Responses of extracellular enzyme activities and microbial community in both the rhizosphere and bulk soil to long-term fertilization practices in a fluvo-aquic soil

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ABSTRACT

The influence of inorganic or organic fertilization on soil microbial ecology has been emphasized recently, but less is known about rhizosphere effects on extracellular enzyme activities and microbial community structure. Eleven extracellular enzymes involved in C, N, P, and S cycling and microbial community structure in both the rhizosphere and bulk soil samples from a long-term (31-year) fertilizer experimental field at the wheat reproductive stage were investigated by microplate fluorometric assay and phospholipid fatty acid analysis (PLFA), respectively. The samples were taken from six treatments: control (CK, without fertilization), fertilizer N (N), fertilizer N and P (NP), fertilizer N, P and K (NPK), organic manure (M), and organic manure plus fertilizer N, P and K (MNPK). Responses to inorganic or organic fertilizers in the rhizosphere were significantly different from those in the bulk soil. Except for NO_3^--N, thus, nutrient concentrations were generally higher in the rhizosphere than in the bulk soil. M and MNPK treatments greatly increased organic C, total N, NH_4^+-N and total S. Inorganic fertilizers (N, NP, and NPK) generally maintained or reduced most enzyme activities in the rhizosphere, but markedly increased these enzyme activities in the bulk soil. However, organic treatments (M and MNPK) enhanced most enzyme activities in both the rhizosphere and bulk soil. Higher total PLFA and lower ratios of bacteria to fungi and of actinomycetes to fungi were observed in the rhizosphere compared with the bulk soil. In the bulk soil, the ratios of bacteria to fungi and of actinomycetes to fungi were highest in the N treatment and lowest in the M treatment. However, in the rhizosphere there were no statistically significant differences in the abundance of bacteria, fungi and actinomycetes between the inorganic and organic treatments. Organic fertilization increased total PLFA and Gram+ to Gram− bacteria ratio in both the rhizosphere and bulk soil. Our results indicated that changes in fertilization regime had a greater impact on the bulk soil microbial community than in the rhizosphere.

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1. Introduction

The rhizosphere, the volume of soil adjacent to and affected by plant roots (Sørensen, 1997), plays an important role in plant growth and soil fertility (Rovira, 1969). As soil microbes are often limited by energy in soils, root exudates such as organic acids, sugars and amino acids may stimulate the growth of microbial populations and the activities of extracellular enzymes capable of influencing biogeochemical cycling of C, N, P and S (Fontaine and Barot, 2005; Rovira, 1969; Stevenson and Cole, 1999). Fertilization, which is widely used to enhance soil fertility and crop yield, significantly affects soil biochemical and biological properties. The influence of fertilization on soil microbial ecology has been emphasized recently (Marschner, 2003; Yevdokimov et al., 2008; Zhong et al., 2010). However, most investigations have been conducted at a bulk soil scale or in short-term experiments, and as a result, there is

still little available information on rhizosphere effects on extracellular enzyme activities and microbial community structure in agricultural soils as influenced by long-term practices.

From a functional perspective, the activities of extracellular enzymes produced by both microbes and plant roots are the primary biological mechanism of organic matter decomposition and nutrient cycling (Wittmann et al., 2004). Organic matter addition often leads to a rapid increase in the activities of various enzymes and reactivation of biogeochemical cycles in bulk soil (Bastida et al., 2007; Madejon et al., 2001). Inorganic N, P and K fertilizers also impact on the activities of soil enzymes (Böhme et al., 2005; Goyal et al., 1999). Most hydrolytic enzyme activities were increased by addition of N fertilizer in a forest soil, but the phenol oxidase activity dropped 40% compared to control plots (Saiya-Cork et al., 2002). Weand et al. (2010) emphasized that the effect of added N on enzymatic activities in a soil changes depending on the nature of the dominant substrates (labile or recalcitrant). Compared to numerous studies on enzyme activity in bulk soil, less effort has been expended on determining how long-term fertilization affects rhizosphere

enzyme changes. In general, soil enzyme activities are lower in bulk soil than in the rhizosphere, as a result of microbial activity induced by root exudates, or because of the release of enzymes from roots (Badalucco and Kuikman, 2001). However, Phillips and Fahey (2008) found that rhizosphere effects on microbial activities and nutrient availability could be reduced by fertilizer addition in nutrient-poor forest soil, which he considered to be a result of fertilizer-induced shifts in the belowground C supply.

Most studies have found obvious changes in soil microbial communities after addition of organic or inorganic fertilizer amendments (Enwall et al., 2005; Marschner, 2003; Peacock et al., 2001). It is generally recognized that organic manure addition tends to increase the total microbial biomass, though the responses of specific groups such as Gram-positive bacteria, Gram-negative bacteria and fungi vary. For instance, organic manure additions often result in increased or altered fungal populations (Bastida et al., 2007; Elfstrand et al., 2007), altered populations of arbuscular mycorrhizal fungi (Corkidi et al., 2002), shifts in Gram-positive and Gram-negative bacteria (Marschner, 2003; Peacock et al., 2001), and increased fungi/bacteria ratios (Elfstrand et al., 2007). Importantly, the response of the microbial community structure to organic manure additions tends to be based on differences in the carbon amount or quality of the organic amendments (Elfstrand et al., 2007). Changes in the soil microbial community structure are also observed after additions of inorganic N, P and K fertilizers (Phillips and Fahey, 2008; Yevdokimov et al., 2008; Zhang et al., 2007). Many studies have indicated that rhizosphere community structure and function are mainly influenced by soil and plant factors (Carelli et al., 2000; Marschner et al., 2004). However, the ecological consequences of the application of various fertilizers in the rhizosphere are unclear, because of the poor understanding of how changes in nutrient availability impact on plant and soil microbial processes (Hobbie et al., 2002; Phillips and Fahey, 2007). Fertilizer additions possibly result in decreased carbon allocation to roots and subsequent decreases in microbial respiration in the rhizosphere (Phillips and Fahey, 2007). In another study, Buyer et al. (2010) reported that a vetch cover crop increased the amount and proportion of Gram-negative bacteria, fungi, and arbuscular mycorrhizal fungi in the rhizosphere of tomato plants.

The present study was conducted to examine how enzyme activity and microbial community structure differs between the rhizosphere and bulk soil in a farmland ecosystem, and how each responds to long-term fertilization. Since Marx et al. (2001) and Saiya-Cork et al. (2002) used fluorometric MUB-linked substrates to measure soil enzymes, this method has become popular in soil studies, because it is very sensitive and allows a high-throughput analysis of enzymatic activities (Deforest, 2009; Wittmann et al., 2004). Phospholipid fatty acid (PLFA) profiles were used to estimate the microbial community structure. We hypothesized that the rhizosphere and bulk soil would have different microbial communities with distinct enzyme activities after long-term fertilizer treatments, and that fertilization would influence rhizosphere effects on microbial community structure and function.

2. Material and methods

2.1. Field design and sampling

The study was conducted in the North China Plain, which is a major grain producing area in China. The calcareous fluvo-aquic soil is a widespread soil type in the North China Plain. In order to illustrate the effect of long-term fertilization on soil quality and food production, a long-term field experiment (incorporating application of inorganic/organic fertilizers and a control treatment) was initiated in 1979 at Malan Farm, Hebei province, China (37°55′N,115°13′E). At the start of the experiment, the soil had a pH (H$_2$O) of 7.8, 1.1% organic matter, 1.80 g kg^{-1} total N, and 5.0 and 87.0 mg kg^{-1} of available P

and K, respectively. The site has a temperate and monsoonal type climate with annual average temperature and precipitation being 12.6 °C and 490 mm, respectively.

The experiment had winter wheat and summer maize rotations with a completely randomized design with twelve treatments and three replicates (Xia et al., 2008). The plot size was 80 m^2. For this study, six treatments were selected as follows:

1) Soil without fertilizer (control, CK)
2) Inorganic fertilizer treatment (N) corresponding to 150 kg N (urea) ha^{-1}.
3) Inorganic fertilizer treatment (NP) corresponding to 150 kg N (urea) ha^{-1} and 150 kg P$_2$O$_5$ (superphosphate) ha^{-1}.
4) Inorganic fertilizer treatment (NPK) corresponding to 150 kg N (urea) ha^{-1}, 150 kg P$_2$O$_5$ (superphosphate) ha^{-1} and 150 kg K$_2$O (KCl) ha^{-1}.
5) Farmyard manure compost (M), 3.75×10^4 kg ha^{-1}, containing straw bedding impregnated with liquid and solid manure.
6) Farmyard manure compost and inorganic fertilizer treatments (MNPK) corresponding to 3.75×10^4 kg cattle manure compost ha^{-1}, 150 kg N (urea) ha^{-1}, 150 kg P$_2$O$_5$ (superphosphate) ha^{-1} and 150 kg K$_2$O (KCl) ha^{-1}.

The manure compost had 120 g kg^{-1} organic matter; 5.0 and 2.2 g kg^{-1} total N and P, respectively, and about 50% water content. Manure, P and K were applied as basal fertilizers, while 40% of the N was applied as a basal dressing and 60% top-dressed on the wheat crop at the reviving growth stage.

In this study, rhizosphere soil was defined as the vegetated soil within the densely rooted portion of the soil profile, and bulk soil as the unvegetated soil immediately surrounding the root mat (Kourtev et al., 2002). Soil samples were collected at the wheat reproductive stage in early May 2010, then rhizosphere effects tend to be most pronounced (Cheng et al., 2003). The random sampling method was used to ensure representative sampling from the different treatments. One composite bulk or rhizosphere soil sample, consisting of 20 paired cores from rhizosphere or neighboring bulk soils, was collected from each treatment. The samples were immediately transported to the laboratory. Plants roots were removed by passing through a 2 mm mesh sieve, and the samples were then stored at room temperature for chemical analysis, at 4 °C for extracellular enzyme analysis, and at −70 °C for PLFA analysis (i.e. the soil was freeze-dried before the determination of PLFAs).

2.2. Chemical analysis

Soil pH was measured with a compound electrode (PE-10, Sartorious, Germany) using a soil to water ratio of 1:2.5. Soil organic C was determined by dichromate oxidation, and total N and total S by element analyzer (Elementar Analysensysteme GmbH, Germany). Ammonium N (NH$_4^+$-N) and nitrate N (NO$_3^-$-N) contents were determined by extracting the soil with 0.01 M KCl solution (1:10, w/v) for 30 min, and determining NH$_4^+$ and NO$_3^-$ concentrations by flow injection autoanalyzer (FLA star 5000 Analyzer, Foss, Denmark). Available P was determined by the Olsen method (Olsen and Sommers, 1982) and available K was analyzed by ammonium displacement of the exchangeable cations.

2.3. Extracellular enzyme activities

The activities of all extracellular enzymes except urease, phenol oxidase and peroxidase were measured using MUF-linked or AMC-linked model substrates yielding the highly fluorescent cleavage products 4-methylumbelliferyl (MUF) or 7-amino-4-methylcoumarin (AMC) upon hydrolysis (Deforest, 2009; Saiya-Cork et al., 2002; Wittmann et al., 2004) (Table 1). The method is very sensitive and allowed a high throughput analysis of enzymatic activities (Wittmann et

Table 1
Extracellular enzymes assayed in both the rhizosphere and bulk soil, their enzyme commission number (EC) and corresponding substrate (L-DOPA = L-3,4-dihydroxy-phenylalanine, 4-MUB = 4-methylumbelliferyl).

Enzyme	Substrate	EC
Urease	Urea	3.5.1.5
Phosphatase	4-MUB-phosphate	3.1.3.1
Sulfatase	4-MUB-sulfate	3.1.6.1
β-glucosidase	4-MUB-β-D-glucoside	3.2.1.21
β-cellobiosidase	4-MUB-β-D-cellobioside	3.2.1.91
N-Acetyl-glucosaminidase	4-MUB-N-acetyl-β-D-glucosaminide	3.2.1.30.
β-xylosidase	4-MUB-β-D-xyloside	3.2.1.37
α-glucosidase	4-MUB-α-D-glucoside	3.2.1.20
L-leucine aminopeptidase	L-Leucine-7-amino-4-methylcoumarin	3.4.11.1
Phenol oxidase	L-DOPA	1.10.3.2
Peroxidase	L-DOPA	1.11.1.7

al., 2004). Specifically, each equivalent of 1.0 g dry mass of fresh soil was added into a 100 ml centrifuge tube, and it was homogenized with 50 mL of 50 mM acetate buffer using a ploytron, then the mixture was poured into a round wide-mouth beaker. An additional 50 mL of acetate buffer washed the centrifuge tube and was poured into the same beaker. A magnetic stirrer was used to maintain a uniform suspension. The buffer, sample suspension, 10 μM references and 200 μM substrates (Table 1) were dispensed into the wells of a black 96-well microplate according to the strict volume and order described by DeForest (2009). The microplates were covered and incubated in the dark at 25 °C for 4 h and the fluorescence quantified using a microplate fluorometer (Scientific Fluoroskan Ascent FL, Thermo) with 365 nm excitation and 450 nm emission filters (Saiya-Cork et al., 2002). The activities were expressed in units of nmol $h^{-1} g^{-1}$.

The non-fluorometric enzymes, phenol oxidase and peroxidase, were measured spectrophotometrically in the clear 96-well microplate using the substrate of L-3, 4-dihydroxyphenylalanine (L-DOPA). The dispensed volume and the order of buffer, sample suspension, 25 mM L-DOPA and 0.3% H_2O_2 were the same as for the fluorometric enzymes (Deforest, 2009). The microplates were covered and incubated in the dark at 25 °C for 20 h, and the activities were assayed by measuring the absorbance at 450 nm using the microplate fluorometer and expressed in unites of nmol $h^{-1} g^{-1}$. Urease activities was determined using urea as the substrate as described by Lu (2000). The determination was based on the product of indophenol blue, which was determined colorimetrically at 578 nm using a spectrophotometer (UV-2550, SHIMADZU). The activities were expressed as nanomoles of ammonium released per hour per gram of soil (nmol $h^{-1} g^{-1}$).

2.4. Microbial community structure

Differences in the microbial community and microbial biomass between the rhizosphere soil and bulk soil were determined by phospholipid fatty acid (PLFA) analysis following the procedure described by Wu et al. (2009). Briefly, three-gram freeze-dried soil samples were used to extract the PLFAs with a single-phase mixture of chloroform: methanol: citrate buffer (15.2 mL at a 1:2:0.8 volume ratio). The extracted fatty acids in the chloroform were fractionated into neutral lipids, glycolipids, and polar lipids using a silica-bonded phase column (SPE-Si, Supelco, Poole, UK) with chloroform, acetone and methanol, respectively. The recovered polar lipids were trans-esterified to the fatty acid methyl esters (FAMES) by a mild alkaline methanolysis. FAMES were quantified by gas chromatograph (N6890, Agilent) and identified with an MIDI SHERLOCKS microbial identification system (Version 4.5, MIDI, Inc., Newark, DE). Nonadecanoic acid methyl ester (19:0) was added as the internal standard. Concentrations of PLFAs were expressed in units of nmol g^{-1}.

Total microbial biomass was determined using the total concentration of PLFAs (nmol g^{-1}). The abundance of individual PLFAs was indicated by their % mole abundance in each sample. PLFAs were divided into various taxonomic groups based on previously published PLFA biomarker data (Bossio et al., 1998; Frostegrd et al., 1993; Green and Scow, 2000; Turpeinen et al., 2004; Vestal and White, 1989; Zelles, 1997). Specifically, 15:0, 16:0, 17:0, 16:1 w5c, 16:1 w7c, 16:1 w9c, 17:1 w8c, 18:1 w5c, 18:1 w7c, a 15:0, a 17:0, cy 17:0, cy 19:0 w8c, i 14:0, i 15:0, i 16:0, i 17:0 and i 19:0 were used to represent bacterial biomarkers. The polyunsaturated PLFA 18:2 w6, 9 was chosen to indicate fungal biomarkers. The fatty acids 16:0 (10Me), 17:0 (10Me) and 18:0 (10Me) were considered the biomarkers of actinomycetes, and cy17:0, cy19:0, 16:1 w5c, 16:1 w9c, 16:1 w7c, 17:1 w8c, 18:1 w5c, 18:1 w7c, and i 14:0, i 15:0, i 16:0, i 17:0, a 15:0, a 17:0 were considered to be Gram-negative and Gram-positive bacteria biomarkers, respectively.

2.5. Statistical analysis

Statistical procedures (ANOVA and principal component analysis (PCA)) were carried out with SAS and Canoco for Windows (version 4.5) softwares, respectively, and some other complemental calculations were carried out using MS Excel 2003. For each variable measured in the rhizosphere or bulk soil, the data were analyzed by one-way ANOVA using Fisher's least significant differences (LSD, $P = 0.05$) to determine significant differences among treatment means. Two-way ANOVA was used to determine statistical differences by soil fractions (rhizosphere and bulk soil) and fertilizer treatments. Stepwise multiple regression analysis (using a probability of p<0.05 to accept) was applied to detect the factors influencing microbial groups.

3. Results

3.1. Soil pH and nutrient concentrations

Soil pH values were not affected by long-term fertilizer treatments, but were lower in the rhizosphere than in the bulk soil. The concentration of total or available nutrients tended to be greater in the rhizosphere than in the bulk soil, except for NO_3-N (Table 2), which was lower. Long-term organic fertilization (M and MNPK) significantly increased soil organic C, total N, NH_4^+-N and total S in both the rhizosphere and bulk soil. In general, inorganic fertilizer addition mainly affected the available nutrients. N fertilizer treatments (N, NP, NPK and MNPK) markedly increased NO_3-N concentrations in the bulk soil, and P fertilizers (NP, NPK and MNPK) also increased available P in both the rhizosphere and bulk soil. The treatments without K fertilizer (CK, N and NP) showed lower concentrations of available K compared to the NPK, M and MNPK treatments in both the rhizosphere and bulk soil.

3.2. Enzyme activities

The activities of 11 soil extracellular enzymes in both the rhizosphere and bulk soil were quantified in the different fertilizer treatments. Both the rhizosphere and fertilizer treatments had strong effects on most enzyme activities, the only exceptions being L-leucine aminopeptidase and phenol oxidase, where the rhizosphere did not affect the activities of L-leucine aminopeptidase ($P = 0.9095$) and phenol oxidase ($P = 0.1110$) (Table 3).

In order to clarify changing patterns of extracellular enzyme activities under the different fertilization treatments, we calculated the percent difference of enzyme activities in the different fertilized treatments compared to the corresponding control (CK) in the rhizosphere and bulk soil, respectively (Fig. 1). The activities of extracellular enzymes presented different variation trends for the different sites (rhizosphere and bulk soil) after the long-term application of inorganic or organic fertilizers. The actual activities of

Table 2
Soil pH and nutrient concentrations after long-term fertilization in the rhizosphere and bulk soil. Data are means ± S.E., n = 3. Different letters indicate significant differences among fertilizer treatments at the $p < 0.05$ level.

Treatments		CK	N	NP	NPK	M	MNPK
pH	Bulk soil	8.27 ± 0.04 a	8.22 ± 0.03 a	8.26 ± 0.12 a	8.23 ± 0.05 a	8.26 ± 0.04 a	8.12 ± 0.14 a
	Rhizosphere	8.08 ± 0.03 a	8.09 ± 0.08 a	8.15 ± 0.06 a	8.10 ± 0.02 a	8.05 ± 0.06 a	8.12 ± 0.02 a
Total N (g kg^{-1})	Bulk soil	1.45 ± 0.12 b	1.45 ± 0.04 b	1.54 ± 0.07 b	1.44 ± 0.10 b	1.89 ± 0.12 a	1.95 ± 0.15 a
	Rhizosphere	1.76 ± 0.12 c	1.65 ± 0.01 c	1.76 ± 0.05 c	1.83 ± 0.07 c	2.54 ± 0.15 b	2.75 ± 0.13 a
Organic C (g kg^{-1})	Bulk soil	9.54 ± 0.67 b	9.74 ± 0.68 b	9.57 ± 0.64 b	9.77 ± 0.39 b	11.84 ± 0.94 a	11.9 ± 0.32 a
	Rhizosphere	11.03 ± 0.74 b	10.56 ± 0.78 b	10.64 ± 0.33 b	10.76 ± 0.25 b	15.11 ± 0.82 a	15.01 ± 1.1 a
Total S (mg kg^{-1})	Bulk soil	330.77 ± 18.60 c	334.97 ± 10.67 c	309.99 ± 6.22 c	325.68 ± 20.86 c	394.36 ± 24.14 b	452.62 ± 19.52 a
	Rhizosphere	476.74 ± 31.06 bc	482.38 ± 39.12 bc	440.50 ± 23.77 d	451.40 ± 42.99 dc	533.02 ± 41.41 ab	554.41 ± 22.59 a
NH$_4^+$-N (mg kg^{-1})	Bulk soil	1.66 ± 0.28 c	1.55 ± 0.89 c	1.99 ± 0.63 bc	3.24 ± 0.54 ab	4.07 ± 0.43 a	3.73 ± 1.06 a
	Rhizosphere	3.27 ± 1.04 bc	1.93 ± 0.53 c	2.54 ± 0.75 c	2.48 ± 1.25 c	5.53 ± 1.25 ab	6.34 ± 0.44 a
NO$_3$-N (mg kg^{-1})	Bulk soil	10.52 ± 2.95 bc	23.13 ± 3.85 a	17.28 ± 4.94 ab	14.03 ± 5.80 bc	7.21 ± 2.73 c	16.84 ± 5.92 ab
	Rhizosphere	10.42 ± 3.06 bc	15.19 ± 0.48 a	7.71 ± 1.85 bcd	7.49 ± 1.24 cd	6.19 ± 0.97 d	11.73 ± 3.67 ab
Available P (mg kg^{-1})	Bulk soil	2.65 ± 0.66 d	2.80 ± 0.48 d	23.53 ± 3.60 b	22.35 ± 5.01 b	11.57 ± 1.91 c	49.10 ± 3.83 a
	Rhizosphere	4.51 ± 0.33 d	3.93 ± 0.97 d	37.16 ± 14.13 bc	46.06 ± 12.49 b	22.27 ± 1.66 c	69.47 ± 14.89 a
Available K (mg kg^{-1})	Bulk soil	110.39 ± 13.62 c	120.03 ± 21.44 bc	101.42 ± 21.84 c	149.02 ± 18.93 abc	163.20 ± 40.23 ab	197.20 ± 17.63 a
	Rhizosphere	141.49 ± 10.30 cd	120.03 ± 11.03 de	97.72 ± 21.77 e	166.03 ± 14.10 c	211.04 ± 33.21 b	278.40 ± 33.81 a

extracellular enzymes were generally greater in the rhizosphere than in the bulk soil (data not shown). Long-term fertilization, especially the application of manure compost (M and MNPK), significantly increased most enzymes activities, except for sulfatase, L-leucine aminopeptidase, phenol oxidase and peroxidase, in the bulk soil. For example, MNPK treatments enhanced β-glucosidase and β-cellobiosidase activities in the bulk soil by approximately 148% and 179%, respectively. However, in the rhizosphere, changes in most enzyme activities were distinctively different from those in the bulk soil. The activities of urease, phosphatase, β-glucosidase, β-cellobiosidase, N-Acetyl-glucosamini-dase, β-xylosidase, and α-glucosidase in the rhizosphere tended to decrease or remain unchanged in the inorganic treatments (N, NP and NPK). In contrast, these enzyme activities were enhanced by organic fertilizers (M and MNPK), but the degree of change was smaller compared with the bulk soil. In addition, the activities of L-leucine aminopeptidase were only repressed by inorganic treatments in the rhizosphere (Fig. 1). Sulfatase was mainly affected by inorganic fertilizers, which tended to decrease its activity in both the rhizosphere and bulk soil. Phenol oxidase and peroxidase only showed minor changes compared to the other hydrolytic enzymes.

3.3. PLFA analysis

PLFA analysis was used to determine the microbial community structure in both the rhizosphere and bulk soil. A total of 70 PLFAs were detected and 68 PLFAs were used as measures of total microbial biomass and the abundance of microbial groups. The soil fractions (rhizosphere or bulk soil), fertilizations and their interactions significantly affected total PLFA and different microbial groups

(Table 4). Total PLFA, which ranged from 31.57 to 139.15 nmol g^{-1}, were typically higher in the rhizosphere than in the bulk soil in all treatments (Fig. 2A). Compared to the bulk soil, the rhizosphere had a higher abundance of fungi and a lower abundance of actinomycetes in all treatments (Figs. 2C and D). Because of these changes, the ratios of bacteria to fungi and of actinomycetes to fungi in the bulk soil were significantly greater than in the rhizosphere. Additionally, the ratio of Gram+ to Gram− bacteria tended to be higher in the bulk soil than in the rhizosphere under CK treatment (Fig. 2B).

Total PLFA was significantly increased by organic fertilization (M and MNPK), especially in the rhizosphere, which was almost doubled compared with other treatments, but remained unchanged by inorganic fertilization (N, NP and NPK) (Fig. 2A). However, the responses of microbial groups in the rhizosphere to inorganic or organic fertilizers were distinctively different from those in the bulk soil. In the bulk soil, bacteria and actinomycetes were more abundant in the inorganic treatments but lower in the M treatment (Figs. 2B and D), whereas fungi were more abundant in the M treatment and less abundant in the N treatment (Fig. 2C). Consequently, the ratios of bacteria to fungi and of actinomycetes to fungi were highest in the N treatment and lowest in the M treatment. In the rhizosphere, however, there were no statistically significant differences in the abundances of bacteria, fungi and actinomycetes between the inorganic and organic treatments, although bacteria abundance commonly increased and fungi abundance generally decreased in all fertilized treatments (inorganic or organic) compared to CK (Fig. 2B, C and D). In addition, the ratio of Gram+ to Gram− bacteria was enhanced by organic fertilization (M and MNPK) in both the rhizosphere and bulk soil (Fig. 2B).

Table 3
Two ways ANOVA analysis of 11 extracellular enzymes activities in 2 soil fractions (rhizosphere and bulk soil), 6 fertilizer treatments each with 3 replicates (n = 36). The data in bold indicated that enzyme was not affected by soil fractions, fertilizer treatments or their interaction (P>0.05).

|	Soil fractions (Rhizosphere or bulk)		Fertilizer treatments		Soil fractions × Fertilizer treatments	
	F	P	F	P	F	P
Urease	170.4	<0.0001	47.28	<0.0001	7.67	0.0003
Phosphatase	327.01	<0.0001	71.44	<0.0001	16.32	<0.0001
Sulfatase	121.00	<0.0001	23.56	<0.0001	3.00	0.0326
β-glucosidase	625.60	<0.0001	80.54	<0.0001	9.96	<0.0001
β-cellobiosidase	336.93	<0.0001	49.67	<0.0001	15.44	<0.0001
N-Acetyl-glucosaminidase	118.82	<0.0001	17.68	<0.0001	7.06	0.0004
β-xylosidase	375.68	<0.0001	32.48	<0.0001	10.19	<0.0001
α-glucosidase	831.91	<0.0001	64.95	<0.0001	15.44	<0.0001
L-leucine aminopeptidase	**0.01**	**0.9095**	33.18	<0.0001	12.90	<0.0001
Phenol oxidase	**2.76**	**0.1110**	14.39	<0.0001	2.80	0.042
Peroxidase	24.29	<0.0001	24.97	<0.0001	6.88	0.0005
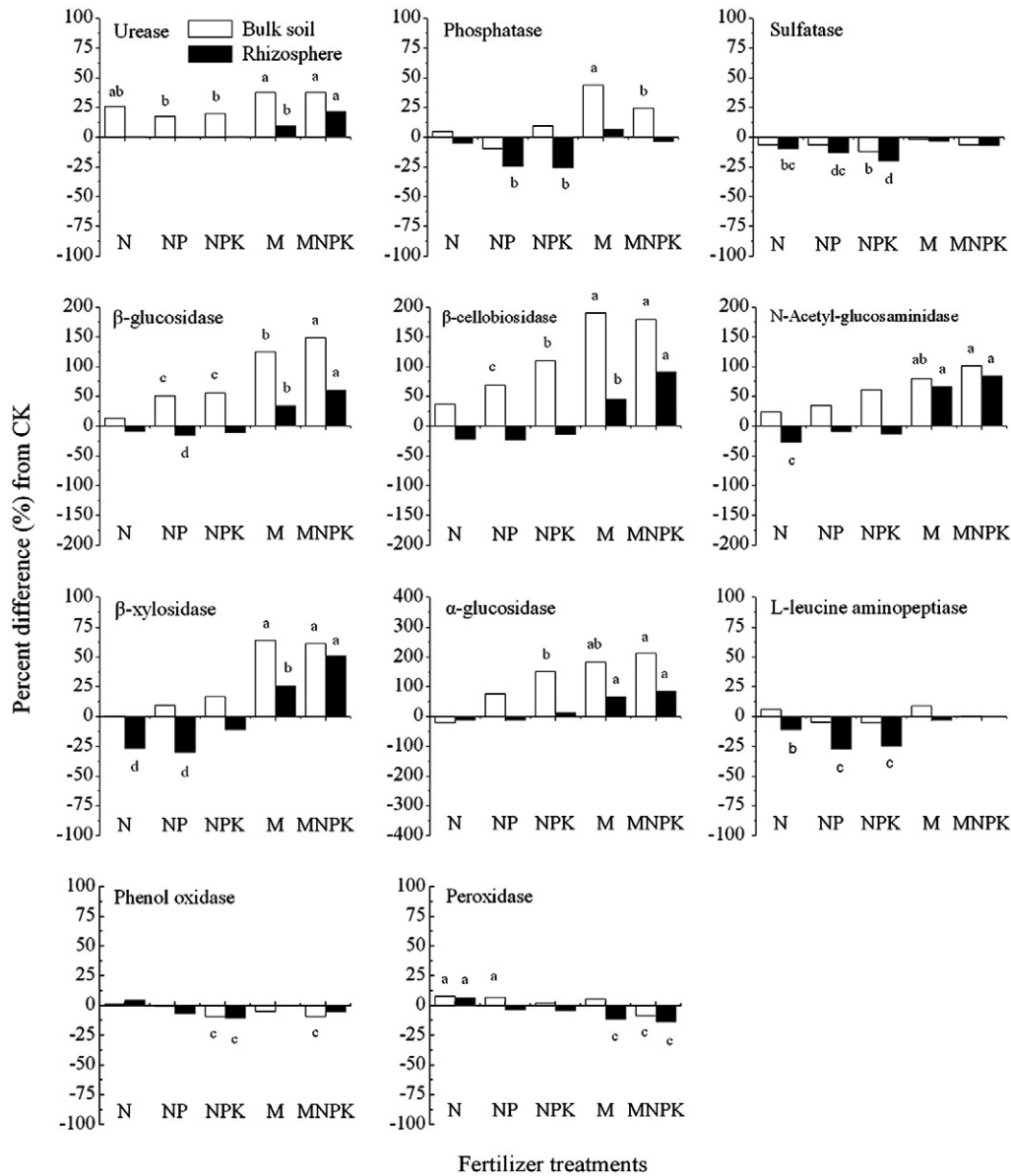
Fig. 1. Percent difference of extracellular enzyme activities in the fertilized treatments compared to the corresponding control treatments (CK) calculated as [(fertilized − CK)/CK × 100]. The lower-case letters indicate significant differences from the corresponding CK at the p<0.05 level.

The PCA was conducted with 68 PLFAs that were present either in the rhizosphere or in the bulk soil. The PC1 and PC2 accounted for 30.7% and 17.0% of the total variation, respectively. PC scores on these axes were well separated on the basis of soil fractions and fertilization

treatments (Fig. 3). However, there are relatively small changes between CK and inorganic fertilizer treatments (N, NP and NPK) in both the bulk soil and rhizosphere. The ANOVA of PC1 scores showed significant effects of fertilizer treatments (P<0.0001) (Table 5), with

Table 4
Two ways ANOVA analysis of microbial groups in 2 soil fractions (rhizosphere and bulk soil), 6 fertilizer treatments each with 3 replicates (n = 36).

	Soil fractions (Rhizosphere or bulk)		Fertilizer treatments		Soil fractions × Fertilizer treatments	
	F	P	F	P	F	P
Total PLFA	600.63	<0.0001	193.97	<0.0001	34.72	<0.0001
Bacterial	4.92	0.0372	9.12	<0.0001	4.68	0.0046
G+/G−[a]	22.59	<0.0001	43.55	<0.0001	5.43	<0.0001
Fungi	312.5	<0.0001	6.84	0.0005	10.84	<0.0001
Bacteria/fungi	854.09	<0.0001	37.00	<0.0001	40.69	<0.0001
Actinomycetes	132.9	<0.0001	6.16	0.001	4.93	0.0035
Actinomycetes/fungi	682.79	<0.0001	32.43	<0.0001	28.76	<0.0001

[a] G+/G− = Gram positive bacteria : Gram negative bacteria ratio.
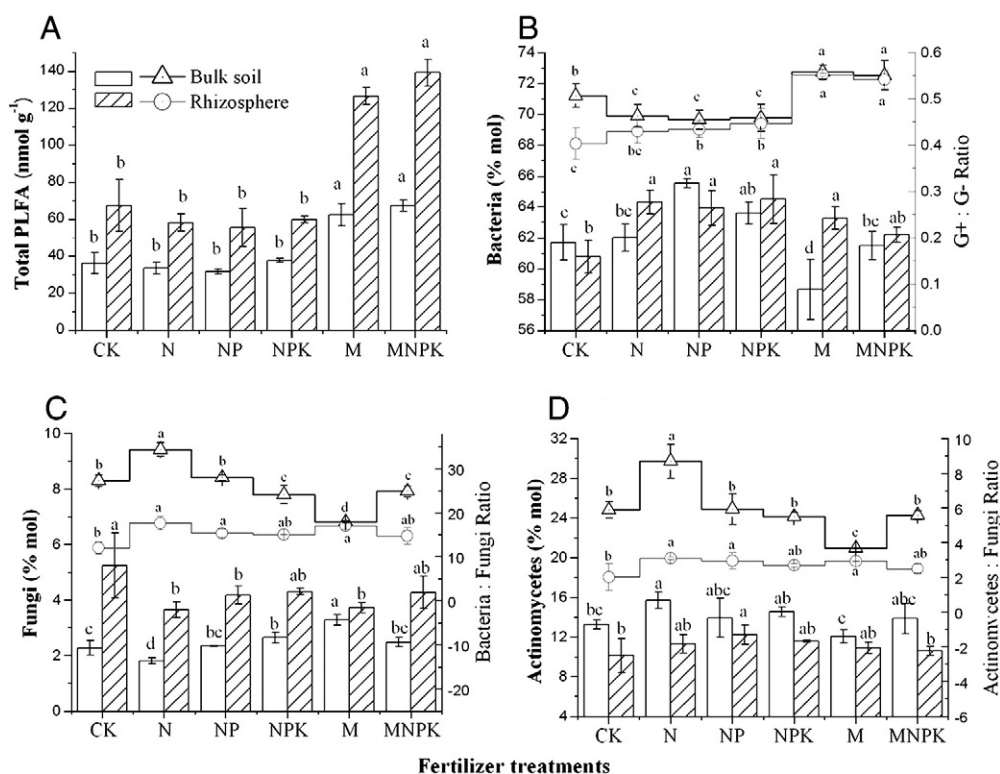
Fig. 2. Comparisons of total PLFA (A); Bacteria (B, histograms) and G+:G− Ratio (B, lines); Fungi (C, histograms) and Bacteria: Fungi Ratio (C, lines) and Actinomycetes (D, histograms) and Actinomycetes:Fungi Ratio (D, lines). Vertical bars represent the SE (n = 3) and lower case letters indicate significant differences among fertilizer treatments in the rhizosphere or bulk soil at the p<0.05 level.

PC scores increasing by organic fertilization. Soil fractions significantly influenced PC1 scores, with rhizosphere higher than bulk soil (P<0.0001). Fertilizer treatments and soil fractions also significantly influenced PC2 scores. Organic fertilizers had a similar, positive impact on PC2 scores as those in PC1. The PC2 scores of rhizosphere, however, were significantly lower than in the bulk soil (P=0.0004). PC loadings for individual PLFAs were showed in Fig. 3B. These data and PC scores in Fig. 3A indicated that, in the rhizopshere, proportions of saturated fatty acids (13:0, 14:0, 15:0, 17:0, a13:0, a15:0, i12:0, i13:0, i14:0, i15:0, i16:0) and hydroxylated fatty acids (10:0 2OH, 11:0 3OH, 15:0 2OH, 16:0 2OH, 18:1 2OH, 11:0 3OH, i15:0 3OH, i17:0 3OH) increased in organic fertilizer treatments, whilst the proportions of monounsaturated fatty (16:1 w5c, 16:1 w7c, 16:1 w9c, 17:1 w8c, 18:1 w7c and 18:1 w9c) and polyunsaturated fatty acids (18:2 w6,9c and 20:4w6,9,12,15c) increased in inorganic fertilizer treatments and CK. In the bulk soil, PC loadings indicated that a large number of the PLFAs detected changed in proportion between the inorganic and organic treatments. In particular, Methyl branched fatty acids [16:0 (10 M) and 18:0 (10 M)] (biomarkers of actinomycetes) increased in inorganic fertilizer treatments and CK.

3.4. Stepwise multiple regression analysis

Stepwise multiple regression analysis showed that organic C was an important factor, which markedly affected total PLFA and the Gram+ to Gram− ratio in both the rhizosphere and bulk soil (Table 6). In the bulk soil, Gram+ bacteria were significantly correlated with total N, and fungi was significantly correlated with pH and NH_4^+-N, with the ratio of bacteria to fungi being affected by NH_4^+-N and NO_3^--N. In the rhizosphere, however, only bacteria (Gram+ or Gram− bacteria) were affected by nutrient factors such as total N and NH_4^+-N concentrations in the rhizosphere.

4. Discussion

After 31 years application of fertilizers, organic C, and total N and S were significantly increased by organic amendments. This is in agreement with other long-term experiments that have found that organic fertilizers can promote the accumulation of organic matter and the major soil macronutrients of N, P, and K, and increase both soil microbial biomass and activity (Bastida et al., 2007; Elfstrand et al., 2007). However, long-term inorganic amendments did not impact on total nutrients, but significantly enhanced the concentrations of available ones (Table 2). These results illustrate that organic manure plays a more important role in improving soil fertility and sustainable land use than do mineral fertilizers (Mäder et al., 2002). It is well known that there are markedly different microenvironments between the rhizosphere and bulk soil due to root exudations, plant absorption and rhizosphere microorganisms (Neumann and Römheld, 2002). Our results also show that the rhizosphere had higher nutrient levels and a lower pH compared to the bulk soil in all treatments. This result is consistent with Wang and Zabowski (1998), who suggested that the main cause of increased nutrient concentration in the rhizosphere was root-induced organic matter decomposition or mineral weathering. However, the concentrations of NO_3-N in fertilizer N treatments (N, NP, NPK and MNPK) in the bulk soil were significantly higher than in the rhizosphere (Table 2), which could be associated with high nitrification rate and plant roots uptake, respectively. On the same soil type, Chu et al. (2007) found long-term N application could greatly promote soil nitrification functions. At the same time, Nitrogen uptake by plant roots can directly decrease the concentration of NO_3-N in the rhizosphere (Wang and Zabowski, 1998).

The ANOVA showed that the soil fractions (rhizosphere and bulk soil) significantly affected most enzyme activities (Table 3). These enzyme activities were not equally distributed between the rhizosphere and bulk soil, and higher activities were found in the
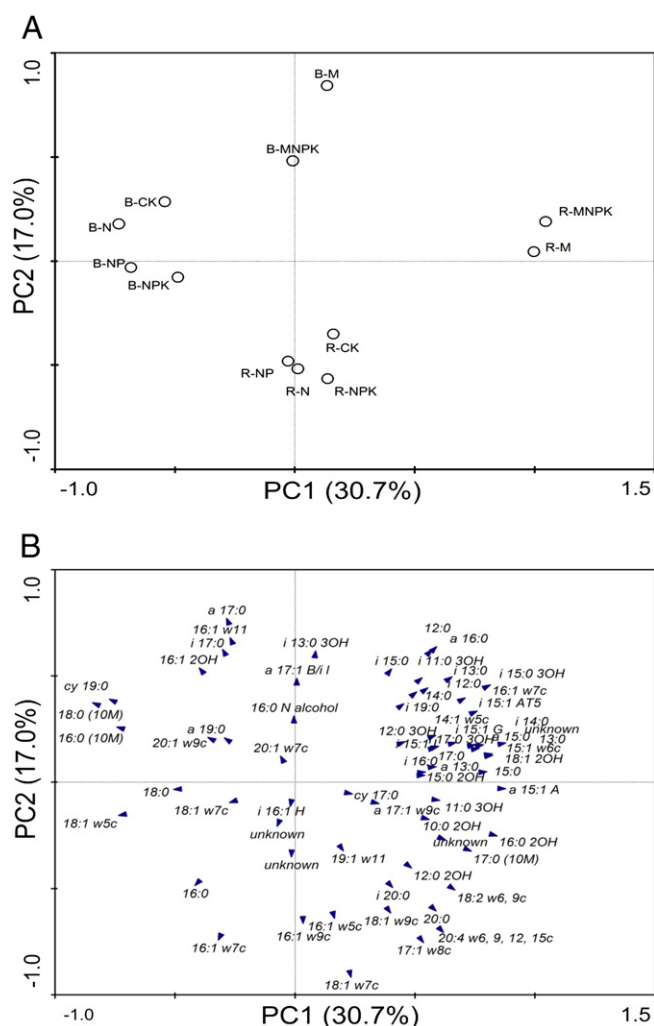
Fig. 3. Plot of first two principle components (PC1 and PC2) grouped in 6 treatments and 2 soils (A) and plot of two principle components (PC1 and PC2) among 68 PLFAs from both the rhizosphere and bulk soil (B), R- and B- represent rhizosphere and bulk soil, respectively.

and chitin (Saiya-Cork et al., 2002). Microorganisms might assimilate N for enzyme production, leading to increased soil enzyme activity (Weand et al., 2010). However, compared to the stimulation effects of inorganic fertilizers on enzyme activities in the bulk soil, most enzymes in the rhizosphere were suppressed or remained unchanged by inorganic fertilizers (Fig. 1). Several studies have reported that application of inorganic fertilizers often reduced rhizosphere effects on soil organic matter (SOM) decomposition and nutrient mineralization (Liljeroth et al., 1994; Merckx et al., 1987). The mechanisms responsible for these effects are complicated, as shown in several studies (Cheng, 1999; Kuzyakov, 2002; Phillips and Fahey, 2007). In forest soils, Phillips and Fahey (2008) also found that mineral NPK fertilizer reduced rhizosphere effects on microbial biomass and activity, N mineralization rates and phosphatase activity. Probably, the decreased enzyme activity in the rhizosphere is associated with changing rhizosphere effects on SOM decomposition and nutrient mineralization, because these processes are completed by various enzymes.

Many studies have indicated that soil enzyme activities are often enhanced by organic amendments and are significantly correlated with soil organic carbon contents (Bastida et al., 2007; Kanchikerimath and Singh, 2001). The biodegradation of SOM enhances microbial activity and induced the synthesis of enzymes in a short-term experiment (Benitez et al., 2005). Our PCA analysis of PLFAs also confirmed that organic fertilization and rhizosphere effects were two major factors affect soil microbial communities (Fig. 3). In addition, extracellular enzymes are likely to be continuously accumulated in soil humic matter (Nannipieri et al., 2002). After 31 years application of organic manure (M and MNPK), organic C and most enzyme activities were significantly increased in the bulk soil (Table 2, Fig. 1). At the same time, this positive effect was also present in the rhizosphere, where enzyme activities were suppressed by inorganic fertilizers. This is a noteworthy finding and suggests that, compared to inorganic fertilizers, the long-term biochemical and biological responses of the soil to organic fertilizer will accelerate nutrient turnover in the rhizosphere and promote plant growth and sustainable land use. Compared to the strong changes in other hydrolytic enzyme activities, the responses of oxidative enzymes and sulfatase to our fertilizer treatments were relatively weak in both the rhizosphere and bulk soil. Bastida et al. (2007) reported that low amounts of organic amendments (65 t ha^{-1}) did not change the activity of humus-associated o-diphenol oxidase. Differences in enzyme activity were also found to be directly associated with the type of humic compound in the soil (Benitez et al., 2005).

As expected, the biomass and composition of PLFAs changed markedly in the rhizosphere compared with the bulk soil. Total PLFA biomass in the rhizosphere significantly increased in comparison with the bulk soil, which can be attributed to root-induced shifts in microbial activity (Neumann and Römheld, 2002). Higher PLFA biomass was also associated with higher extracellular enzyme activities in the rhizosphere. The ratio of Gram+ to Gram− bacteria in CK treatment was higher in the bulk soil than in the rhizosphere; the ratios of bacteria to fungi and of actinomycetes to fungi were lower in the rhizosphere than in the bulk soil in all treatments (Fig. 2). This result confirms several recent studies showing that root exudates

rhizosphere in all fertilizer treatments (data not shown). This "rhizosphere effect" may be linked to substrate availability and microbial activity (Badalucco and Kuikman, 2001). Greater enzyme activities, in turn, will induce higher decomposition, which would be a reason for the higher availability of nutrients in the rhizosphere (Table 2) (Badalucco and Kuikman, 2001).

The results further showed that enzymes activities in the rhizosphere and bulk soil were changed by fertilization, but not in the same way. Our results and other studies indicate that inorganic fertilizers, especially N fertilizers, usually enhance the activities of most enzymes (e.g. urease and glycosidase) in bulk soil involved in N mineralization and decomposition of storage carbohydrates, cellulose

Table 5
Two ways ANOVA analysis of PC scores for PLFAs in 2 soil fractions (rhizosphere and bulk soil), 6 fertilizer treatments each with 3 replications (n = 36).

	Soil fractions (Rhizosphere or bulk)		Fertilizer treatments		Soil fractions × Fertilizer treatments	
	F	P	F	P	F	P
PC1	333.30	< 0.0001	44.91	< 0.0001	3.52	0.0173
PC2	17.72	0.0004	7.90	0.0002	2.12	0.1011

Table 6
Stepwise regressions between microbial properties (dependents variable Y) and soil nutrients (independent variable X) in both the rhizosphere and bulk soil from the long-term fertilization experiment.

Dependents (Y)	Variables related (X)	R^2
Bulk soil		
Total PLFA	Organic C	0.98**
G+[b]	Total N	0.76*
G−	ns[a]	
G+/G−	Organic C	0.80*
Fungi	pH, NH_4^+-N	0.97**
Bacteria/fungi	NH_4^+-N, NO_3^--N	0.97**
Actinomycetes	ns	
Rhizosphere		
Total PLFA	Organic C	0.98***
G+	Total N, NH_4^+-N	0.97**
G−	NH_4^+−N	0.96***
G+/G−	Organic C	0.92**
Fungi	ns	
Bacteria/fungi	ns	
Actinomycetes	ns	

* Significant at P = 0.05.
** Significant at P = 0.01.
*** Significant at P = 0.001.
[a] No variable was detected by stepwise regression analysis to be correlated with a corresponding microbial property.
[b] G+ = Gram positive bacteria; G− = Gram negative bacteria.

are preferentially utilized by Gram− bacteria and fungi, leading to increased growth in the rhizosphere (Bird et al., 2010; Esperschütz et al., 2009). More importantly, we found that responses of the microbial community to inorganic or organic fertilizers in the rhizosphere differed from those in the bulk soil.

Long-term fertilization strongly affects the microbial community structure in bulk soil (Marschner, 2003; Zhong et al., 2010). The ratios of bacteria to fungi and of actinomycetes to fungi were highest in the N treatments, but least in the M treatment. In general, a high ratio of fungi to bacteria is indicative of more sustainable land use (De Vries et al., 2006). However, compared to the bulk soil, there were no statistically significant differences in the ratios of bacteria to fungi and of actinomycetes to fungi in the rhizosphere between the inorganic and organic treatments. Lovell et al. (2001) reported that the microbial community in the *S. alterniflora* rhizosphere did not respond dramatically to changing environmental factors (e.g. physical or chemical disruptions), due to a highly structured and physically non-perturbed rhizosphere habitat. This is supported by stepwise regression analysis in our study, which showed that changing soil fertilizations had a greater impact on bulk soil microbial groups than those in the rhizosphere (Table 6).

Although there were no statistically significant differences in the abundance of bacteria in the rhizosphere between inorganic and organic treatments, the ratio of Gram+ to Gram− bacteria was significantly increased by organic fertilization, which also happened in the bulk soil (Fig. 2B). Gram negative bacteria, which are sensitive to copiotrophic conditions (Esperschütz et al., 2009; Kieft et al., 1994), are often stimulated by added organic matter resulting in a low ratio of Gram+ to Gram− bacteria (Buyer et al., 2010; Larkin et al., 2006). However, several studies have shown that organic treatments also lead to a high ratio of Gram+ to Gram− bacteria (Marschner, 2003). Bird et al. (2010) reported gram positive bacteria initially had a low preference for belowground ^{13}C labeled SOM compared with other microbial groups, but this preference was increasingly enhanced over time. Stepwise regression analysis confirmed that organic C was a key factor controlling the ratio of Gram+ to Gram− in both the rhizosphere and bulk soil. In addition, it should be noted that observed changes in bacterial community structure may be also caused by bacteria added with the organic manure (Marschner, 2003).

5. Conclusions

Long-term fertilization had a great impact on soil extracellular enzyme activities and the microbial community. However, the response in the rhizosphere to added inorganic or organic fertilizers was significantly different from that in the bulk soil. The rhizosphere effect, which is generally thought to enhance most extracellular enzyme activities, was suppressed by inorganic fertilizers, whereas organic manure tended to increase most enzyme activities in both the rhizosphere and bulk soil. The influence of fertilization on the microbial community was mainly observed in the bulk soil. In the rhizosphere, however, total PLFA and the ratio of Gram+ to Gram− bacteria were significantly increased by organic fertilization. Long-term applications of organic fertilizers enhanced soil fertility and microbial activity, while root exudates probably play an important role in mediating the degree to which fertilization affects the soil microbial community and extracellular enzyme activities.

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