly between the different levels of plant richness in our study (Table 2), and the lack of a close correlation between phenolics and microbial properties (Table S2) suggested that phenolics probably had a smaller effect on the microbial community; however, the content of some phenolic compounds (e.g., p-cresol and hydroquinone) increased with plant richness. Thus, phenolic composition may have been limited in soil leachate; only five phenolic compounds were identified, of which hydroquinone was the most abundant.

Mixtures of plant species do not merely indicate an increase in plant richness, but most likely the presence of competition between neighbors. In our study, the higher leachate diversity of the 2- and 3-species mixtures compared with that of the monocultures of the three plant species indicated that the increase in leachate diversity caused by a higher plant diversity was attributed to the inclusion of one of the three plant species (Table S1). The presence of potential competitors for belowground resources has been shown to increase the release of root exudates into the soil (Semchenko et al. 2014), which may explain the greater amount of leachate in the soil of the 2- species and 3-species mixtures than that in the monocultures. Such behavior is expected to improve the competitive ability of plants by increasing the amount of root exudates in the soil (Mallik et al. 2016). An increased amount of soil leachate can be interpreted as an effort to enhance resource uptake and is, therefore, in line with the prediction that the presence of competitors should trigger the "selfish" behavior of attempting to acquire resources before neighbors (Semchenko et al. 2014). The fact that soil leachate are regulated by competition was confirmed via the identification of specific leachate compounds. A previous study demonstrated that glucose can serve as the main source of C utilized by a wide range of microbial populations and plays critical roles in driving and shaping the selection of microbes (Yuan et al. 2017). In our study, a greater amount of glucose in the grass mixtures than in the monocultures (Table 2) suggested that more C substrates could be utilized by microbes at a higher plant diversity. Some of the exudates, such as hydroquinone, could be involved in regulating root proliferation in response to neighbor presence and identity (Badri and Vivanco 2009), and an increase in plant species richness



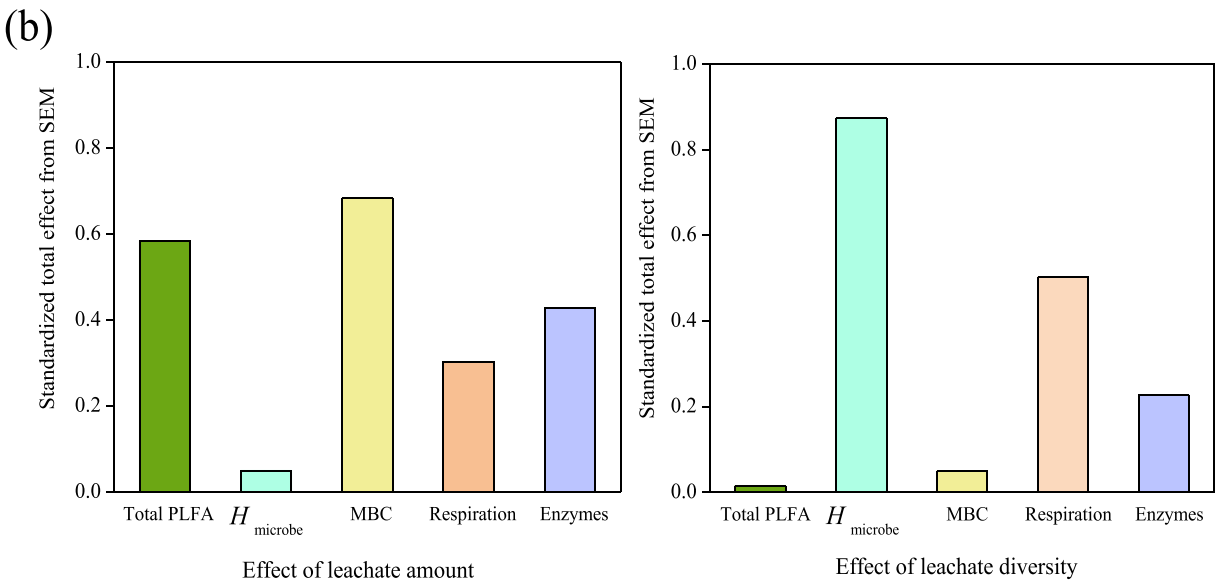


Fig. 6 The structural equation model (SEM) examining the multivariate effects of soil leachates on soil microbial community. Solid and dash arrows indicate the significant and non-significant pathways, respectively. The numbers are the standardized path coefficient. *,**, and ***indicated the significance at the $p < 0.05$, $p < 0.01$,and $p < 0.001$, respectively. R^2 values represent the proportion of the variance explained for each endogenous variable. MBC: microbial biomass C; $H_{microbe}$: microbial Shannon diversity

probably indicates a greater competition between plant species. Azelaic acid is assumed to be limited to bioenergetic use in microbes, and glycine has the lowest energy efficiency in microbes (Keiluweit et al. 2015). Differential exudation is a plausible mechanism by which plants could regulate their interactions with neighboring plants and microbes, as exemplified by the correlation found between leachate compounds and microbial properties in this study (Table S2).

Effect of plant diversity on microbial biomass and diversity

The abundance and diversity of soil microorganisms largely depend on abiotic and biotic factors, such as soil type (Murugan et al. 2014), microclimate (Kuzyakov and Blagodatskaya 2015), plant age (Chaparro et al. 2014), or plant species (Khlifa et al. 2017). Total PLFAs, indicative of microbial biomass, increased significantly with plant richness (Table 3). This finding was in agreement with the results of previous studies illustrating positive effects of plant diversity on microbial biomass (Eisenhauer et al. 2010; Steinauer et al. 2015, 2016), and suggests that plant-derived resources affects microbial biomass (Cline et al. 2018). A study assessing potential C-source use by bacterial communities in a field experiment found a log-linear increase in bacterial abundance with increasing plant richness, as well as an increase in bacterial 'functional' diversity (Stephan et al. 2000). Previous studies have assumed that microbial growth was mainly determined by inputs of C and N substrates into soils, so the production of plant biomass was assumed to affect microbial biomass (Eisenhauer et al. 2017; Mommer et al. 2010; Ravenek et al. 2014). Our experiment demonstrated that microbial biomass was also influenced by the type and number of plant species. For example, plant richness significantly affected microbial biomass, including bacterial, fungal, and total PLFAs, and the increased plant diversity increased PLFA content (Table 3), possibly because the increase in resource heterogeneity was due to the root exudates in the leachate. Eisenhauer et al. (2017) found less microbial biomass when grass species, such as *Festuca rubra*, *Phleum pratense*, and *Anthoxanthum odoratum* were grown separately than when grown together, because the microbial communities changed when mixed-species litter assemblages were provided. Based on this evidence, we can assume that the higher amount and diversity of soil leachate in the mixed cultures than the monocultures could increase resource heterogeneity for soil microorganisms. This assumption was supported by the regression results showing that the change in PLFAs of total microorganisms was linearly correlated with the variation in the amount and diversity of leachate (Figs. 3 and 5). This suggests that soil leachate are one pathway for plant diversity to affect soil microbial communities. The increased amount and diversity of leachate caused by higher plant richness could increase the spatial and temporal provision of substrates, which may have been much patchier at low than at high species diversity, and therefore could have contributed to the higher microbial biomass per unit of soil (Spehn et al. 2000). The soil microorganisms generally responded positively to the soil leachate, but the dominant microbial groups responded differently. For example, the change in fungal PLFAs was more linearly correlated with the variation in the diversity of leachate, but bacterial PLFAs were more correlated with the amount of leachate. This finding suggested that the effect of soil leachate on microbial groups was variable, and the fungal community may have been influenced more by diversity of leachate, but the bacterial community may have been affected more by the amount of leachate.

Plant richness had a significant effect on the microbial community diversity, which is consistent with the study by Steinauer et al. (2016) who reported that the Shannon diversity index increased with plant diversity in a pot experiment. Evenness indicates more equally distributed abundances of microbial species with higher than lower plant richness (Table 3). H_{microbe}, however, increased along the richness gradient, suggesting a more diverse microbial community in a more diverse plant community, in contrast to the findings by Steinauer et al. (2016) that plant richness did not significantly affect microbial richness. H_{microbe} and E_{microbe} were also linearly correlated with the amount and diversity of leachate (Figs. 3 and 5), indicating a direct effect of soil leachate on microbial diversity, which supported our hypothesis that the variation in soil leachate caused by a change in plant diversity could affect microbial community diversity. Results from the OLS regression (Table 4) and SEM (Fig. 6) confirmed the contribution of the amount and diversity of soil leachate to the microbial community and indicated that leachate amount probably had a larger effect on microbial biomass than leachate diversity, but that leachate diversity had a larger effect on microbial diversity, even though the amount and diversity of soil leachate were mainly responsible for the

changes in microbial-community biomass, diversity, and evenness. The results of Shannon diversity and evenness measurements in our experiment suggest that the diversity of soil leachate probably contributed to microbial diversity more than the exudate amount, which had a greater influence on the microbial biomass.

Effect of plant diversity on microbial activities

Plant richness had significant effects on microbial activities, including microbial biomass C, basal respiration, and the activities of soil enzymes involved in the C and N cycles. Microbial biomass C, as a component of soil organic matter, has been used to represent microbial activity because of its rapid response to soil changes (Zhang et al. 2011). The effect of plant richness on microbial biomass C was significant in our study; it was significantly correlated with the amount of soil leachate (Fig. 2), suggesting that soil leachate amount strongly affected the ability of microbes to fix C. This finding agreed with the results of a field experiment by Lange et al. (2015) where different plant-induced changes in microbial biomass and activity were caused by variations in the release of labile C from roots. Basal respiration, an indicator of microbial use of C substrates, has often been used for measuring microbial activity (Conant et al. 2004; Zhang et al. 2011). The significant effect of plant richness on basal respiration, and the linear relationship between plant richness and soil leachate (amount and diversity), indicated that plant diversity could alter microbial respiration by affecting soil leachate.

Higher BG activity in the treatments with higher plant richness suggested a higher availability of cellulose substrates to soil microorganisms (Table 3). The activities of enzymes that degrade cellulose are stimulated by the amount of available substrate (Lynd et al. 2002). An experiment investigating the effects of plant richness on soil microbial functions documented consistent results on enhanced activities of BG as plant richness increased (Steinauer et al. 2015). Increased activity of NAG, an enzyme involved in chitin degradation, also suggests an increasing proportion of fungal biomass with higher plant richness in our experiment. Higher NAG activity may thus be a good indicator of higher rates of chitin processed as C and N sources by soil fungi and bacteria (Brzezinska et al. 2009). This finding was supported by the significant correlations between the activities of BG and NAG and the PLFAs of bacteria and fungi in our study. In contrast to BG and NAG, the activity of AP, an important enzyme involved in the cycling of soil P, tended to increase as richness level decreased, possibly because some chemical compounds in soil leachate can negatively affect functional microbial groups involved in P transformation, but this requires further investigation. Enzymatic activity in soil is generally strongly influenced by soil leachate, especially for some functional microbial groups involved in C and N transformation, which was supported by the significant linear correlation between the amount of soil leachate and the activities of BG and NAG.

Soil leachate are assumed to have a large impact on microbial activity, but changes in microbial biomass C and the activities of BG and NAG were interestingly significantly correlated with the variation in the amount but not the diversity of leachate, suggesting that microbial activity is influenced more by the amount than the diversity of leachate in the soil. These findings support the important role of soil leachate in microbial activities.

Conclusions

Our results support our first hypothesis that plant diversity drives changes in soil microbial communities by altering the amount and diversity of soil leachate. Changes in plant richness significantly affected microbial biomass C, enzymatic activities, abundances of microbial groups, microbial diversity, and the amount and diversity of soil leachate. In support of our second hypothesis, we found a closer correlation between leachate diversity and microbial diversity than between leachate amount and microbial diversity, suggesting that microbial diversity was determined more by leachate diversity than leachate amount, which had a large impact on microbial biomass and activity. These findings provide experimental evidence that soil leachate represent a crucial association between plant diversity and soil microorganisms, and provide new insights into the associations between plants and soil microbes.

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