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Distribution of soil nutrients, extracellular enzyme activities and microbial communities across particle-size fractions in a long-term fertilizer experiment

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ABSTRACT

Soils were particle-size fractionated to evaluate changes in carbon and nitrogen contents, enzyme activities and microbial community composition in response to 33 years of fertilization. This study focused on yellow–brown paddy soil and the particle-size fractions of >2000, 2000–200, 200–63, 63–2 and 2–0.1 μm. Microplate fluorometric assays and phospholipid fatty acid analysis (PLFA) were used to determine soil biological characteristics under no fertilizer (control, CK), fertilizer N (N), fertilizer N and P (NP), fertilizer N, P and K (NPK), organic manure plus fertilizer N, P and K (NPKM) and organic manure (M) treatments. The results showed that fertilizer and soil fraction individually and interactively ($P < 0.05$) affected soil C, N contents, enzyme activities and microbial communities except for α-glucosidase activity, bacterial relative abundance and the G+:G− ratio. Particularly, organic treatments significantly increased soil organic carbon (SOC) and total nitrogen (N) contents of all five fractions. The highest C and N contents and enzyme activities were observed in the 200–63 μm fraction, except for phosphatase and sulfatase, which showed the highest activities in the 2–0.1 μm fraction. The highest activities of β-glucosidase, β-cellobiosidase, α-glucosidase, aminopeptidase, phenol oxidase and peroxidase in each fraction were obtained in the organic treatments (NPKM and M). Activities of phosphatase, sulfatase, *N*-acetyl-glucosaminidase and β-xylosidase in the 2000–200 μm fraction were highest under NPK treatment. PLFA analysis showed that the >63 μm fraction contained higher abundance of total PLFAs than that in the 63–0.1 μm fraction. Organic treatments significantly enhanced total PLFAs abundance in >2000 μm fraction, but decreased PLFAs abundance in the 2000–200 μm fraction compared with the NPK treatment. Larger fractions (>2000 μm and 2000–200 μm) held relatively lower G+:G− ratios and higher fungi:bacteria ratios, which indicated better soil conditions in these fractions. Principal component analysis showed a smaller variability of microbial community composition among treatments than particle-size fractions. Most treatments of larger fractions (>2000 μm and 2000–2 μm) were well separated from the other fractions. Redundancy analysis showed total N, C:N ratio, phosphatase, sulfatase, *N*-acetyl-glucosaminidase and β-cellobiosidase activities significantly affected the composition of the microbial community. Significant correlations were also obtained between enzyme activities with SOC, total N and C:N ratio. We concluded that the long-term application of organic fertilizers contributed to improvements in the soil organic carbon and total nitrogen and most of the enzyme activities, especially for the 200–63 μm fraction, along with abundant and diverse microbial community composition in larger particles.

1. Introduction

Soils consist of particles of sand, silt and clay which bound into aggregates of various sizes by organic and inorganic agents. The distribution and stability of aggregates, and of the pores within and between them, affect soil properties and the composition and activity of soil biotic communities (Tisdall, 1994; Mikha and Rice, 2004). At the same time, aggregate formation and stabilization are

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affected by various factors, including climate conditions, mineral composition and types and amount of soil organic carbon (SOC) (Degens and Harris, 1997; Denef and Six, 2005). The main agents of aggregate formation and stabilization are organic materials, including persistent cementing agents, such as humic matter involved in stabilizing microaggregates, and transient bonding agents (e.g., polysaccharides derived from plants and microorganisms) as well as temporary binding agents (e.g., fungal hyphae, fine roots, bacterial cells) related to formation and stabilization of macroaggregates (Tang et al., 2011). Therefore, the determination of SOC and the soil nitrogen content (total N) within different particle-size fractions is important for evaluating the effects of different fertilizer managements on soil quality.

Continuous use of imbalanced fertilizers under intensive rice–wheat cultivation in the Yangtze Plain has adverse impacts on the soil. Integrated nutrient management practice is seen as a viable option in restoring the soil physical structure and chemical fertility (Gattinger et al., 2012), improving soil organic C and therefore, sustaining the system productivity (Seufert et al., 2012) and reducing global warming potential (Cavigelli et al., 2013). The scientific literature is replete with studies on the effects of organic matter on soil structure and other properties, but frequently at the bulk soil scale. Little attention has been given to understanding the soil mechanism properties at the particle-size scale, which might manifest quite differently, but largely influence the bulk soil properties (Das et al., 2014). Therefore, the specific locations of enzyme activities within the soil matrix have attracted attention, especially as the area of these enzyme activities is affected by SOM quality (Kandeler et al., 1999) and turnover (Stemmer et al., 1998). Additionally, extracellular enzyme activities in soils play an important role in the degradation of polymeric material and the supply of low molecular weight substrates to microorganisms (Nannipieri et al., 2012). To date, it is unknown how soil nutrients and extracellular enzyme activities are distributed across particle-size fractions after different fertilizer treatments in a long-term experiment.

Definitely, balanced fertilizer application can influence both soil organic matter content and quality, substantially increasing microbial diversity in soils, thus improving soil functions and increasing the resistance of soil to environmental stress (Wardle and Ghani, 1995; Pankhurst et al., 1996; Kennedy and Gewin, 1997; Degens et al., 2000; Nsabimana et al., 2004). Microbial communities in soils produce extracellular enzymes to acquire energy and resources from complex biomolecules in the soil environment (Burns, 1982). These enzymes are of interest on an ecosystem scale because they catalyze important transformations in the carbon (C), nitrogen (N) and phosphorus (P) cycles (Wallenstein and Burns, 2011). It is well known that the addition of easily decomposable substrates to soil rapidly stimulates the soil microflora, resulting in a significant increase in aggregate stability (De Gryze et al., 2005; Abiven et al., 2007). Denef et al. (2001) and De Gryze et al. (2005) showed that fungi significantly affected macroaggregates formation. Besides the physical effects of enmeshment of macroaggregates by hyphae, extracellular polysaccharides can be produced by hyphae, attaching microaggregates and binding them together into stable macroaggregates (Neufeldt et al., 1999). Additionally, the hydrophobicity of microbial extracellular polysaccharides contributes to the stabilization of macroaggregates by decreasing their wettability (Liu et al., 2010). Besides fungi, bacteria also exude extracellular polysaccharides to bind soil particles and increase inter-particle cohesion (Degens and Harris, 1997). Increases in the fungi: bacteria ratio have been linked to increases in soil C and the C:N ratio across landscapes (Fierer et al., 2009 De Vries et al., 2012) and in response to organic amendments (Bernard et al., 2012). Other studies have shown increases in phospholipid fatty acid biomarkers for arbuscular mycorrhizal fungi (AMF) in response to long-term organic management (Bossio et al., 1998; Moeskops et al., 2010, 2012). Studies also supports that fungal communities has been suggested as a means of increasing agroecosystem N retention and other functions (De Vries and Bardgett, 2012; Jackson et al., 2012). However, the relationships among soil C and N pools, extracellular enzyme activities and microbial community composition and structure in soils amended with inorganic and organic fertilizers are still poorly understood.

As stated previously, aggregate formation could be influenced by many different factors, so it is still necessary and meaningful to conduct experiments in yellow–brown paddy soil, which is a typical paddy soil in the Yangtze Plain of China. Our objective was to elucidate the distribution of organic C and total N, enzyme activities and microbial community composition within different particle-size fractions to determine the significant positive effects of adequate fertilization. It is thus reasonable to predict that the application of balanced fertilization in combination with organic amendments to a long-term rice–wheat rotation would significantly impact aggregation, increase extracellular enzyme activities and enrich the microbial community, thereby resulting in a better soil structure and a higher soil quality for crops.

2. Materials and methods

2.1. Field design and sampling

The long-term field fertilizer experiment was initiated in 1981 at South Lake station (30°37′N, 114°20′1″E), Hubei Province, China, where rice–wheat rotation is the common cropping system. The site is located in the northern subtropical to middle subtropical transitional geographic climate zone with an annual average total accumulated temperature of 5189.4 °C (>10 °C/day) and precipitation of 1300 mm. The tested yellow–brown paddy soil belongs to Udalfs with clay loam texture (USDA soil classification). At the beginning of the experiment in 1981, the soil had a pH (H_2O) of 6.3, organic matter of 27.43 g kg^{-1}, total N, P, K of 1.801 g kg^{-1}, 1.004 g kg^{-1} and 30.22 g kg^{-1}, respectively. The concentrations of available P and K were 5.0 mg kg^{-1} and 98.5 mg kg^{-1}. Six treatments (three replicates each) were randomly implemented in 18 plots (40 m^2 each) under a rotation of winter wheat and rice. Treatments consisted of soil without fertilizer (control, CK), fertilizer N (N), fertilizer N and P (NP), fertilizer N, P and K (NPK), organic manure plus fertilizer N, P and K (NPKM) and organic manure (M). For the NPKM treatment, fertilizer N, P and K were applied in the form of urea (300 kg N ha^{-1} per year), superphosphate (150 kg P_2O_5 ha^{-1} per year) and potassium chloride (150 kg K_2O ha^{-1} per year), respectively, while no PK or K was applied for the N and NP treatments, respectively. Organic manure was applied as pig manure (H_2O 69%) with properties of 15.1 g kg^{-1} total N, 20.8 g kg^{-1} P_2O_5 and 13.6 g kg^{-1} K_2O (22,500 kg ha^{-1} per year).

Sixty percent of chemical fertilizers were applied to rice and the other 40% were applied during the wheat season, while organic manure was applied equally (50:50) to the two crops. All fertilizer P and K and manure during the wheat season and the rice season were applied once as basal dressing. Meanwhile 40% of fertilizer N was applied as a basal fertilizer, 40% during tillering stage and 20% during booting stage in rice season. The amounts of N fertilizer applied to wheat were 50% as basal fertilizer, 25% for overwintering period and 25% during the jointing stage. Manure and mineral fertilizers were evenly broadcasted onto the soil surface and immediately incorporated into the plowed soil (0–20 cm depth) by tillage before sowing. According to the experimental design, the nutrient application rates of the other treatments were equal to the nutrients applied in the NPKM treatment.

Soil samples from the three replicates of each treatment were collected 1 week before wheat harvesting in May 17th, 2014. Four soil cores (5 × 10 × 18 cm) were collected at a depth of 0–20 cm from each plot. Moist soils were gently broken apart along the natural breakpoints and passed through a 5 mm sieve to remove visible organic debris. The 5 mm sieve was used rather than a 2 mm sieve because of the unique viscid characteristic of the paddy soil. If soils were forced through a 2 mm sieve, the natural structure of the soil would be destroyed. After thorough mixing, the field-moist soil was used for particle-size fractionation.

2.2. Fraction procedure

Soil samples were dispersed by low-energy sonication and the particle size fractions were separated by a combination of wet sieving and centrifuging as described by Stemmer et al. (1998). Briefly, the soil–water suspension was dispersed by low-energy sonication (output energy of 0.2 kJ/g) and subsequently fractionated by a combination of wet sieving and repeated centrifugation to avoid disruption of microaggregates. Finally, five fractions were obtained for each sample: large macroaggregates (>2000 μm), coarse sand-sized fraction (2000–200 μm), fine sand-sized fraction (200–63 μm), silt-sized fraction (63–2 μm), and clay-sized fraction (2–0.1 μm). Field-moist soils (140 g equivalent dry weight for each sample) were suspended in 400 mL of distilled water and then equally placed into four 150 mL glass beakers. The large macroaggregates, coarse and fine sand particle size fraction (>63 μm) were separated by manual wet sieving with a maximum of 700 mL of cooled distilled water. Silt-sized particles were separated from the clay fraction by four centrifugation steps at 150 × *g* for 5 min and at 15 °C. Between each centrifugation the pellets were resuspended in water and centrifuged again to purify the silt fraction. The combined supernatants were centrifuged at 3900 × *g* for 30 min to obtain clay-sized particles and the resulting same size soil fractions from the glass beakers of the same sample were pooled together. The above procedures were repeated until we got enough soil samples for all the tests. The fractions were then stored at room temperature for chemical analysis, at 4 °C for extracellular enzyme analysis and at −80 °C for PLFA analysis (the soil was freeze-dried before the determination of PLFAs).

2.3. Soil analysis

Soil organic C and total N were determined by dichromate oxidation (Kalembasa and Jenkinson, 1973) and Kjeldahl digestion (Bremner and Mulvaney, 1982), respectively.

2.4. Enzyme activity

The activities of all extracellular enzymes tested except phenol oxidase and peroxidase were measured using MUB-linked or AMC-linked model substrates yielding the highly fluorescent cleavage products 4-methylumbelliferyl (MUB) 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 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 polytron homogenizer, 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 μmol h^{-1} g^{-1}.

Table 1
Extracellular enzymes assayed in all the particle-size fractions, their enzyme commission number (EC) and corresponding substrate (L-DOPA = L-3, 4-dihydroxyphenylalanine, 4-MUB = 4-methylumbelliferyl).

Enzyme	Substrate	EC
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
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

2.5. PLFA profiles

Differences in the microbial community and microbial biomass among the various nutrients managements were determined by phospholipid fatty acid (PLFA) analysis following the procedure described by Wu et al. (2009). Briefly, 3 g 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 (N6850, 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 estimated 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). Specifically, 16:0, 17:0, 16:1ω5c, 16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c, 18:1ω7c, a15:0, a17:0, cy17:0, cy19:0ω8c, i14:0, i15:0, i16:0, i17:0 and i19:0 were used to represent bacterial biomarkers. The polyunsaturated PLFAs 18:2ω6,9c, 18:1ω9c and 18:3ω6c(6,9,12) (Hill et al., 2000; Dong et al., 2014; Ai et al., 2015) were chosen to indicate fungal biomarkers. The fatty acids 16:0(10Me), 17:0(10Me) and 18:0 (10Me) were considered as biomarkers of actinomycetes. We used 17:0, i14:0, i15:0, i16:0, i17:0, a15:0, a17:0 as gram-positive bacteria biomarkers; cy17:0, cy19:0ω8c, 16:1ω5c, 16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c, 18:1ω7c as gram-negative bacteria biomarkers.

2.6. 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 Origin 8, Adobe Illustrator CS4 and MS Excel 2010. Redundancy analysis (RDA) was also carried by Canoco for Windows (Version 4.5) but with the Monte Carlo permutations test (499 permutations) to determine whether the microbial community composition could be correlated to enzyme activities and properties of soil. For each variable measured in the 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 and fertilizer treatments.

3. Results

3.1. Distribution and recovery of soil fractions

Physical fractionation procedures according to particle size yielded high recovery rates (95.20–97.30%, Table 2). The three main fractions of soil aggregates, obtained by ultrasonic fractionation, were 63–2 μm, > 2000 μm and 2–0.1 μm, which represented approximately 34%, 24% and 17%, respectively, of the total soil fractions. Compared with CK, the NPKM treatment had a significantly decreased proportion of the large macroaggregates (>2000 μm) fraction and an increased proportion of the remaining soil fractions (2000–2 μm).

3.2. Soil fraction C and N

Differences in SOC and total N in the different particle-size fractions were very pronounced between fertilizer treatments, with SOC concentrations in the 200–63 μm fraction nearly double that of the >200 μm fraction and triple that of the 63–0.1 μm fraction (Fig. 1). The fine sand fraction (200–63 μm) had a significantly higher total N concentration than the other fractions (2000–200 μm > 2–0.1 μm >> 2000 μm > 63–2 μm). NPKM and M treatments both significantly increased total N contents compared to NPK treatment in each fraction. Significant effects of fraction and fertilizer treatments ($P < 0.001$) on C:N ratio in particle-size fractions (Table 3) followed the expected pattern, with lower values in the smaller fractions (63–2 μm and 2–0.1 μm) compared with the larger fractions (>63 μm). Organic managements (NPKM and M) were characterized by a significant decrease in the C:N ratio in the >2000 μm and 63–2 μm fractions.

3.3. Patterns of soil enzyme activities

The activities of ten enzymes showed different trends for the five particle-size fractions after the long-term application of inorganic and organic fertilizers (Table 4). Both soil fractions and fertilizer treatments had strong effects on soil enzyme activities, and their interaction also had a remarkable effect on the enzyme activities, with the only exception being α-glucosidase, where the interaction of fraction and fertilization did not affect the activity of α-glucosidase ($P = 0.0873$) as assessed by two-way ANOVA (Table 3). β-cellobiosidase activity was most affected by particle-size fractions ($F = 790.19$).

The maximum phosphatase and sulfatase activities were found in the 2–0.1 μm fraction of the organic treatments (Table 4). The β-glucosidase, β-cellobiosidase, *N*-acetyl-glucosaminidase, β-xylosidase, α-glucosidase, aminopeptidase, phenol oxidase and peroxidase activities were significantly higher in the 200–63 μm fraction than in the other fractions. The highest activities of these ten enzymes in each fraction were measured in the organic treatments (NPKM or M), with the exception of phosphatase, sulfatase, *N*-acetyl-glucosaminidase and β-xylosidase activities in the 2000–200 μm fraction which were highest in NPK treatment. Phenol oxidase and peroxidase showed minor changes in activity compared with the other hydrolytic enzymes, where the differences in activity could reach several fold among fractions.

3.4. Microbial communities within soil fractions (PLFA analysis)

We measured microbial composition via phospholipid fatty acid (PLFA) analysis on physically separated soil particle-size fractions. A total of 76 PLFA individuals were detected and used as measures of total microbial biomass and the relative abundance of specific microbial groups. Both particle-size fractions and fertilizer managements yielded large differences in total PLFA and different microbial groups, except for the ratio of G+:G−, which fertilization did not significantly affect ($P = 0.598$) and the relative abundance of bacteria ($P = 0.1521$), which were not significantly affected by the interaction of fraction and fertilization (Table 6).

Total PLFAs, which ranged from 41.70 to 205.74 nmol g^{-1}, were typically higher in the >2000 μm and 2000–200 μm fractions and lower in the 63–2 μm and 2–0.1 μm fractions (Table 5). At the same time, total PLFAs were significantly increased to varying extent by organic fertilization (NPKM and M), especially in the >2000 μm fraction, where the total PLFAs were almost 4× higher than in the control and inorganic fertilization treatments (CK and N). However, the response of concentration of total PLFAs to inorganic or organic fertilizers in the 2000–200 μm fraction was distinctly different from the responses in other fractions, where it was abundant in theNPK treatment but less abundant in the organic (NPKM and M) treatments. Relative abundance of microbial groups under six treatments in all the fractions were also calculated, which showed a tendency of

Table 2
Particle-size distribution of soils using ultrasonic fractionation under different fertilizer managements. The percentage recovery is reported for ultrasonic fractionation.

Treatments	Particle-size fractions					
	Large macroaggregates >2000 μm	Coarse sand 2000–200 μm	Fine sand 200–63 μm	Silt 63–2 μm	Clay 2–0.1 μm	Recovery
CK	25.66 ± 1.18ab	13.63 ± 0.61bc	4.95 ± 0.25b	33.94 ± 0.56bc	17.12 ± 0.71ab	95.30 ± 1.85a
N	21.82 ± 0.58b	11.90 ± 0.35c	6.23 ± 0.59ab	35.89 ± 0.45ab	19.61 ± 1.28a	96.78 ± 0.41a
NP	26.08 ± 0.44a	15.72 ± 2.21ab	5.09 ± 0.18b	31.78 ± 1.34c	16.53 ± 1.15b	95.20 ± 1.22a
NPK	27.04 ± 0.98a	12.28 ± 0.43c	4.48 ± 1.51b	34.48 ± 0.72bc	16.97 ± 0.51ab	95.25 ± 0.42a
NPKM	17.35 ± 1.95c	18.36 ± 1.13a	7.15 ± 0.33a	38.79 ± 1.05a	15.65 ± 0.49b	97.30 ± 2.08a
M	24.05 ± 0.43ab	18.13 ± 0.84a	5.61 ± 0.69ab	32.55 ± 0.97c	15.06 ± 0.65b	95.41 ± 1.27a

Data are means ± standard error, $n = 3$. Different lower case letters indicate significant differences ($P < 0.05$) among fertilizations within each fraction (Fisher's LSD test).

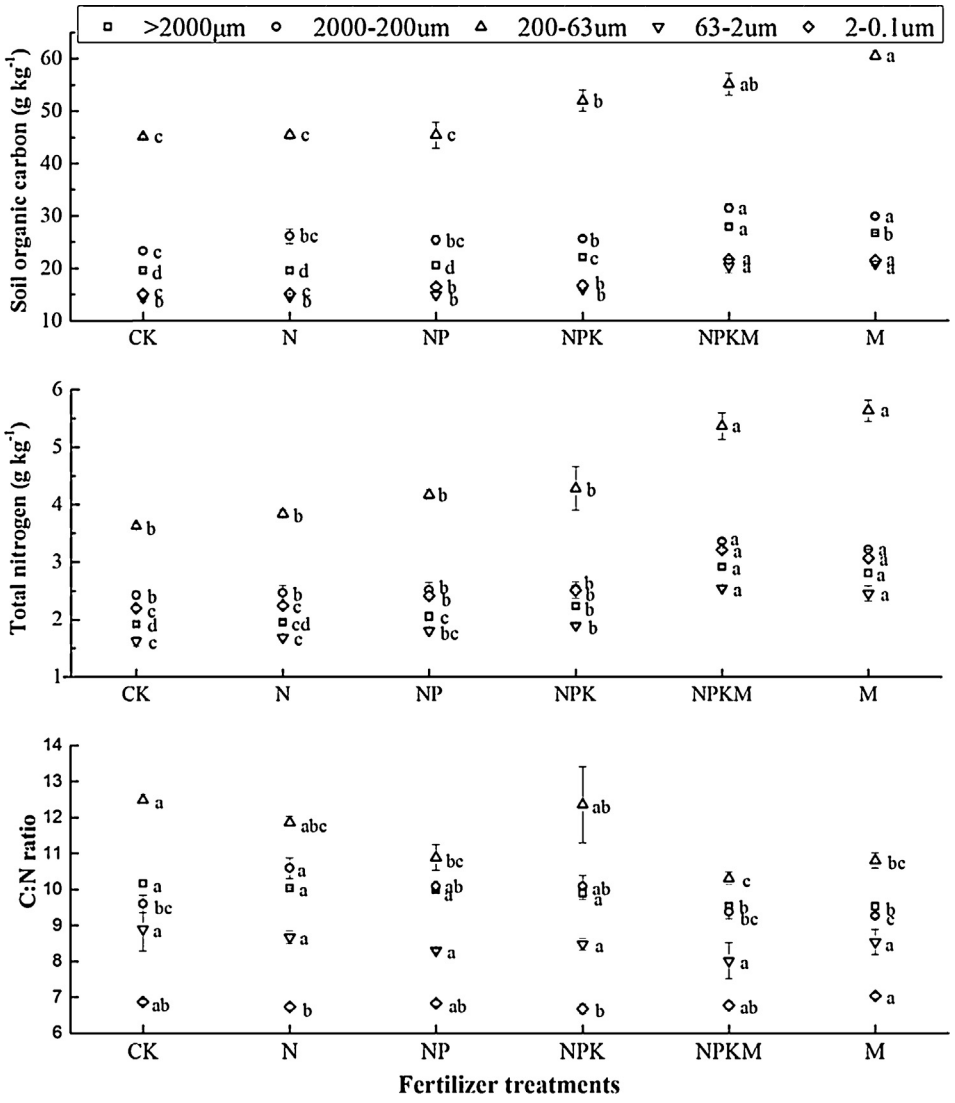

Fig. 1. Content of soil organic C, total nitrogen and C:N ratio in particle-size fractions under different treatments. Different lower case letters indicate significant differences ($P < 0.05$) among fertilizer treatments within each fraction (Fisher's LSD test).

Table 3
Two-way ANOVA analysis of soil organic carbon, total nitrogen and 10 extracellular enzymes activities in five soil particle-size fractions, six fertilizer treatments each with three replicates ($n = 90$). The data in bold indicated that enzyme was not affected by soil fractions, fertilizer treatments or their interaction ($P < 0.05$).

	Particle-size fraction		Fertilizer treatment		Particle-size fraction × Fertilizer treatment	
	F	*P*	*F*	*P*	*F*	*P*
Soil organic carbon	1382.46	<0.0001	83.70	<0.0001	3.89	<0.0001
Total nitrogen	480.39	<0.0001	106.21	<0.0001	3.14	0.0003
C:N ratio	209.37	<0.0001	6.19	<0.0001	2.10	0.0145
Phosphatase	391.48	<0.0001	60.94	<0.0001	6.67	<0.0001
Sulfatase	272.92	<0.0001	10.17	<0.0001	3.20	0.0003
β-Glucosidase	476.41	<0.0001	24.85	<0.0001	2.30	0.0069
β-Cellobiosidase	790.19	<0.0001	31.93	<0.0001	2.14	0.0126
N-acetyl-glucosamidase	504.83	<0.0001	17.65	<0.0001	3.51	<0.0001
β-Xylosidase	333.62	<0.0001	21.84	<0.0001	2.26	0.0081
α-Glucosidase	226.59	<0.0001	19.92	<0.0001	**1.58**	**0.0873**
Aminopeptidase	298.66	<0.0001	127.19	<0.0001	2.58	0.0024
Phenol oxidase	64.59	<0.0001	52.65	<0.0001	3.73	<0.0001
Peroxidase	99.57	<0.0001	83.19	<0.0001	4.23	<0.0001

bacteria > actinomycetes > fungi (Table 5). The relative abundance of bacteria and fungi were higher in larger fractions (>63 μm), while actinomycetic proportion tended to be more abundant in small fractions (<63 μm). Small changes of relative abundance of microbial groups among treatments in the <200 μm fraction were observed when compared to that of larger fraction (>200 μm). The

Table 4
Soil extracellular enzyme activities in particle-size fractions in different soils.

	Particle-size fraction				
	>2000 μm	2000–200 μm	200–63 μm	63–2 μm	2–0.1 μm
Phosphatase					
CK	648.72 ± 65.98b	1313.90 ± 18.57b	2077.85 ± 49.53c	1669.34 ± 49.00c	1816.69 ± 91.27d
N	780.52 ± 81.78b	1513.24 ± 92.86ab	2193.54 ± 45.29bc	1830.57 ± 49.01bc	1945.65 ± 60.58d
NP	1027.09 ± 60.43a	1510.05 ± 63.64ab	2236.95 ± 71.71bc	1899.90 ± 73.69bc	2837.40 ± 96.54c
NPK	1106.19 ± 37.41a	1658.11 ± 98.96a	2420.72 ± 104.01ab	2073.73 ± 53.18b	2992.80 ± 33.13bc
NPKM	1190.01 ± 56.46a	1590.23 ± 106.75a	2636.04 ± 125.45a	2419.63 ± 41.01a	3247.66 ± 22.73a
M	1163.24 ± 44.03a	1587.82 ± 49.56a	2619.97 ± 220.96a	2405.04 ± 141.13a	3148.32 ± 75.72 ab
	E	D	B	C	A
Sulfatase					
CK	13.45 ± 0.75c	56.18 ± 0.56abc	58.35 ± 3.94b	30.44 ± 2.35c	71.40 ± 0.69b
N	17.53 ± 4.09bc	59.28 ± 3.58ab	65.40 ± 1.87b	35.28 ± 2.14bc	72.46 ± 3.10b
NP	21.27 ± 0.96ab	59.78 ± 1.35ab	67.11 ± 6.20b	35.53 ± 0.28bc	81.63 ± 0.40ab
NPK	22.45 ± 1.23ab	61.31 ± 5.62a	69.04 ± 2.74b	37.81 ± 1.99bc	82.30 ± 5.24ab
NPKM	24.87 ± 1.53a	47.39 ± 0.45c	69.14 ± 1.36b	55.90 ± 2.73a	84.43 ± 7.58ab
M	22.60 ± 1.75ab	51.47 ± 2.73bc	84.80 ± 7.21a	50.75 ± 1.39a	92.01 ± 7.21a
	E	C	B	D	A
β-Glucosidase					
CK	167.69 ± 18.72c	496.41 ± 13.02b	1116.46 ± 26.68c	422.68 ± 3.27b	219.31 ± 24.13b
N	218.86 ± 61.53bc	630.65 ± 39.88a	1173.25 ± 15.69bc	430.30 ± 14.90b	220.83 ± 19.67b
NP	265.24 ± 20.50bc	651.22 ± 2.84a	1189.78 ± 52.00bc	448.43 ± 11.18b	360.41 ± 21.52a
NPK	284.78 ± 16.90ab	679.38 ± 25.35a	1203.16 ± 112.01bc	485.19 ± 12.18b	367.37 ± 19.81a
NPKM	369.17 ± 13.57a	632.82 ± 28.00a	1358.94 ± 46.67ab	740.31 ± 33.81a	412.31 ± 8.34a
M	375.39 ± 7.21a	665.15 ± 21.46a	1495.81 ± 155.35a	777.77 ± 49.40a	386.96 ± 19.11a
	C	B	A	B	C
β-Cellobiosidase					
CK	34.51 ± 1.24c	118.95 ± 11.28d	391.51 ± 5.23b	82.81 ± 1.62b	35.24 ± 6.07b
N	42.57 ± 10.81c	144.50 ± 4.79cd	411.25 ± 32.90b	84.98 ± 0.22b	37.99 ± 5.32b
NP	70.53 ± 9.44b	154.81 ± 2.41bc	418.55 ± 39.24b	92.08 ± 1.71b	58.27 ± 3.13ab
NPK	72.16 ± 2.57b	185.28 ± 6.87ab	449.50 ± 21.11b	98.59 ± 4.33b	58.47 ± 7.25ab
NPKM	101.39 ± 3.72a	193.13 ± 19.34a	522.69 ± 32.35a	171.34 ± 9.70a	73.57 ± 7.18a
M	99.97 ± 3.38a	208.77 ± 9.82a	551.21 ± 25.11a	175.58 ± 4.84a	72.08 ± 12.69a
	D	B	A	C	D
N-acetyl-glucosaminidase					
CK	54.88 ± 5.62d	204.77 ± 37.91b	480.04 ± 15.51b	130.82 ± 4.58b	100.85 ± 5.02c
N	68.52 ± 13.34cd	266.15 ± 28.25ab	521.69 ± 9.71ab	132.44 ± 4.27b	102.40 ± 14.69c
NP	94.97 ± 12.51c	266.60 ± a17.87b	528.48 ± 19.72ab	137.92 ± 5.66b	163.44 ± 8.16b
NPK	98.74 ± 6.19bc	342.28 ± 27.78a	546.50 ± 41.46ab	144.31 ± 5.70b	164.68 ± 11.96b
NPKM	137.00 ± 7.40a	224.37 ± 19.47b	561.18 ± 12.86a	261.47 ± 12.66a	198.16 ± 0.62ab
M	128.59 ± 4.72ab	237.63 ± 19.63b	585.91 ± 40.06a	260.53 ± 23.15a	220.79 ± 24.61a
	D	B	A	C	D
β-Xylosidase					
CK	24.91 ± 3.45c	98.15 ± 7.91a	206.47 ± 2.33b	123.15 ± 3.19b	41.16 ± 5.55b
N	32.83 ± 7.22bc	102.04 ± 5.83a	221.60 ± 11.55b	127.72 ± 1.63b	42.87 ± 6.08b
NP	46.13 ± 6.93b	104.39 ± 2.74a	222.40 ± 14.54b	127.83 ± 3.24b	62.53 ± 3.41a
NPK	46.50 ± 1.46b	120.59 ± 4.24a	231.86 ± 28.57b	132.54 ± 3.52b	65.82 ± 4.68a
NPKM	67.10 ± 3.73a	114.80 ± 18.89a	281.04 ± 6.20a	194.31 ± 5.20a	73.94 ± 4.79a
M	64.29 ± 1.97a	118.47 ± 23.18a	304.55 ± 25.43a	207.23 ± 15.83a	74.79 ± 3.20a
	D	C	A	B	D
α-Glucosidase					
CK	20.20 ± 2.56c	65.05 ± 1.93a	117.31 ± 1.25c	60.92 ± 1.03b	45.38 ± 4.42b
N	24.54 ± 6.81bc	65.80 ± 2.73a	125.01 ± 6.26bc	62.82 ± 1.43b	46.50 ± 4.60b
NP	32.34 ± 0.61b	67.36 ± 2.18a	129.51 ± 7.62bc	63.90 ± 0.47b	71.16 ± 4.47a
NPK	32.39 ± 1.94b	74.98 ± 5.07a	130.26 ± 17.93bc	66.49 ± 0.54b	77.37 ± 3.18a
NPKM	45.91 ± 1.15a	71.75 ± 3.10a	146.12 ± 2.24ab	96.85 ± 4.65a	85.34 ± 2.44a
M	43.61 ± 2.08a	72.40 ± 3.52a	156.71 ± 15.21a	94.58 ± 6.98a	82.24 ± 11.18a
	C	B	A	B	B
Aminopeptidase					
CK	170.61 ± 12.42c	354.94 ± 4.55b	647.96 ± 51.53b	335.74 ± 10.80b	255.78 ± 16.17b
N	178.10 ± 6.96c	358.05 ± 3.94b	683.96 ± 45.45b	338.05 ± 9.78b	256.19 ± 19.64b
NP	247.99 ± 18.49b	373.61 ± 19.32b	698.75 ± 31.86b	354.00 ± 11.88b	318.10 ± 17.55b
NPK	244.99 ± 9.50b	370.91 ± 10.90b	702.74 ± 3.97b	363.01 ± 13.41b	329.39 ± 25.58b
NPKM	475.92 ± 15.87a	526.80 ± 28.08a	979.43 ± 29.81a	672.33 ± 39.38a	593.53 ± 55.96a
M	437.56 ± 35.91a	491.81 ± 39.79a	1036.61 ± 24.34a	679.64 ± 15.05a	519.81 ± 52.30a
	D	BC	A	B	C
Phenol oxidase					
CK	1.31 ± 0.10c	2.32 ± 0.27d	5.12 ± 0.31b	6.72 ± 0.28cd	5.28 ± 0.26b

Table 4 (*Continued*)

	Particle-size fraction				
	>2000 μm	2000–200 μm	200–63 μm	63–2 μm	2–0.1 μm
N	1.34 ± 0.07c	2.30 ± 0.81d	5.27 ± 0.36b	6.63 ± 0.20d	5.63 ± 0.66b
NP	3.25 ± 0.65b	4.52 ± 0.11c	6.00 ± 0.38b	7.75 ± 0.26abc	5.94 ± 0.61b
NPK	3.05 ± 0.58b	4.88 ± 0.30bc	7.26 ± 0.78b	7.40 ± 0.19bcd	6.24 ± 0.43ab
NPKM	7.36 ± 0.09a	6.66 ± 0.72a	9.81 ± 1.54a	8.18 ± 0.28ab	7.59 ± 0.54a
M	6.79 ± 0.12a	6.18 ± 0.54ab	10.99 ± 0.21a	8.64 ± 0.56a	6.59 ± 0.20ab
	C	C	A	A	B
Peroxidase					
CK	4.82 ± 0.30b	5.10 ± 0.12c	8.71 ± 0.77b	7.72 ± 0.53c	2.97 ± 0.31d
N	5.04 ± 0.53b	5.84 ± 0.48bc	8.77 ± 0.32b	7.80 ± 0.10c	3.09 ± 0.26d
NP	5.35 ± 0.46b	5.91 ± 0.24bc	9.09 ± 1.12b	9.54 ± 0.43b	5.08 ± 0.13c
NPK	5.68 ± 0.32b	6.39 ± 0.67b	11.09 ± 1.45b	9.61 ± 0.27b	5.25 ± 0.52c
NPKM	10.86 ± 0.70a	8.90 ± 0.29a	14.84 ± 1.27a	11.20 ± 0.57a	10.25 ± 0.62a
M	10.34 ± 0.48a	8.12 ± 0.33a	17.50 ± 0.26a	10.61 ± 0.52ab	8.41 ± 0.37b
	C	C	A	B	C

Data are means ± standard error, $n = 3$. Different capital letters indicate significant differences ($P < 0.05$) among the five fractions; different lower case letters indicate significant differences ($P < 0.05$) among six treatments within each fraction (Fisher's LSD test).

Table 5
Microbial community composition of different particle-size fractions under different fertilizer managements.

Microbial PLFA composition	Treatments	Particle-size fractions				
		>2000 μm	2000–200 μm	200–63 μm	63–2 μm	2–0.1 μm
Total PLFAs (nmol g^{-1})	CK	62.38 ± 2.92c	157.39 ± 7.15b	103.82 ± 6.25a	44.64 ± 6.80b	47.85 ± 4.06c
	N	64.19 ± 4.02c	159.12 ± 9.85b	94.81 ± 5.93a	41.70 ± 4.59b	50.37 ± 2.29c
	NP	150.82 ± 6.11b	169.37 ± 4.97ab	111.85 ± 6.83a	47.19 ± 2.48ab	65.07 ± 12.23bc
	NPK	172.10 ± 4.22b	188.70 ± 6.10a	116.20 ± 5.05a	45.03 ± 6.99b	65.31 ± 10.28bc
	NPKM	205.74 ± 9.73a	112.73 ± 3.01c	99.58 ± 13.07a	52.66 ± 5.92ab	94.92 ± 5.77a
	M	202.87 ± 9.23a	101.40 ± 2.30c	112.34 ± 2.95a	61.64 ± 1.22a	90.54 ± 9.06ab
		AB	A	BC	D	CD
Bacterial PLFA (mol %)	CK	60.10 ± 0.63d	57.93 ± 0.73a	61.66 ± 1.11a	57.84 ± 0.62a	57.97 ± 0.70bc
	N	62.01 ± 1.38cd	59.43 ± 0.43a	61.65 ± 2.16a	55.65 ± 0.44a	57.01 ± 0.29c
	NP	64.63 ± 0.17b	60.06 ± 1.23a	64.57 ± 0.28a	57.72 ± 1.00a	60.91 ± 0.44ab
	NPK	63.63 ± 1.44bc	59.06 ± 0.18a	60.59 ± 1.43a	56.68 ± 1.39a	61.33 ± 1.49a
	NPKM	67.01 ± 0.67a	60.68 ± 0.89a	61.14 ± 1.65a	58.07 ± 0.52a	60.53 ± 0.57ab
	M	65.33 ± 1.11ab	59.92 ± 1.75a	59.99 ± 2.57a	56.71 ± 1.24a	59.90 ± 1.39abc
		A	C	B	D	C
Fungal PLFA (mol %)	CK	2.33 ± 0.05c	8.51 ± 0.12a	2.76 ± 0.16ab	1.99 ± 0.14b	2.02 ± 0.09c
	N	2.28 ± 0.05c	8.88 ± 0.51a	2.81 ± 0.17ab	1.76 ± 0.08b	1.96 ± 0.11c
	NP	7.98 ± 0.38b	9.27 ± 0.33a	2.76 ± 0.14ab	2.19 ± 0.32ab	1.97 ± 0.09c
	NPK	8.02 ± 0.07b	9.34 ± 0.14a	2.55 ± 0.08b	1.96 ± 0.08b	2.15 ± 0.08bc
	NPKM	8.84 ± 0.27a	2.66 ± 0.43b	2.95 ± 0.16ab	2.76 ± 0.33a	2.39 ± 0.17ab
	M	8.86 ± 0.29a	2.27 ± 0.11b	3.03 ± 0.13a	2.39 ± 0.13ab	2.68 ± 0.08a
		A	A	B	B	B
Actinomycetic PLFA (mol %)	CK	17.55 ± 0.24a	13.23 ± 0.32b	15.88 ± 0.09a	18.29 ± 0.51a	18.26 ± 0.94a
	N	17.74 ± 0.58a	13.53 ± 0.07b	16.07 ± 0.84a	17.18 ± 0.19ab	16.93 ± 0.24ab
	NP	15.21 ± 0.67b	14.22 ± 0.48b	16.91 ± 0.22a	18.23 ± 0.53a	17.70 ± 0.02ab
	NPK	14.19 ± 0.14b	13.62 ± 0.10b	15.32 ± 0.27a	17.27 ± 0.53ab	16.76 ± 0.22b
	NPKM	15.71 ± 0.22b	16.50 ± 0.18a	16.69 ± 0.72a	18.11 ± 0.25a	17.57 ± 0.12ab
	M	15.35 ± 0.71b	16.41 ± 0.46a	15.74 ± 0.50a	16.52 ± 0.45b	16.94 ± 0.25ab
		B	C	B	A	A

Data are means ± standard error, $n = 3$. Different capital letters indicate significant differences ($P < 0.05$) among the five fractions; different lower case letters indicate significant differences ($P < 0.05$) among treatments within each fraction (Fisher's LSD test).

relative abundance of fungi in 2000–200 μm fraction showed higher in inorganic treatments, while that of actinomycetes showed higher in organic treatments. The results of fungi and actinomycetes in >2000 μm fraction were in contrary with that of 2000–200 μm fraction. Significant increase of gram–positive bacterial relative abundance in the 2000–200 μm fraction of organic treatments (Fig. 2A) and significant decrease of gram-negative bacterial proportion (Fig. 2B) could explain the significant increase in the G+:G− ratio in the organic treatments compared with the inorganic treatments (Fig. 2C). Additionally, relative abundance of gram-negative bacterial was excessively low in the 63–2 μm fraction, which resulted in a remarkable increase in the G+:G− ratio in this fraction. We should note that the fungi: bacteria ratios of the >2000 μm and 2000–200 μm fractions showed the opposite tendency with the G+:G− ratios.

Principal component analysis (PCA) was conducted with PLFAs that were presented in the five particle-size fractions. PC1 and PC2 accounted for 49.70% and 13.10% of the total variation, respectively. PC scores on these axes were well separated on the basis of soil fractions and fertilizer treatments and the 30 plots

Table 6
Two-way ANOVA analysis of microbial groups in five soil particle-size fractions, six fertilizer treatments each with three replicates ($n = 90$). The data in bold indicated that microbial group was not affected by soil fractions, fertilizer treatments or their interaction ($P < 0.05$).

	Particle-size fraction		Fertilizer treatment		Particle-size fraction × Fertilizer treatment	
	F	*P*	*F*	*P*	*F*	*P*
Total PLFA	240.96	<0.0001	26.83	<0.0001	23.85	<0.0001
Bacteria	28.17	<0.0001	4.34	0.0020	**1.41**	**0.1521**
Fungi	710.76	<0.0001	39.06	<0.0001	110.17	<0.0001
Actinomycetes	45.92	<0.0001	6.63	<0.0001	5.31	<0.0001
G+/G−	546.9	<0.0001	**0.74**	**0.5980**	5.46	<0.0001
Fungi/bacteria	588.87	<0.0001	30.3	<0.0001	92.76	<0.0001

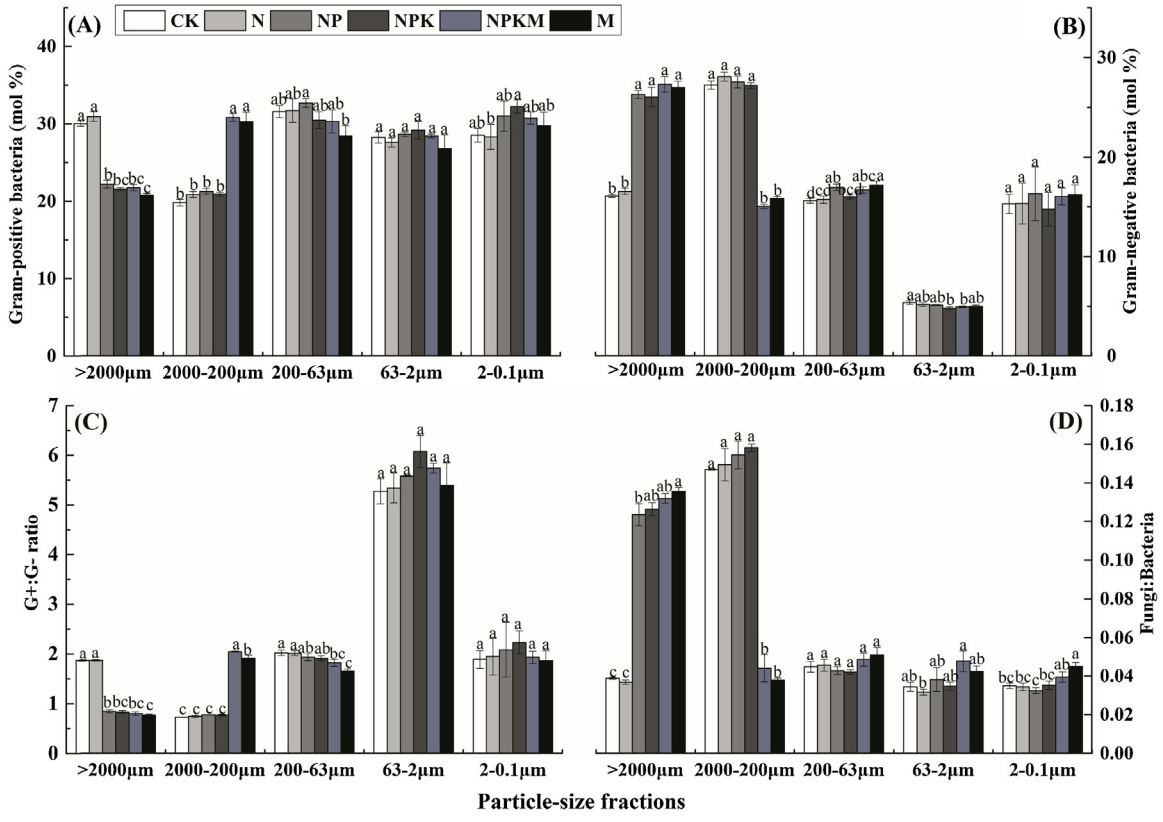

Fig. 2. Comparisons of Gram-positive bacteria (A); Gram-negative bacteria (B); G+:G− ratio (C) and Fungi: bacteria ratio (D). Vertical bars represent the S.E. ($n = 3$) and lower case letters indicate significant differences among fertilizer treatments ($P < 0.05$). G+:G− = Gram-positive bacteria:Gram-negative bacteria ratio.

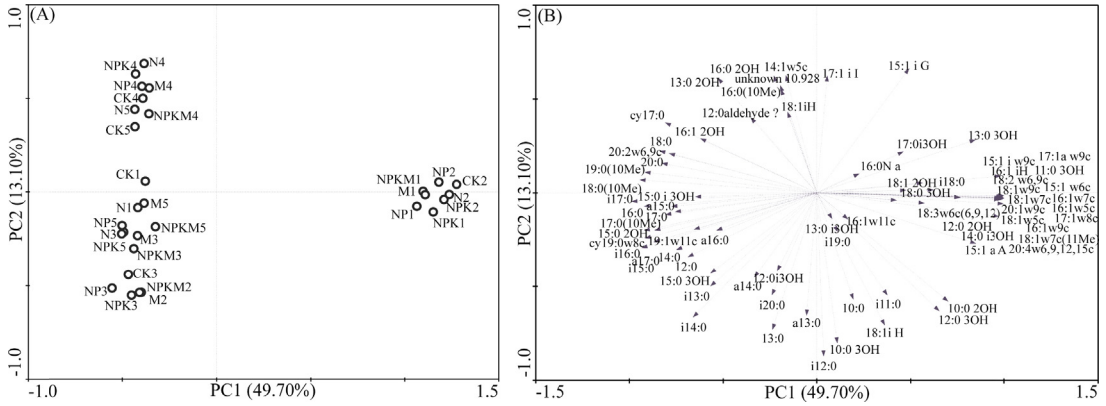

Fig. 3. Plot of the first two principle components (PC1 and PC2) grouped in six fertilizer treatments and five particle-size fractions (A) and plot of two principle components among PLFA individuals (B) from all the fractions. Numbers (1–5) following the treatment indicate the particle sizes of >2000 μm, 2000–200 μm, 200–63 μm, 63–2 μm, 2–0.1 μm, respectively.

were divided into three sections (Fig. 3A). Most of the >2000 μm and 2000–200 μm fractions were well separated along PC1. The 63–2 μm fractions and CK and N treatments of 2–0.1 μm fractions were separated as one group along PC2. All of the 200–63 μm fractions, the inorganic treatments of the >2000 μm and the other treatments of 2–0.1 μm fractions were grouped against PC1 by the principal component analysis. For PLFAs from all the fractions, the proportions of monounsaturated fatty acids (15:1ω6c, 16:1ω5c, 16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c, 18:1ω7c and 18:1ω9c) and polyunsaturated fatty acids (18:3ω6c (6,9,12), 18:2ω6,9c and 20:4ω6,9,12,15c) increased in the larger fractions (>200 μm). The proportions of saturated fatty acids (14:0, 17:0, 18:0, a15:0, a16:0, a17:0, i13:0, i14:0, i15:0, i16:0, i17:0), cyclopropane fatty acids (cy17:0 and cy19:0ω8c) and methyl branched fatty acids (16:0 (10Me), 17:0 (10Me), 18:0(10Me) and 19:0(10Me)) (biomarkers of actinomycetes) increased against PC1 (Fig. 3B).

3.5. Correlations

The redundancy analysis (RDA) was carried out for enzyme activities and soil properties of soil fractions of the six treatments. Soil properties were used as environmental variables (Fig. 4). The first and second axes accounted for 60.00% and 11.90% of the total variation between enzyme activities and soil C and N pools. The enzyme activities showed significant correlation with SOC ($F = 3.651$, $P = 0.016$), total N ($F = 37.229$, $P = 0.002$) contents and C:N ratio ($F = 10.084$, $P = 0.002$).

Results of the RDA between soil properties and microbial community composition are shown in Fig. 5. The first and second axes accounted for 24.40% and 4.50%, respectively, of the total variation in microbial community composition and structure. The microbial community composition was significantly correlated with total N content ($F = 4.386$, $P = 0.024$) and C:N ratio ($F = 3.771$, $P = 0.034$), which explained 11.40% and 11.90% of total variance, respectively. Conversely, SOC ($F = 1.845$, $P = 0.196$) had no significant correlation with microbial community composition of different particle-size fractions of the six fertilizer treatments.

The RDA showed the relationships of soil enzyme activities with microbial community composition (Fig. 6), and the first and second axes accounted for 54.10% and 12.90%, respectively, of the total variation. Soil enzyme activities, including phosphatase ($F = 5.416$, $P = 0.018$), sulfatase ($F = 6.094$, $P = 0.016$), *N*-acetyl-glucosaminidase ($F = 9.317$, $P = 0.004$), β-cellobiosidase ($F = 9.097$, $P = 0.006$) activities of different soil fractions were significantly correlated with microbial community after long-term fertilization. However, peroxidase ($F = 1.851$, $P = 0.168$), β-xylosidase ($F = 2.769$, $P = 0.074$), phenol oxidase ($F = 0.642$, $P = 0.438$), β-glucosidase ($F = 0.904$, $P = 0.37$), aminopeptidase ($F = 0.618$, $P = 0.49$) and α-glucosidase ($F = 0.429$, $P = 0.582$) were not significantly correlated with microbial community after long-term fertilization.

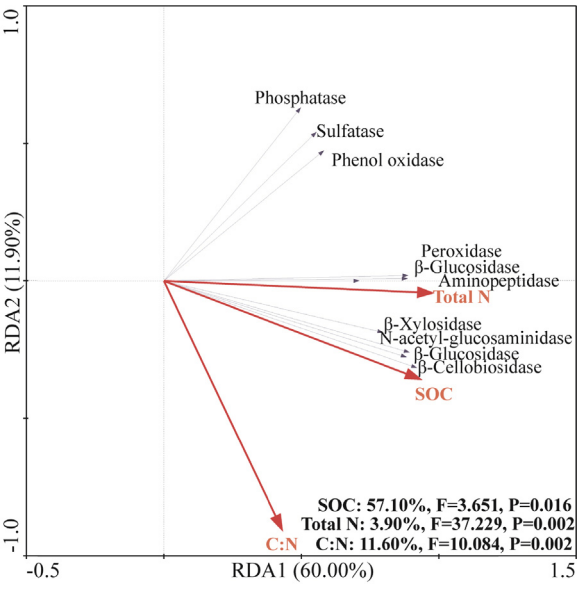

Fig. 4. Correlations of soil enzyme activities to soil properties as determined by redundancy analysis (RDA) and corresponding explained proportion of variability was shown in the lower right corner.

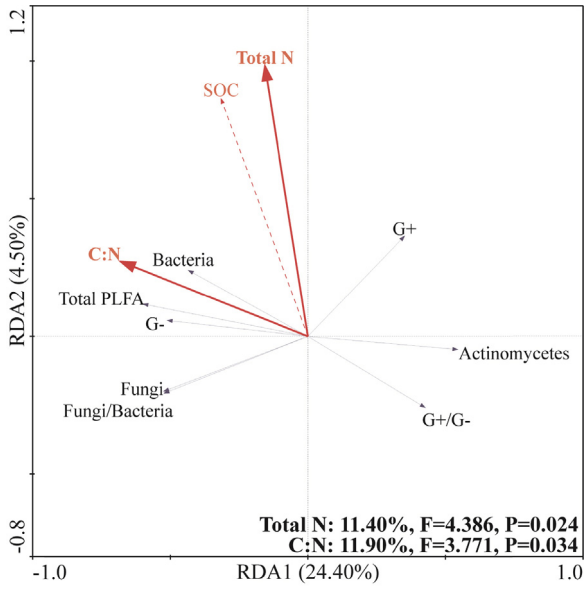

Fig. 5. Correlations of soil properties and microbial community composition indicated by PLFA groups as determined by redundancy analysis (RDA) and corresponding explained proportion of variability was shown in the lower right corner.

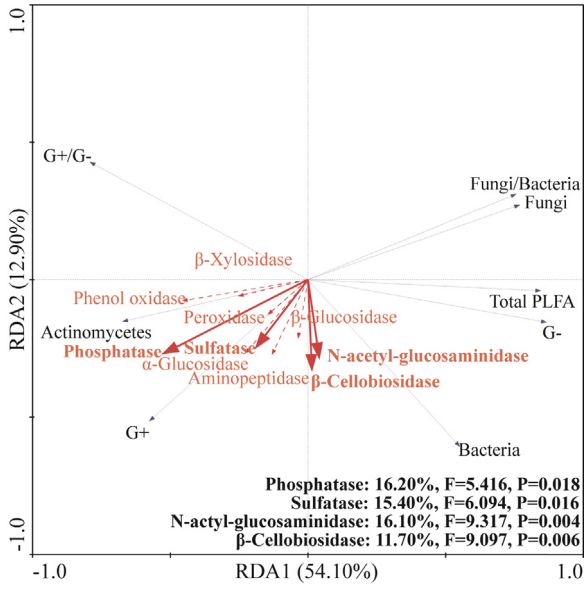

Fig. 6. Correlations of soil enzyme activities and microbial community composition indicated by PLFA groups as determined by redundancy analysis (RDA) and corresponding explained proportion of variability was shown in the lower right corner.

4. Discussion

This study explored the effects of inorganic fertilizers and organic amendment on the relationships between soil microbial community composition, enzyme activities and soil C and N pools in different soil particle fractions of yellow–brown paddy soil in the Yangtze Plain region of China. We investigated soil particle-size fractions by ultrasonic fractionation. Consistent differences in biological characteristics and elemental chemistry among soil fractions and across fertilizer managements indicated that the physical fractions represented different C and N pools with potentially different turnover rates, which could lead to changes in soil biological processes. Significant benefits of organic fertilizer application were also observed. However, the drivers for the structure and activity of microbial communities appear to differ depending on the spatial scale of each experiment (Martiny et al., 2011), this is why large scale studies are required to evaluate predictors of microbial community composition and structure.

4.1. Fertilizer effects on soil particle-size fractions and nutrient pools

It is well known that the addition of easily decomposable substrates to soil rapidly stimulates the soil microflora and determines the proportions of different particle-size fractions, resulting in a significant improvement in soil structure (De Gryze et al., 2005; Abiven et al., 2007). In this experiment, this is confirmed by the increased proportion of the 2000–2 μm fraction and the decreased proportion of the >2000 μm and 2–0.1 μm fractions in the organic treatments (NPKM and M) (Table 2). Better aggregation contributes to C storage by creating a more complex soil structure and limiting C accessibility to decomposers (Smith et al., 2014).

Silveira (2005) stated SOC balance was mainly determined by the net result of carbon input and decomposition rates in soil. Our results also revealed that 33 years of organic fertilizer application (NPKM and M) to the yellow–brown paddy soil significantly increased SOC and total N contents across all the fractions. This confirmed that appropriate use of manures and/or crop residues within management systems can enhance soil microbial biomass, activity and diversity (Mandal et al., 2007), which can also increase the levels of soil nutrients in turn. At the microenvironment scale, coarse sand fractions are characterized by high concentrations of labile C and N originating predominately from plant residues, whereas silt and clay fractions are usually characterized by high concentrations of relatively stable organic C and N (Elliott, 1986; Six et al., 2000). Moreover, high SOC contents are usually found in silt and clay fractions, whereas low contents are observed in the sand fractions of most soils, such as in Calcaric Phaeozems (Kandeler et al., 2000), Calcic Chernozems and Cambisols (Stemmer et al., 1998) and Humic Dystrudepts (Chiu et al., 2006). In the present study, however, we detected the highest SOC concentrations in fine sand fraction (200–63 μm), followed by the coarse sand fraction, which generally agrees with previous findings in Hydragric Anthrosol in which crop residues were returned to the paddy soil (Jiang et al., 2011). It has been demonstrated that the organic C (fresh or labile) derived from crop residues is first incorporated into the coarse sand fraction during the initial decomposition period and subsequently accumulates and becomes stable in silt or clay soils (Angers et al., 1997; Six et al., 2000). Consequently, it is possible that higher fresh SOC contents could first accumulate in larger fractions, particularly in soils receiving large amounts of crop residues (Chen et al., 2014). The C:N ratio indicates the degradation of fresh plant residues, which is important in the process of C sequestration (Potter et al., 1998). Organic particles of >200 μm, which consist predominantly of particulate residues from plant material, decompose very rapidly, while the organic material in smaller size fractions is characterized by an increase in the degree of humification and a decrease in the C:N ratio (Kanazawa and Filip, 1986). It is possible that smaller fractions contain carbon and nitrogen that has undergone more degradation than the macroaggregate fraction and therefore have comparatively less diversity occluded in the microaggregate fractions contributing to the C:N ratio (An et al., 2010). This may explain the relatively high C:N ratio in the >63 μm fraction and the low C:N ratio in the 63–0.1 μm fraction.

4.2. Fertilizer effects on soil extracellular enzyme activities in soil particle-size fractions

Extracellular enzymes are unevenly distributed through the soil, and depending on their location, they may be more or less sensitive to environmental changes (Nannipieri et al., 2002). Soil particle-size fractionation is one way of dividing the bulk enzyme activity into functionally meaningful components, which is particularly useful when investigating effects of long-term management on soil enzymes (Stemmer et al., 1999). The techniques used for soil fractionation are diverse and, as a consequence, the quality and numbers of fractions generated are variable (Kandeler et al., 1999). It is therefore difficult to make comparisons between experiments. Most fractionation techniques include steps for aggregate disruption and wet sieving. As a result of these procedures, enzymes previously located in the soil solution or detached during the fractionation process may be washed out and lost from the analysis. Only enzymes that were previously immobilized (adsorbed on organic matter or clay minerals, incorporated into humic matter, or associated with living microorganisms) remain in the assay (Perez Mateos and Gonzalez Carcedo, 1985).

The general pattern of soil enzyme activities is often dominated by the amount and quality of organic substances as well as by various physical and chemical protection mechanisms (Allison and Jastrow, 2006; Nannipieri et al., 2012; Lagomarsino et al., 2012). Our findings affirmed this observation that the majority of the variation in potential enzyme activities could be explained by soil characteristics related to nutrient availability, which are well known to be strongly influenced by fertilizer managements (Table 3). In the present study, the highest enzyme activities were observed in the 200–63 μm fraction of NPKM or M treatments, except for phosphatase and sulfatase which were highest in the 2–0.1 μm fraction. The 200–63 μm sized fractions of NPKM and M soils both contained higher SOC and total N contents than the other fractions. Long-term application of organic manures expectedly contributed to increased enzyme activities with the exception of phosphatase, sulfatase and β-xylosidase, which showed higher activities in the 2000–200 μm fraction in the NPK treatment. This phenomenon probably occurred because those enzymes were predominantly associated with the larger fractions and therefore poorly protected during the fractionation procedure, especially under the organic treatments (Qin et al., 2010). Strong effects of soil fractions and fertilizations on activities of all tested enzymes were determined (Table 3). The results further showed that the enzyme activities of soil fractions were differentially altered by fertilization. Additionally, RDA was carried out to evaluate the correlation between soil enzyme activities and soil properties (Fig. 4). The cosine of the angle between the soil enzyme activity and soil property shows the type of relationship between these factors. SOC, total N and the ratio of C:N all showed significant positive correlations. Therefore, our results were in agreement with previous conclusions that long-term compost amendment could increase SOC by increasing organic C in all fractions, thus changing the soil enzyme activities of different soil fractions (Yu et al., 2012). Differences in enzyme activity can also depend on the

type of humic compounds in soil (Benitez et al., 2005; Nannipieri et al., 2002).

4.3. Fertilizer effects on microbial community composition in soil particle-size fractions

Consistent trends in microbial compositional shifts and C and N pools among fertilizer treatments across different particle-size fractions further supported the strong relationships between microbial community composition and substrate microsite heterogeneity. Balanced fertilizer applications, especially with organic manure addition, enhanced the microbial biomass as reflected by total PLFA as well as PLFAs associated with specific functional groups (Table S1). This trend appeared to correspond to the significantly higher concentrations of SOC and total N under organic treatments. Total PLFAs were typically higher in the >2000 μm and 2000–200 μm fractions and lower in the 63–2 μm and 2–0.1 μm fractions. During the research of soil bonding agents, Chatterjee and Jain (1970) found microbial agents contributed to the formation of macroaggregates (2000–200 μm) and the important agents of microaggregate (<200 μm) were sesquioxides of iron and aluminum. While the abundance of PLFAs in the 2000–200 μm fraction was found to be significantly higher than in the smaller fractions, the application of organic applications treatments significantly decreased the abundance of the statistical PLFA groups in the 2000–200 μm fraction ($P < 0.05$) (Table S1). Previous studies found that the coarse sand fraction (2000–200 μm) was always characterized by high concentrations of labile C and N originating predominantly from plant residues and the relatively loose structure under organic treatments, which may cause active microorganisms to be easily washed away during the fractionation procedure (Qin et al., 2010; Chen et al., 2014). Additionally, bacteria dominate in rice paddy and may compete with fungi for substances, resulting in greater stress on the fungal community, which is convinced by the significant lower relative abundance of fungi under organic treatments in Table 5. The significant reduction in the fungi: bacteria ratio in the 2000–200 μm fraction of the organic treatments compared with the NPK treatments was not surprising.

Reduced fungal abundance with decreasing soil aggregate or particle size has been documented across soil and vegetation types (Kandeler et al., 1999, 2000; Poll et al., 2003; Schutter and Dick, 2002) and has been attributed to decreased bioavailability of C substrates in the smaller size fractions (Briar et al., 2011; Chiu et al., 2006). The higher relative abundance of fungi in the larger aggregates is likely because of more favorable substrate properties (Huygens et al., 2008), higher C:N ratios, which are known to favor fungal colonization (Bossuyt et al., 2005; Eiland et al., 2001; Six et al., 2006; Waring et al., 2013), and restricted access to smaller pore sizes (Heijnen and Van Veen, 1991; Six et al., 2006). Even though the highest C:N ratio in the 200–63 μm fraction did not result in the highest fungal abundance, the relatively higher C:N ratio in the >200 μm fraction compared with the 63–0.1 μm fraction was in accord with the trend described above. Fungi may preferentially colonize larger soil fractions as they have been shown to grow well in soils with high porosity and low bulk density, which are facilitated by a well-developed soil structure (Harris et al., 2003). The smaller C:N ratios for the silt and clay-sized fractions (63–2 μm and 2–0.1 μm) also suggested differences in the degree of alteration of SOM and the preferential enrichment of microbial products and microbially-processed SOM in mineral-associated fractions.

Conversely, bacterial enrichment is often reported for the smallest size fractions because of the favorable bacterial microclimate (Ranjard and Richaume, 2001). We found distinct groups of bacteria dominated the different aggregate fractions. Specifically, gram-positive bacteria were more abundant than gram-negative bacteria in silt and clay fractions, and gram-negative bacteria were more abundant in the larger fractions (>200 μm). These differences were consistent with the general understanding of gram-positive and gram-negative substrate preference and observed C:N ratios of the different particle-size fractions. Gram-negative bacteria preferentially use fresh plant inputs as C sources, whereas gram-positive bacteria are thought to favor older and more microbially-processed SOM (Fierer et al., 2003; Kramer and Gleixner, 2006; Potthast et al., 2012). The greater G+:G− ratios in the silt and clay sized fractions compared with the larger fractions suggested that the silt and clay fractions are depleted in easily-decomposable substrate. Studies have shown that a higher fungi:bacteria ratio can reflect the relative abundance of the microbial population, which is an important indicator of a stronger soil ecosystem buffering capacity and more sustainable land use (Bossio et al., 1998; De Vries et al., 2006). In the present study, relatively lower G+:G− ratio and higher fungi:bacteria ratios were found in the larger fractions (>2000 μm and 2000–200 μm), which indicated better soil conditions and stronger ecosystem buffering capacity for crops.

PCA plots showed that the relatively more diverse microbial community in the larger fractions (>200 μm) and the relatively less diverse microbial community in the smaller fractions (63–2 μm) were well separated. The microbial communities of particles of 200–63 μm and most 2–0.1 μm treatments together with organic treatments of 2000–200 μm were separated into one group. As mentioned previously, enzyme activities and microbial abundance were significantly decreased in the 2000–200 μm fractions when compared with other fractions and this phenomenon was confirmed by the PCA plots. As shown in Fig. 3A, the 2000–200 μm fractions of the NPKM and M treatments were grouped with the 200–63 μm and 2–0.1 μm fractions instead of being grouped with the >2000 μm and 2000–200 μm fractions of the other treatments along PC1. PCA analysis also indicated fatty acids, which mostly were biomarkers of gram-negative bacteria and fungi, were positively correlated with PC1and strongly affected >2000 μm and 2000–200 μm fractions. This result was convinced by the higher relative abundance of fungi and gram-negative bacteria of organic treatments of >2000 μm and inorganic treatments of 2000–200 μm fraction. Fatty acids, which mostly represented gram-positive bacteria and actinomycetes, were negatively correlated with PC1. The treatments, affected by gram-positive bacteria and actinomycetes in PCA plot (Fig. 3A), contained higher proportion of these microbial groups (Table 5 and Fig 2A).

RDA was also carried out to detect the relationships between soil properties, enzyme activities and microbial community composition (Figs. 5 and 6). Total N, C:N ratio, phosphatase, sulfatase, *N*-acetyl-glucosaminidase and β-cellobiosidase activities were significantly correlated with microbial community composition after long-term fertilization, whereas no significant correlations were detected between SOC, phenol oxidase, β-xylosidase, peroxidase, β-glucosidase, aminopeptidase and α-glucosidase activities with microbial community composition. Ling et al. (2014) stated that these enzyme activities might be affected by specific functional microbial communities rather than by the overall microbial community. It is worth noting that phosphatase, sulfatase and *N*-acetyl-glucosaminidase activities, which were significantly related to the microbial community composition, were lower in the organic treatments but higher in the NPK treatment of 2000–200 μm fraction. This may partly explain the decline in microbial biomass in the 2000–200 μm fraction of the organic treatments. The consistent changes again confirmed the strong relationship between enzyme activities and microbial communities.

The high variability in results among studies may be because of soil-specific properties such as mineralogy and differences in soil C and nutrient content (Chiu et al., 2006), or differences in the methods used to characterize microbial biomass and community composition. Most studies on the relationship between soil physical fractions and microbial communities have been conducted in temperate, agricultural soils, so their applicability to other ecosystems is limited. Further work across environmental gradients and a diversity of soil types will increase understanding of the relationship between soil structure and microbial biomass, composition and, ultimately, function.

5. Conclusion

Soil particle-size fractions and fertilizer regimes individually and interactively ($P < 0.05$) impacted soil carbon and nitrogen contents, enzyme activities and microbial communities of yellow–brown paddy soil in China. The long-term application of organic fertilizer contributed to the improvement of soil C, N contents and most enzyme activities. The highest SOC, total N and activities of most enzymes were existed in 200–63 μm fraction. Larger fractions (>63 μm) held higher microbial abundance and better soil conditions for microorganisms than smaller sizes as reflected by phospholipid fatty acid analysis. Strong relationships between total N, C:N ratio, phosphatase, sulfatase, *N*-acetyl-glucosaminidase and β-cellobiosidase activities with soil microbial community composition were also found. Thus, we recommend soil fractionation as a promising approach that offers potential in analyzing the relationship between soil functional diversity and soil fertility. Nevertheless, further information is necessary about functions of the observed uncultured bacteria and the relationship between functional microbial activity and enzyme activities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2015.05.005.

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