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Fatty-Acid Profiles and Enzyme Activities in Soil Particle-Size Fractions under Long-Term Fertilization

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A long-term experiment was established in 1981 to examine the influence of mineral and organic fertilizer on soil organic carbon (SOC), total nitrogen (TN), enzyme activities, and microbial community composition. In this study, we considered 33 yr of the following fertilizer treatments: no fertilizer (control, CK), fertilizer N (N), fertilizer N and P (NP), fertilizer N, P and K (NPK), manure plus fertilizer N, P and K (NPKM), and manure (M). We focused on yellow-brown paddy soil and its particle-size fractions of >2000 μm (large macroaggregate sized), 2000–200 μm (coarse sand sized), 200–63 μm (fine sand sized), 63–2 μm (silt sized), and 2–0.1 μm (clay sized). Nutrient concentrations and enzymes, affected by fertilizer treatment and particle fraction, were unevenly active throughout the soils which showed significantly highest concentration and activity in the fine sand fraction, except sulfatase (Sul). However, the coarse sand fraction contributed the largest SOC, TN, and enzyme pools to bulk soil, followed by silt-sized and large macroaggregate-sized fractions. Compared with NPK, NPKM, and M treatments significantly improved SOC, TN, phosphatase (Pho), β-glucosidase (βG), β-cellobiohydrolase (βCB), N-acetyl-glucosaminidase (NAG), β-xylosidase (βX), phenol oxidase (PhOx), peroxidase activities, and the total phospholipid fatty acids (PLFAs) abundance of soil fractions. Manure also accelerated SOC, TN, and most enzymes accumulation in coarse sand fraction at the expense of clay fraction. Principal component analysis (PCA) of microbial community composition showed a smaller variability in particle-size fractions than treatments which suggested a considerable effect of soil nutrient availability on microbial community composition. Redundancy analysis (RDA) also convinced SOC, TN, C/N ratio, α-glucosidase (αG), Sul, βG, βCB, and PhOx activities significantly governed microbial community in this study. Our results conveyed long-term application of organic fertilizers contributed to the increase of SOC, TN, and most enzyme activities in bulk soil and particle fractions, along with abundant and diverse microbial community in fine sand fraction and other organic treated soil fractions.

Abbreviations: αG, α-glucosidase; βCB, β-cellobiohydrolase; βG, β-glucosidase; βX, β-xylosidase; AMC, 7-amino-4-methylcoumarin; CK, control with no fertilizer; FAMES, fatty acid methyl esters; L-DOPA, L-3, 4-dihydroxyphenylalanine; LAP, L-leucine aminopeptidase; M, manure; MUB,4-methylumbelliferyl; NAG, N-acetyl-glucosaminidase; N, fertilizer nitrogen; NP, fertilizer N and P; NPK, fertilizer NPK; NPKM, manure plus fertilizer N, P, and K; Perox, peroxidase; Pho, phosphatase; PhOx, phenol oxidase; PLFA, phospholipid fatty acid; RDA, redundancy analysis; SOC, soil organic carbon; Sul, sulfatase; TN, total nitrogen.

Core ideas

- **Organic treatments significantly enhanced soil organic C, total N, and most enzymes activities.**
- **The highest soil organic C, total N, and enzyme activities were existed in 200- to 63-μm fraction.**
- **Soil organic C, total N, C/N ratio, α-glucosidase, sulfatase, β-glucosidase, β-cellobiohydrolase, and phenol oxidase activities were significantly correlated with phospholipid fatty acids.**

The rice (*Oryza sativa* L.)–wheat (*Triticum aestivum* L.) rotation is the major cropping system in South Asia and China, occupying about 13.5 million ha in South Asia and about 4.5 million ha in China (Ladha et al., 2003; Dawe et al., 2004). This crop rotation, providing stable food grains for more than 20% of the world's population (Kumari et al., 2011), is extremely

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important for the food security of Asia and China (Jing et al., 2010). The interchange between cultivation of rice and wheat leads to anoxic conditions when soils are flooded during most of the rice-growing season and aerobic conditions when soils are drained during the wheat-growing season. Thus, nutrient cycling induced by microbial activities are relatively more diverse and complex in this variable environment (Ge et al., 2008).

Soil provides a heterogeneous environment for microorganisms, with a non-uniform distribution of organic matter, N, and other substrates (Balser et al., 2006; Six et al., 2000). Soil particles determine soil type and affect the pore size and the ability of soil to absorb water and organic matter, creating diverse habitats with characteristic microclimatic conditions (Stemmer et al., 1998). Previous research has reported that SOC and TN associated with sand fractions were more sensitive to fertilization management than those associated with silt- and clay-sized fractions (Liang et al., 2014). Generally, coarse sand is characterized by a high C/N ratio, indicating low rates of decomposition, whereas clay particles are dominated by microbially derived metabolites and characterized by a low C/N ratio, reflecting a high degree of humification of organic matter (Christensen, 2001; Gerzabek et al., 2006).

The SOC and TN are considered important indicators of soil fertility and productivity (Ai et al., 2013). However, their losses or gains are difficult to measure directly over short-term periods because of their generally high background stocks, small annual changes, and natural variability (Bosatta and Ågren, 1994). In contrast, enzyme activities, which are crucial for biological and biochemical processes and can serve as proxies for organic matter degradation and litter mineralization (Badiane et al., 2001), respond much more quickly to changes in agricultural practices (Ng et al., 2014). Usually, readily degradable substrates such as cellulose or proteins are depolymerized mainly by hydrolases, whereas oxidative enzymes are involved in the decay of recalcitrant compounds (Burns et al., 2013). For instance, phenol oxidase is involved in lignin degradation and plays an important role in soil C stabilization by favoring humic substance formation through the catalysis of phenol oxidation reactions (Weand et al., 2010). β-Cellobiohydrolase breaks down cellulose into cellobiose and βG hydrolyzes polymers of plant residues such as cellobiose. The scientific literature is replete with studies about the effects of fertilizers on soil enzyme activities, but frequently at the bulk soil scale. Little attention has been given to understanding the soil mechanism properties at the particle scale, which might manifest quite differently, but have a considerable influence on the bulk soil properties (Das et al., 2014). Therefore, enzymes involved in C, N, P, and S cycling in different particle-size fractions should be considered to reveal the spatial heterogeneity and localization of extracellular enzyme to improve our understanding of biochemical processes after application of different fertilizers.

Soil microbial communities are sensitive to fertilization and the responses of microbial communities to manure and/or mineral fertilizer in bulk soils have been well studied in the literature (Lazcano et al., 2013). Now ecologists are also looking to soil particles as a means to detect differences in soil microbial communities and activities at the microscale. Wet sieving is the accepted tool for isolating different soil fractions for biological analyses including extracellular enzyme assays (Allison and Jastrow, 2006; Lagomarsino et al., 2009), terminal restriction fragment length polymorphism (Mummey and Stahl, 2004), phospholipid fatty acid analysis (PLFA; Kong et al., 2011), and pyrosequencing (Davinic et al., 2012). Sessitsch et al. (2001) found that bacterial diversity in soils from temperate climatic regions was highest in the clay fraction while the sand and silt fractions were mainly colonized by fungi. Some authors have also reported decreasing fungal abundance (Chiu et al., 2006; Huygens et al., 2008) and fungi/bacteria ratio with decreasing particle-size (Kandeler et al., 2000; Briar et al., 2011), whereas others have found no change in this ratio between different soil physical fractions (Huygens et al., 2008; Chiu et al., 2006). The high variability in results among studies could be attributed to soil-specific properties such as mineralogy and differences in soil C and nutrient content (Chiu et al., 2006), or to differences of the methods used to characterize microbial biomass and community composition. Therefore, in this yellow-brown paddy soil of China, information regarding the activity and composition of the microbial community in different particle-size fractions under numerous fertilizations is needed.

Therefore we performed a study on biochemical and biological properties of different soil particle-size fractions of yellow-brown paddy soil in a long-term fertilizer experiment. Physical fractionation based on low-energy sonication was applied to obtain soil particle-size fractions. We measured nutrient contents, enzyme activities, and PLFA profiles within different particle-size fractions of soils that had been under 33 yr of organic and/or inorganic fertilization management in the Yangtze Plain of China. Our objectives were as follows: (i) to determine the changes of SOC, TN, and extracellular enzyme activities among bulk soil and particle-size fractions; (ii) to evaluate the effects of fertilization on microbial community composition and structure within particle-size fractions; and (iii) to identify the main factor(s) affecting microbial community composition and structure within different particle-size fractions. We hypothesized that 33-yr application of different fertilization especially manure addition would have distinct effects on soil fertility, enzyme activities, and microbial community. The results will help to select the best fertilization management practice for maintaining and improving soil health and quality under rice–wheat cropping system.

MATERIALS AND METHODS

Field Design and Sampling

The long-term field fertilizer experiment was initiated in 1981 at South Lake station (30°37′ N lat., 114°20′1″ E long.), 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 d^{-1}) and precipitation of 1300 mm. The tested yellow-brown paddy soil, belongs to Udalfs (USDA soil classification), had a clay loam texture with 15% sand, 36% silt, and 49% clay in 1987. At the beginning of the experiment, the soil had a pH (H_2O) of 6.3, 27.43 g kg^{-1} organic matter, 1.801 g kg^{-1} total N, 1.004 g kg^{-1} total P, 30.22 g kg^{-1} total K, and 5.0 and 98.5 mg kg^{-1} of available P and K, respectively. Six treatments (three replicates each) were randomly implemented in 18 plots (40 m^2 each) under a rotation of winter wheat and middle-season rice. Treatments consisted of soils under CK, N, NP, NPK, NPKM, and M. Chemical fertilizers were applied as annual rate of 150 kg N ha^{-1}, 75 kg P_2O_5 ha^{-1}, and 150 kg K_2O ha^{-1}. The N, P, and K fertilizers were applied as urea, superphosphate, and potassium chloride, respectively. For the NPKM treatment, the same rate of chemical fertilizers as NPK treatment were used in addition to 22,500 kg ha^{-1} manure per year, while no PK or K was applied for the N and NP treatments, respectively. Organic fertilizer was applied as pig manure (H_2O 69%) with properties of 15.1 g kg^{-1} N, 20.8 g kg^{-1} P_2O_5, and 13.6 g kg^{-1} K_2O.

Sixty percent of inorganic fertilizers were applied to rice and the other 40% were applied during the wheat season, while manure was applied equally (50:50) to the two crops. All fertilizer P and K and manure were applied once as basal dressing during the wheat season and the middle-rice season, while 40% of fertilizer N was applied as a basal fertilizer, 40% during tillering stage and 20% during booting stage in the middle–rice season. The amounts of N fertilizer applied to winter wheat were 50% as basal fertilizer, 25% for overwintering period, and 25% during the jointing stage. Manure and mineral fertilizers were evenly applied onto the soil surface and immediately incorporated into the plowed soil (0- to 20-cm depth) by tillage before sowing.

Undisturbed soil samples from the three replicates of each treatment were collected 1 wk before rice harvesting in 20 Sept. 2014. Three soil samples (5 × 10 × 18 cm) were collected at a depth of 0 to 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 thoroughly mixing, the field-moist soil was used for particle-size fractionation.

Particle-Size Fractionation

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^{-1}) and subsequently fractionated by a combination of wet sieving and repeated centrifugation. Finally, five fractions were obtained for each sample: large macroaggregate (>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 macroaggregate, coarse and fine sand particle-size fractions (>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 analyses. 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).

Soil Organic Carbon and Total Nitrogen Determination

Soil organic C concentration was determined by dichromate oxidation (Kalembasa and Jenkinson, 1973), while TN was measured using a vario MACRO cube element analyzer (Elementar Analysensysteme GmbH, Hanau, Germany; Ai et al., 2013).

Enzyme Activities

The activities of all extracellular enzymes tested except phenol oxidase and peroxidase were measured using 4-methylumbelliferyl (MUB)-linked or 7-amino-4-methylcoumarin (AMC)-linked model substrates yielding the highly fluorescent cleavage products MUB or AMC on hydrolysis (DeForest, 2009; Saiya–Cork et al., 2002; Wittmann et al., 2004). 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 mM references, and 200 mM substrates 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, ThermoFischer Scientific, Waltham, MA) 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% (w/v) 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 $h^{-1}g^{-1}$. Enzymes in this paper were abbreviated as: Pho, phosphatase; Sul sulfatase; βG, β-glucosidase; βCB, β-cellobiohydrolase; NAG, N-acetyl-glucosaminidase; βX, β-xylosidase; αG, α-glucosidase; LAP, L-leucine aminopeptidase; PhOx, phenol oxidase; and Perox, peroxidase.

Phospholipid Fatty Acid Profiles

Differences in the microbial community and microbial abundance among the treatments were approximately determined by PLFA analysis following the procedure described by Wu et al. (2009). Briefly, 3 g of 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. Fatty acid methyl esters were quantified by gas chromatograph (N6850, Agilent Technologies, Santa Clara, CA) 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 relative abundance (% mole) in each sample. Phospholipids fatty acids were divided into various taxonomic groups based on previously published PLFA biomarker data (Bossio et al., 1998; Frostegård et al., 1993; Green and Scow, 2000). Specifically, 14:0, 15:0, 16:0, 17:0, 18:0, 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, i18:0, 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 i14:0, i15:0, i16:0, i17:0, i18:0, a15:0, and a17:0 as gram-positive bacteria biomarkers; cy17:0, cy19:0ω8c, 16:1ω5c, 16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c, and 18:1ω7c as gram-negative bacteria biomarkers.

Statistical Analysis

Statistical procedures (ANOVA and principal component analysis [PCA]) were performed with SAS and CANOCO 4.5 (Ithaca, NY) softwares, respectively, and some other complemental calculations were performed using Origin 8, Adobe Illustrator CS4, and MS Excel 2010. Redundancy analysis (RDA) was used to determine which environmental factors were related to the composition of soil microbial communities represented by relative abundance of PLFA individuals using CANOCO 4.5. Monte Carlo permutation tests were applied to compute statistical significance ($n = 499$). 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 treatments within each fraction or difference among fractions.

RESULTS

Particle-Size Fraction Distribution

Particle-size fractionation was conducted for 0- to 20-cm layer. The soil weight recovery by the fractionation procedure was about 95.03 to 95.48% (Table 1). Of the fractions obtained by ultrasonic fractionation, the coarse sand fraction was dominant in soils under all treatments, accounting for 28.74 to 40.44% of total soil fractions, followed by the silt-sized fraction and the large macroaggregate, which accounted for 20.36 to 30.25% and 18.34 to 22.63% of the total soil composition, respectively. Compared with NPK, the NPKM treatment had a significantly decreased proportion of large macroaggregate in the total soil fractions. Manure plus fertilizer NPK and M treatments also significantly increased the proportions of the coarse sand fraction and reduced the proportions of the silt and clay fractions.

Table 1. Particle-size distribution of soils using ultrasonic fractionation under different fertilizer managements. The percentage of recovery is reported for ultrasonic fractionation.

Treatments	Particle-size fractions					
	Large macroaggregate >2000 μm	Coarse sand 2000–200 μm	Fine sand 200–63 μm	Silt 63–2 μm	Clay 2–0.1 μm	Recovery
CK	18.34 ± 1.14c†	28.74 ± 1.15b	3.18 ± 0.46ab	29.57 ± 0.91a	15.42 ± 0.72ab	95.25 ± 0.49a
N	18.39 ± 0.32c	29.66 ± 0.53b	2.96 ± 0.33b	30.25 ± 1.05a	13.79 ± 0.68bc	95.04 ± 1.91a
NP	20.00 ± 1.41bc	30.54 ± 1.20b	3.01 ± 0.17b	26.09 ± 1.17b	15.84 ± 0.91a	95.48 ± 0.72a
NPK	22.63 ± 1.10a	31.71 ± 0.60b	3.72 ± 0.57ab	25.32 ± 1.26b	11.96 ± 1.24c	95.34 ± 0.46a
NPKM	18.74 ± 0.98c	39.67 ± 1.85a	4.23 ± 0.34a	24.25 ± 0.41c	8.19 ± 0.31d	95.07 ± 1.14a
M	22.02 ± 0.98ab	40.44 ± 0.15a	4.06 ± 0.18ab	20.36 ± 1.08c	8.15 ± 0.18d	95.03 ± 0.80a
	C	A	E	B	D	

† Data are means ± standard error, $n = 3$. Different lowercase letters indicate significant differences ($P < 0.05$) among fertilizations within each fraction (Fisher's LSD test) and the capital letters indicate significant differences among the five fractions.

Soil Organic Carbon, Total Nitrogen, and Carbon to Nitrogen Ratio in Bulk Soil and Particle-Size Fractions

The concentrations of SOC and TN in the bulk soil were significantly greater under manure treatments (NPKM and M) than those under inorganic treatments. In contrast, the C/N ratio in the bulk soil was not significantly affected by different fertilizer managements (Fig. 1).

Regardless of fertilizer treatment, the differences of SOC, TN, and C/N ratio in the different particle-size fractions were very pronounced, with the highest values obtained in the fine sand fraction ($P < 0.05$; Fig. 1). For the other fractions, the SOC concentrations and C/N ratios were in the order of large macroaggregate > coarse sand > clay and silt, whereas TN content was in the order of clay > coarse sand > large macroaggregate > silt, which resulted in the significant reduction of C/N ratio in the clay fraction. Organic treatments significantly increased the SOC and TN concentrations of the five fractions compared with the NPK treatment, except for TN content of the clay fraction under the M treatment (Fig. 1).

The recovery rates of SOC and TN exceeded 92 and 97%, respectively (Table 2). Even though the fine sand fraction contained high SOC and TN contents, they contributed a minority to the overall soil nutrients (approximately 8.50 and 6.32%, respectively). In contrast, the proportional contribution of the coarse sand fraction to SOC and TN reached 35.70 and 32.15% on average, respectively. The percentage contribution of both SOC and TN under NPKM and M treatments showed a clear increase in the coarse sand fraction and reduction in the clay fraction.

Soil Enzyme Activities in Bulk Soil and Particle-Size Fractions

The activities of 10 soil enzymes involved in C, N, P, and S cycling were determined in bulk soil and the five particle-size fractions under different fertilizer treatments (Fig. 2; Table S1). Enzyme activities showed remarkable variation between fractions and different fertilizers ($P < 0.05$). For most enzyme activities (except Sul, αG, and LAP), there were statistically significant differences between NPKM and NPK treatments in bulk soil and the five particle-size fractions (Table S1). All enzyme (except Sul) activities showed significant differences between particle-size fractions and were highest in the fine sand fraction (Fig. 2). Sulfatase behaved differently from the other enzymes, with the

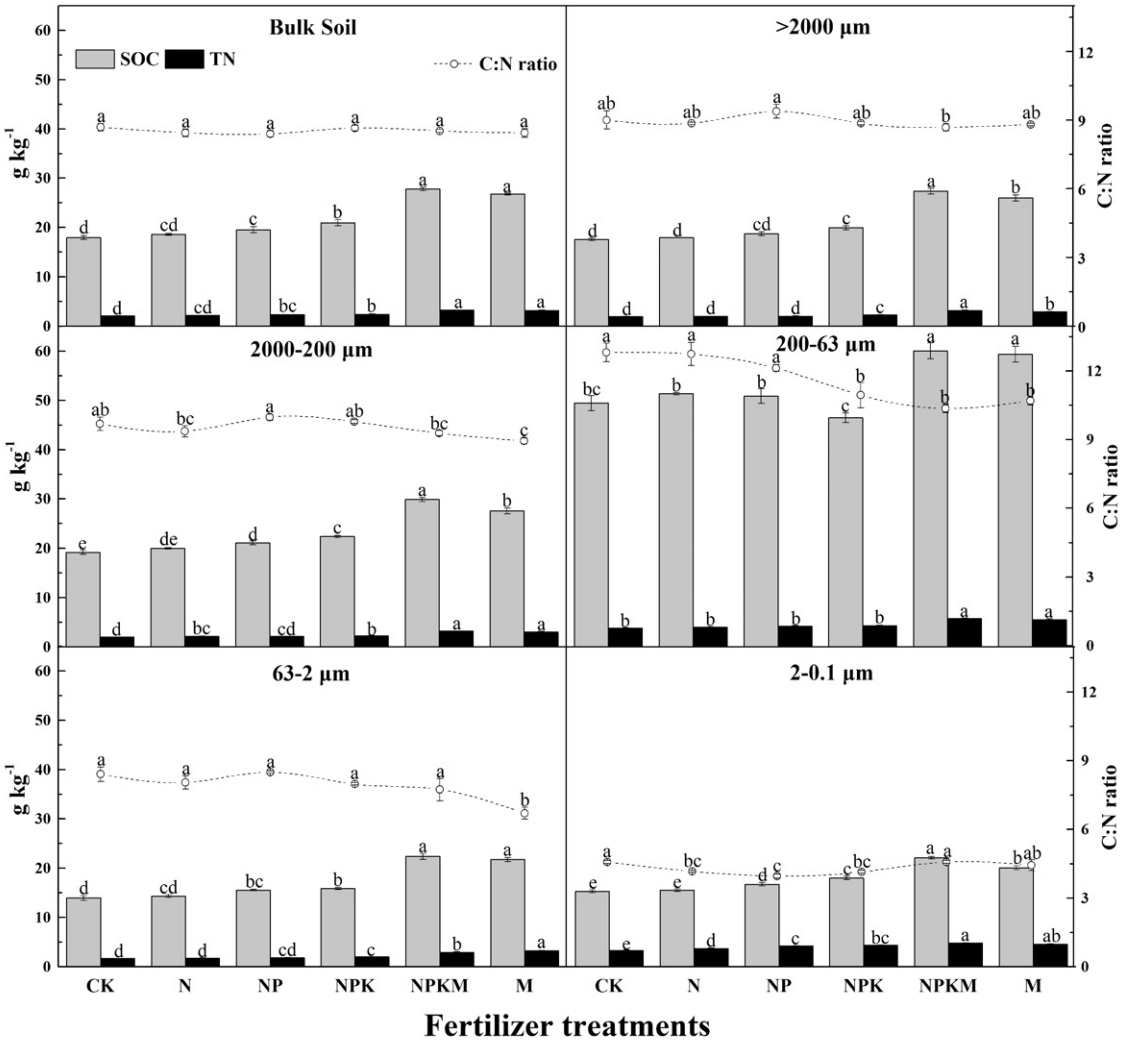


Fig. 1. Concentrations of soil organic C, total N, and C/N ratio in particle-size fractions under different treatments. Different lowercase letters indicate significant differences ($P < 0.05$) among fertilizer treatments within each fraction (Fisher's LSD test).

Table 2. The percentage contribution (%) of the parameters in each particle-size fraction to the bulk soil and the total recovery rates of parameters under the six fertilizer treatments.†

Treatments‡	Particle-size fraction					
	Large macroaggregate > 2000 μm	Coarse sand 2000–200 μm	Fine sand 200–63 μm	Silt 63–2 μm	Clay 2–0.1 μm	Total
SOC						
CK	17.98 ± 1.34c	30.92 ± 2.41b	8.70 ± 1.06a	22.99 ± 0.81a	13.13 ± 0.11a	93.71 ± 2.03a
N	17.75 ± 0.43c	31.91 ± 0.43b	8.18 ± 0.91a	23.35 ± 1.31a	11.51 ± 0.30b	92.70 ± 2.61a
NP	19.18 ± 1.29bc	33.15 ± 2.14b	7.82 ± 0.25a	20.82 ± 1.23ab	13.57 ± 0.69a	94.53 ± 2.04a
NPK	21.57 ± 1.29a	33.97 ± 1.14b	8.24 ± 1.18a	19.22 ± 0.94bc	10.23 ± 0.61b	93.23 ± 0.94a
NPKM	18.43 ± 0.77c	42.64 ± 1.95a	9.10 ± 0.55a	19.52 ± 0.08bc	6.52 ± 0.23c	96.21 ± 1.83a
M	21.34 ± 0.98ab	41.60 ± 1.32a	8.97 ± 0.34a	16.53 ± 0.99c	6.11 ± 0.16c	94.54 ± 1.85a
	B	A	C	B	C	
TN						
CK	17.43 ± 1.55c	27.69 ± 1.78b	5.94 ± 0.84ab	23.84 ± 0.41ab	24.93 ± 0.11b	99.83 ± 3.05a
N	16.92 ± 0.46c	28.84 ± 1.18b	5.38 ± 0.32b	24.55 ± 0.92a	23.29 ± 0.69bc	98.98 ± 2.63a
NP	17.13 ± 0.75c	27.90 ± 1.77b	5.43 ± 0.28b	20.61 ± 1.09b	28.80 ± 1.67a	99.87 ± 0.64a
NPK	21.03 ± 0.84a	30.01 ± 0.32b	6.57 ± 1.07ab	20.93 ± 1.27ab	21.40 ± 1.74c	99.93 ± 1.71a
NPKM	18.13 ± 0.91bc	39.22 ± 2.06a	7.50 ± 0.53a	21.78 ± 1.35ab	12.15 ± 0.49d	98.77 ± 0.70a
M	20.44 ± 0.89ab	39.23 ± 0.97a	7.09 ± 0.36ab	20.88 ± 1.22ab	11.69 ± 0.67d	99.33 ± 0.89a
	B	A	C	B	B	
Pho						
CK	19.49 ± 0.99bc	31.29 ± 1.78b	4.75 ± 0.77a	32.89 ± 2.54a	12.07 ± 0.27ab	100.49 ± 4.11a
N	18.57 ± 1.26c	29.73 ± 0.69b	3.85 ± 0.52a	30.19 ± 2.69ab	9.85 ± 0.21c	92.18 ± 4.20a
NP	19.25 ± 1.62bc	30.70 ± 1.28b	3.69 ± 0.17a	27.50 ± 0.93ab	13.93 ± 1.20a	95.07 ± 1.47a
NPK	22.92 ± 1.64ab	32.65 ± 0.57b	4.82 ± 0.80a	27.75 ± 1.97ab	11.94 ± 0.94b	100.07 ± 1.54a
NPKM	18.57 ± 1.86c	38.68 ± 0.66a	5.30 ± 0.38a	25.56 ± 1.46bc	8.62 ± 0.33c	96.74 ± 2.97a
M	23.78 ± 1.24a	40.16 ± 1.16a	5.23 ± 0.11a	21.24 ± 1.10c	8.06 ± 0.52c	98.47 ± 2.89a
	C	A	E	B	D	
Sul						
CK	17.53 ± 1.52a	34.30 ± 1.33b	3.34 ± 0.52b	21.33 ± 0.61a	14.66 ± 0.84ab	91.15 ± 1.01a
N	18.08 ± 1.37ab	36.00 ± 4.27b	3.05 ± 0.37b	21.69 ± 1.12a	13.15 ± 0.87ab	91.97 ± 7.30a
NP	19.36 ± 2.23a	37.54 ± 1.34b	3.10 ± 0.52b	19.04 ± 1.12ab	15.89 ± 1.21a	94.93 ± 2.61a
NPK	20.20 ± 0.80a	37.47 ± 0.39b	3.95 ± 0.68ab	17.47 ± 0.79b	11.66 ± 2.04bc	90.76 ± 2.01a
NPKM	15.35 ± 0.53a	48.70 ± 2.43a	4.35 ± 0.59ab	17.22 ± 0.73b	8.04 ± 0.50d	93.66 ± 2.88a
M	19.18 ± 2.01a	47.62 ± 2.87a	5.17 ± 0.36a	16.81 ± 0.53b	8.73 ± 0.65cd	97.51 ± 2.19a
	B	A	D	B	C	
βG						
CK	18.37 ± 0.74c	33.37 ± 1.82b	8.58 ± 1.59a	28.86 ± 1.99a	6.91 ± 0.35ab	96.09 ± 3.20a
N	19.31 ± 0.74abc	34.62 ± 0.60b	7.95 ± 0.87a	27.47 ± 2.37ab	6.54 ± 0.33b	95.90 ± 3.32a
NP	22.65 ± 1.67a	36.67 ± 1.73ab	8.53 ± 0.90a	26.57 ± 0.65ab	7.95 ± 0.64a	102.38 ± 2.50a
NPK	21.91 ± 1.00ab	36.99 ± 1.45ab	9.62 ± 1.69a	23.22 ± 3.01bc	6.28 ± 0.26b	98.01 ± 6.41a
NPKM	18.50 ± 1.15bc	41.51 ± 2.94a	10.06 ± 1.26a	24.47 ± 0.65ab	4.40 ± 0.48c	98.94 ± 4.46a
M	21.80 ± 1.83abc	41.20 ± 1.85a	9.73 ± 0.54a	19.35 ± 0.14c	3.53 ± 0.09c	95.61 ± 3.88a
	B	A	D	C	D	
βCB						
CK	18.47 ± 1.21ab	39.74 ± 2.11ab	12.61 ± 2.05a	22.66 ± 0.77a	5.57 ± 0.24a	99.05 ± 1.20a
N	17.40 ± 1.17b	35.91 ± 2.19b	11.10 ± 0.87a	20.93 ± 1.55a	5.04 ± 0.26ab	90.38 ± 2.95a
NP	18.69 ± 0.83ab	39.81 ± 2.55ab	10.66 ± 0.50a	20.08 ± 1.71ab	5.30 ± 0.45ab	94.54 ± 4.24a
NPK	21.86 ± 1.24a	43.68 ± 1.38a	13.80 ± 2.34a	21.03 ± 2.04a	4.45 ± 0.31b	104.82 ± 4.31a
NPKM	20.13 ± 2.63ab	44.03 ± 0.17a	13.46 ± 0.59a	20.88 ± 0.75a	2.97 ± 0.32c	101.47 ± 2.93a
M	21.33 ± 2.24ab	40.56 ± 1.97ab	12.94 ± 0.40a	15.94 ± 1.00b	2.61 ± 0.32c	93.37 ± 4.82a
	B	A	C	B	D	
NAG						
CK	17.64 ± 0.78b	32.07 ± 1.91c	10.37 ± 1.27a	23.36 ± 0.43ab	7.59 ± 0.41a	91.03 ± 1.25a
N	18.23 ± 1.02b	34.12 ± 1.41bc	10.02 ± 1.22a	24.73 ± 0.58a	6.76 ± 0.24a	93.86 ± 3.03a
NP	19.30 ± 1.83ab	35.70 ± 1.69abc	9.88 ± 0.43a	21.70 ± 0.81ab	7.46 ± 0.82a	94.04 ± 0.53a
NPK	22.19 ± 0.75a	37.86 ± 2.98ab	12.27 ± 2.25a	21.29 ± 1.91b	6.17 ± 0.30a	99.77 ± 5.95a
NPKM	18.04 ± 1.73b	39.69 ± 0.16a	10.90 ± 0.26a	22.78 ± 1.23ab	4.52 ± 0.43b	95.93 ± 3.15a
M	21.35 ± 1.87ab	38.48 ± 0.24ab	10.03 ± 0.60a	17.98 ± 0.17c	3.86 ± 0.16b	91.70 ± 2.57a
	B	A	C	B	D	

Table 2. Continued.

Treatments‡	Particle-size fraction					
	Large macroaggregate > 2000 μm	Coarse sand 2000–200 μm	Fine sand 200–63 μm	Silt 63–2 μm	Clay 2–0.1 μm	Total
βX						
CK	15.50 ± 0.61b	37.32 ± 2.70b	9.56 ± 1.80a	23.22 ± 1.11a	6.75 ± 0.26b	92.35 ± 3.17a
N	17.79 ± 1.66ab	38.19 ± 0.90b	8.95 ± 1.22a	23.21 ± 2.18a	6.94 ± 0.41b	95.07 ± 3.59a
NP	20.29 ± 2.02ab	39.83 ± 1.14ab	9.05 ± 0.31a	22.53 ± 0.43a	9.18 ± 1.15a	100.89 ± 2.77a
NPK	22.01 ± 2.69a	37.70 ± 4.90b	10.45 ± 1.53a	21.30 ± 2.74a	7.37 ± 0.24ab	98.83 ± 10.81a
NPKM	18.81 ± 2.47ab	47.34 ± 2.93a	10.63 ± 0.68a	21.10 ± 1.51a	4.70 ± 0.56c	102.58 ± 5.44a
M	19.18 ± 2.02ab	41.73 ± 3.20ab	10.16 ± 0.07a	15.47 ± 0.93b	3.87 ± 0.10c	90.41 ± 5.61a
	B	A	C	B	D	
αG						
CK	18.41 ± 1.26b	29.85 ± 3.56b	6.51 ± 1.15a	27.63 ± 1.68a	14.93 ± 0.36a	97.33 ± 6.08a
N	20.36 ± 1.85ab	33.49 ± 3.76ab	6.20 ± 1.16a	30.94 ± 4.83a	14.21 ± 1.93a	105.20 ± 13.00a
NP	20.79 ± 1.53ab	33.74 ± 5.11ab	6.63 ± 0.43a	27.33 ± 2.25a	15.96 ± 0.71a	104.44 ± 8.50a
NPK	21.35 ± 1.92ab	34.18 ± 0.63ab	6.81 ± 1.29a	24.89 ± 2.47ab	13.28 ± 1.49ab	100.51 ± 2.11a
NPKM	20.08 ± 0.72b	40.87 ± 2.03a	7.04 ± 1.03a	23.96 ± 1.79ab	9.74 ± 0.57bc	101.70 ± 3.41a
M	24.49 ± 1.82a	41.74 ± 3.02a	7.26 ± 0.51a	18.86 ± 1.38b	8.79 ± 0.30c	101.15 ± 5.77a
	C	A	E	B	D	
LAP						
CK	13.92 ± 1.11d	29.94 ± 1.50b	5.39 ± 0.87a	34.52 ± 1.49a	13.71 ± 0.47a	97.49 ± 1.95a
N	14.33 ± 0.70cd	31.34 ± 1.28b	4.82 ± 0.43a	35.09 ± 1.22a	13.39 ± 0.66a	98.96 ± 2.55a
NP	14.95 ± 1.17bcd	30.84 ± 2.48b	4.69 ± 0.16a	30.88 ± 2.08b	15.60 ± 1.17a	96.97 ± 2.74a
NPK	17.34 ± 1.19ab	32.20 ± 1.27b	5.65 ± 0.78a	30.59 ± 1.33b	12.79 ± 1.98ab	98.57 ± 3.21a
NPKM	16.80 ± 0.97bc	38.59 ± 1.27a	4.69 ± 0.45a	29.13 ± 0.61b	10.28 ± 0.87bc	99.48 ± 2.65a
M	19.98 ± 0.44a	40.87 ± 1.36a	5.30 ± 0.21a	23.76 ± 1.28c	9.46 ± 0.44c	99.38 ± 2.87a
	B	A	C	A	B	
PhOx						
CK	18.35 ± 1.47a	23.42 ± 0.54c	4.05 ± 0.58bc	35.48 ± 2.12ab	15.02 ± 1.30ab	96.33 ± 1.88a
N	18.56 ± 1.57a	27.48 ± 0.81c	3.82 ± 0.27bc	38.89 ± 1.01a	13.45 ± 1.55ab	102.21 ± 1.34a
NP	18.58 ± 1.25a	32.40 ± 1.78b	3.16 ± 0.22c	31.08 ± 2.28bc	15.81 ± 1.39a	101.02 ± 2.00a
NPK	19.92 ± 1.07a	32.65 ± 1.49b	4.58 ± 0.70abc	27.79 ± 1.64cd	12.29 ± 2.06b	97.22 ± 2.90a
NPKM	19.27 ± 0.56a	44.20 ± 1.65a	5.66 ± 0.66a	23.06 ± 1.64de	8.49 ± 0.61c	100.67 ± 1.59a
M	20.57 ± 1.04a	47.57 ± 1.62a	5.07 ± 0.23ab	20.10 ± 0.60e	7.05 ± 0.70c	100.36 ± 1.24a
	B	A	C	A	B	
Perox						
CK	14.34 ± 1.37d	28.86 ± 2.36c	4.51 ± 0.85b	38.80 ± 0.30a	12.91 ± 0.78b	99.41 ± 0.73a
N	14.83 ± 0.34cd	30.34 ± 1.50bc	4.30 ± 0.38b	40.79 ± 1.36a	12.33 ± 1.09b	102.59 ± 1.62a
NP	18.04 ± 1.69bc	31.80 ± 4.90abc	4.32 ± 0.25b	33.53 ± 1.56b	15.76 ± 0.68a	103.45 ± 5.27a
NPK	22.31 ± 1.87a	32.36 ± 0.93abc	5.53 ± 0.64ab	31.83 ± 2.73b	11.83 ± 1.16b	103.86 ± 2.52a
NPKM	19.18 ± 0.36ab	40.56 ± 4.82a	7.07 ± 0.41a	26.46 ± 0.95c	7.53 ± 0.28c	100.80 ± 5.38a
M	20.09 ± 0.79ab	39.58 ± 0.88ab	6.92 ± 1.15a	24.54 ± 1.68c	7.85 ± 0.53c	98.98 ± 2.54a
	B	A	D	A	C	

† Data are means ± standard error, $n = 3$. Different capital letters indicate significant differences ($P < 0.05$) among the five fractions; different lowercase letters indicate significant differences ($P < 0.05$) among treatments within each fraction (Fisher's LSD test). Enzyme abbreviation: Pho, phosphatase; Sul, sulfatase; βG, β-glucosidase; βCB, β-cellobiohydrolase; NAG, N-acetyl-glucosaminidase; bX, β-xylosidase; αG, α-glucosidase; LAP, L-leucine aminopeptidase; PhOx, phenol oxidase; Perox, peroxidase.

‡ CK, control; N, fertilizer N; NP, fertilizer N and P; NPK, fertilizer N, P, and K; NPKM, fertilizer N, P, and K plus manure; M, manure.

highest activity obtained in the coarse sand fraction and showed no obvious differences between the treatments in silt fraction. Excluding fine sand fraction, LAP, PhOx, and Perox were highest in the silt fraction and lowest in the large macroaggregate fraction. Activities of Pho, βG, βCB, NAG, and βX were highest in the coarse sand fraction and lowest in the clay fraction. Phenol oxidase and Perox showed minor changes in activity compared with the other hydrolytic enzymes, where the differences in activity could reach several fold between fractions. In general, the average recovery rates of enzyme activities exceeded 90% with some enzymes (such as αG, PhOx, Perox) and under some treatments exceeded 100% (Table 2). The proportions of Pho, Sul, αG, LAP, PhOx, and Perox activities in the fine sand fraction were significantly lower than those in the clay fraction; however, βCB, NAG, and βX activities showed the opposite response.

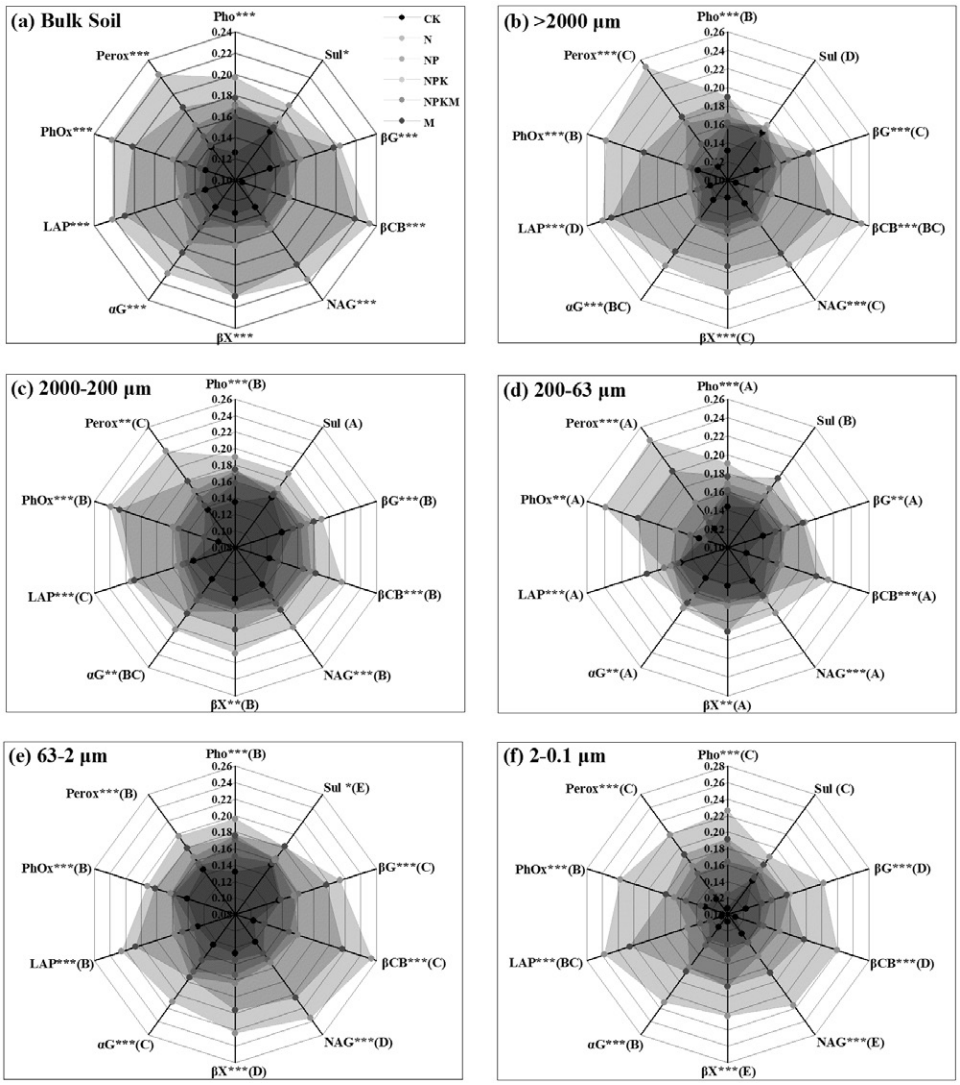


Fig. 2. Radar graphs illustrating the relative response of enzyme activities to different fertilizer treatments in (a) bulk soil and the five fractions: (b) >2000 μm, (c) 2000–200 μm, (d) 200–63 μm, (e) 63–2 μm, (f) 2–0.1 μm. Asterisks indicate significant differences among different treatments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Fisher's LSD test) and the capital letter behind asterisk indicate significant differences among the five fractions. Enzyme abbreviation: Pho phosphatase, Sul sulfatase, βG β-glucosidase, βCB β-cellobiohydrolase, NAG N-acetyl-glucosaminidase, βX β-xylosidase, αG α-glucosidase, LAP L-leucine aminopeptidase, PhOx phenol oxidase, Perox peroxidase.

Soil Microbial Community in Bulk Soil and Particle-Size Fractions

We measured microbial community composition via PLFA analysis in bulk soil and physically separated soil particle-size fractions under organic and/or inorganic fertilizer treatments. In bulk soil, total PLFAs, which ranged from 25.19 to 73.56 nmol g^{-1}, were significantly higher in organic treatments (NPKM and M) than in CK and mineral fertilizer treatments (Table 3) and the absolute biomass of microbial groups showed similar trends (Table S2). Compared with CK, fertilizer treatments increased the relative abundance of bacteria but reduced the proportion of actinomycetes; however there was no significant difference between organic treatments with NPK in bulk soil (Table 3). The proportions of fungi under CK and N treatments were significantly higher than those under NP, NPK, and M treatments, which resulted in the increased fungi/bacteria ratio in CK and N treatments. G+/G− ratios were significantly reduced in the organic treatments because of the clearly reduced proportion of gram-positive bacteria and increased proportion of gram-negative bacteria (Fig. 3c).

Soil particle-size fraction and fertilizer treatment yielded large differences of PLFA profiles in distinctive ways (Tables 3 and S2). The abundance of total PLFAs was highest in the fine sand fraction followed by the >200-μm fraction, and lowest in the silt fraction. Soils amended with NPKM treatment contained significantly higher total PLFAs and absolute abundance of microbial groups than the other treatments of bulk soil and the five fractions (Table S2). The relative abundances of microbial groups under the six treatments in all the fractions were also calculated, which were in the order of bacteria > actinomycetes > fungi (Table 3). The distribution of relative abundance of microbial groups was different. Bacterial relative abundance was significantly higher in the fine sand fraction and lower in the silt fraction compared with the other fractions. Actinomycetes tended to be more abundant in the large macroaggregate and clay fractions and less in the fine sand fraction. No significant difference was found in the relative number of fungi among the five fractions (Table 3). Significantly increased relative abundances of gram-positive bacteria (Fig. 3a) and relatively lower proportions of gram-negative bacteria (Fig. 3b) were observed in the coarse sand, fine sand, and clay fractions, resulting in significantly higher G+/G− ratios in these fractions than the other soil fractions (Fig. 3c). Fungi/bacteria ratios were very similar among the different soil particle-size fractions and the differences were not significant.

Principal component analysis (PCA) was conducted with individual PLFAs (mol %) which were present in bulk soil and the five particle-size fractions. Principal component 1 (PC1) and principal component 2 (PC2) accounted for 53.80 and 8.90% of the total variation, respectively. The PC scores on these axes were well separated on the basis of soil fractions and fertilizer treatments, especially along PC1 (Fig. 4a). Fertilizer treatments of fine sand fraction and organic treatments of bulk soil and the five fractions, except for the silt, were grouped along PC1. In con-

Table 3. Microbial community composition of different particle-size fractions under different fertilizer managements.†

Microbial PLFA composition	Treatments‡	Particle-size fractions					
		Bulk soil	Large macroaggregate > 2000 μm	Coarse sand 2000–200 μm	Fine sand 200–63 μm	Silt 63–2 μm	Clay 2–0.1 μm
Total PLFAs	CK	25.19 ± 0.85e	39.92 ± 0.20d	31.64 ± 0.75f	47.34 ± 1.61e	14.83 ± 0.56e	28.46 ± 0.58f
(nmol g^{-1})	N	33.60 ± 0.83d	39.02 ± 1.57d	41.30 ± 0.75d	63.81 ± 0.98d	20.09 ± 0.37d	36.44 ± 0.58e
	NP	36.84 ± 0.92d	39.15 ± 0.93d	37.29 ± 1.21e	75.19 ± 0.68c	21.39 ± 0.66c	46.05 ± 0.54bd
	NPK	44.26 ± 2.30c	61.82 ± 0.97c	44.09 ± 0.10c	97.45 ± 1.66b	25.87 ± 0.53b	51.19 ± 0.28c
	NPKM	73.56 ± 0.86a	71.80 ± 1.34a	77.26 ± 1.76a	108.78 ± 3.58a	35.80 ± 0.86a	73.62 ± 0.67a
	M	69.00 ± 1.01b	67.47 ± 0.86b	63.94 ± 0.88b	94.51 ± 2.30b	36.67 ± 0.73a	69.56 ± 0.25b
			B	B	A	C	B
Bacterial PLFA	CK	63.16 ± 1.44b	65.13 ± 1.28a	65.86 ± 0.50a	66.29 ± 1.48bc	63.59 ± 0.71ab	64.16 ± 2.05a
(mol %)	N	63.73 ± 1.11b	64.19 ± 0.97ab	64.08 ± 0.97a	65.64 ± 0.64c	61.02 ± 1.35b	65.56 ± 0.84a
	NP	66.12 ± 0.43ab	62.30 ± 0.98b	65.77 ± 0.75a	69.26 ± 0.98ab	63.56 ± 0.47ab	67.13 ± 2.45a
	NPK	65.69 ± 2.21ab	63.71 ± 1.06ab	65.53 ± 1.78a	68.14 ± 1.42abc	62.65 ± 1.25ab	64.54 ± 1.52a
	NPKM	68.70 ± 0.95a	64.89 ± 0.26ab	64.45 ± 0.48a	70.84 ± 1.64a	64.41 ± 0.99a	62.96 ± 0.85a
	M	66.37 ± 0.88ab	63.63 ± 0.55ab	65.44 ± 0.52a	68.84 ± 0.33abc	62.11 ± 0.36ab	66.44 ± 1.04a
			BC	B	A	C	B
Fungal PLFA	CK	3.07 ± 0.11a	2.23 ± 0.03b	2.09 ± 0.04c	2.90 ± 0.04a	2.79 ± 0.02b	3.18 ± 0.12a
(mol %)	N	3.19 ± 0.08a	2.34 ± 0.07b	3.06 ± 0.19ab	2.81 ± 0.04ab	3.33 ± 0.31a	1.63 ± 0.01d
	NP	2.56 ± 0.07c	2.29 ± 0.03b	3.24 ± 0.19a	1.94 ± 0.11d	2.07 ± 0.16c	1.88 ± 0.06c
	NPK	2.62 ± 0.04c	2.08 ± 0.06b	2.87 ± 0.19b	2.09 ± 0.06cd	2.55 ± 0.12bc	3.15 ± 0.05a
	NPKM	3.00 ± 0.10ab	2.05 ± 0.19b	1.94 ± 0.05cd	2.19 ± 0.09c	2.08 ± 0.08c	2.47 ± 0.02b
	M	2.80 ± 0.06bc	3.20 ± 0.06a	1.76 ± 0.09d	2.61 ± 0.04b	2.19 ± 0.08c	2.42 ± 0.01b
			A	A	A	A	A
Actinomycetic PLFA	CK	17.95 ± 0.09a	17.86 ± 1.12a	17.16 ± 0.50ab	14.50 ± 0.29a	17.23 ± 0.16a	17.53 ± 0.17ab
	N	16.14 ± 0.28ab	17.12 ± 1.12a	16.38 ± 0.14b	14.38 ± 0.10a	15.41 ± 0.41c	18.18 ± 0.48a
(mol %)	NP	16.30 ± 0.41ab	18.20 ± 0.09a	17.20 ± 0.03ab	14.84 ± 0.39a	16.11 ± 0.28ab	17.33 ± 0.29abc
	NPK	16.66 ± 1.03ab	17.73 ± 0.90a	14.60 ± 0.41c	15.03 ± 0.49a	16.05 ± 0.22ab	16.40 ± 0.50bc
	NPKM	16.63 ± 0.29ab	17.60 ± 0.98a	17.35 ± 0.64ab	14.70 ± 0.55a	17.73 ± 0.24a	16.11 ± 0.44c
	M	15.78 ± 0.72b	17.78 ± 1.00a	17.59 ± 0.34a	14.76 ± 0.41a	16.26 ± 0.08b	16.47 ± 0.46bc
			A	B	C	B	AB

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

‡ CK, control; N, fertilizer N; NP, fertilizer N and P; NPK, fertilizer N, P, and K; NPKM, fertilizer N, P, and K, plus manure (NPKM); M, manure.

trast, the 63- to 2-μm fraction and inorganic treatments in bulk soil and the other fractions were separated negatively along PC1. For individual PLFAs, the proportions of monounsaturated fatty acids (17:1ω8c, 18:1ω7c, 18:1ω9c), cyclopropane fatty acids (cy17:0, cy19:0ω8c), and methyl branched fatty acids (16:0[10Me], 17:0[10Me], 18:0[10Me], and 19:0[10Me]; biomarkers of actinomycetes) were positively correlated with both PC1 and PC2. The proportions of saturated fatty acids (14:0, 16:0, 17:0, a15:0, a16:0, a17:0, i14:0, i15:0, i16:0), 16:1ω7c, 16:1ω9c and 18:1ω7c were positively correlated with PC1.

Correlations

Redundancy analysis (RDA) was performed for enzyme activities with SOC and TN in bulk soil and fractions of the six treatments. Soil organic C and TN were used as environmental variables (Fig. 5a). The first and second axes accounted for 67.40 and 6.70%, respectively, of the total variation between enzyme activities and soil C and N pools. The enzyme activities showed significant correlation with SOC concentration ($F = 67.960$, $P = 0.002$), TN concentration ($F = 5.805$, $P = 0.004$), and C/N ratio ($F = 8.280$, $P = 0.002$).

Results of the RDA between soil nutrients and microbial community composition (relative abundance of individual PLFAs) are shown in Fig. 5b. The first and second axes accounted for 43.10 and 5.40%, respectively, of the total variation in microbial community composition and structure. The microbial community composition was significantly correlated with SOC ($F = 19.511$, $P = 0.002$), TN content ($F = 4.883$, $P = 0.002$), and C/N ratio ($F = 4.394$, $P = 0.002$), which explained 36.50, 8.20, and 6.70% of the total variance, respectively.

The RDA showed the relationships between soil enzyme activities and microbial community composition (Fig. 5c). The first and second axes accounted for 46.80 and 6.90%, respectively, of the total variation. Soil enzyme activities, including αG ($F = 22.462$, $P = 0.002$), Sul ($F = 3.832$, $P = 0.002$), βG ($F = 4.012$, $P = 0.002$), βCB ($F = 2.995$, $P = 0.004$), and PhOx ($F = 1.818$, $P = 0.028$) activities of different soil fractions were significantly correlated with microbial community composition after long-term fertilization. Conversely, LAP, Pho, βX, and NAG had no significant relationship with microbial community composition in different particle-size fractions of the six fertilizer treatments.

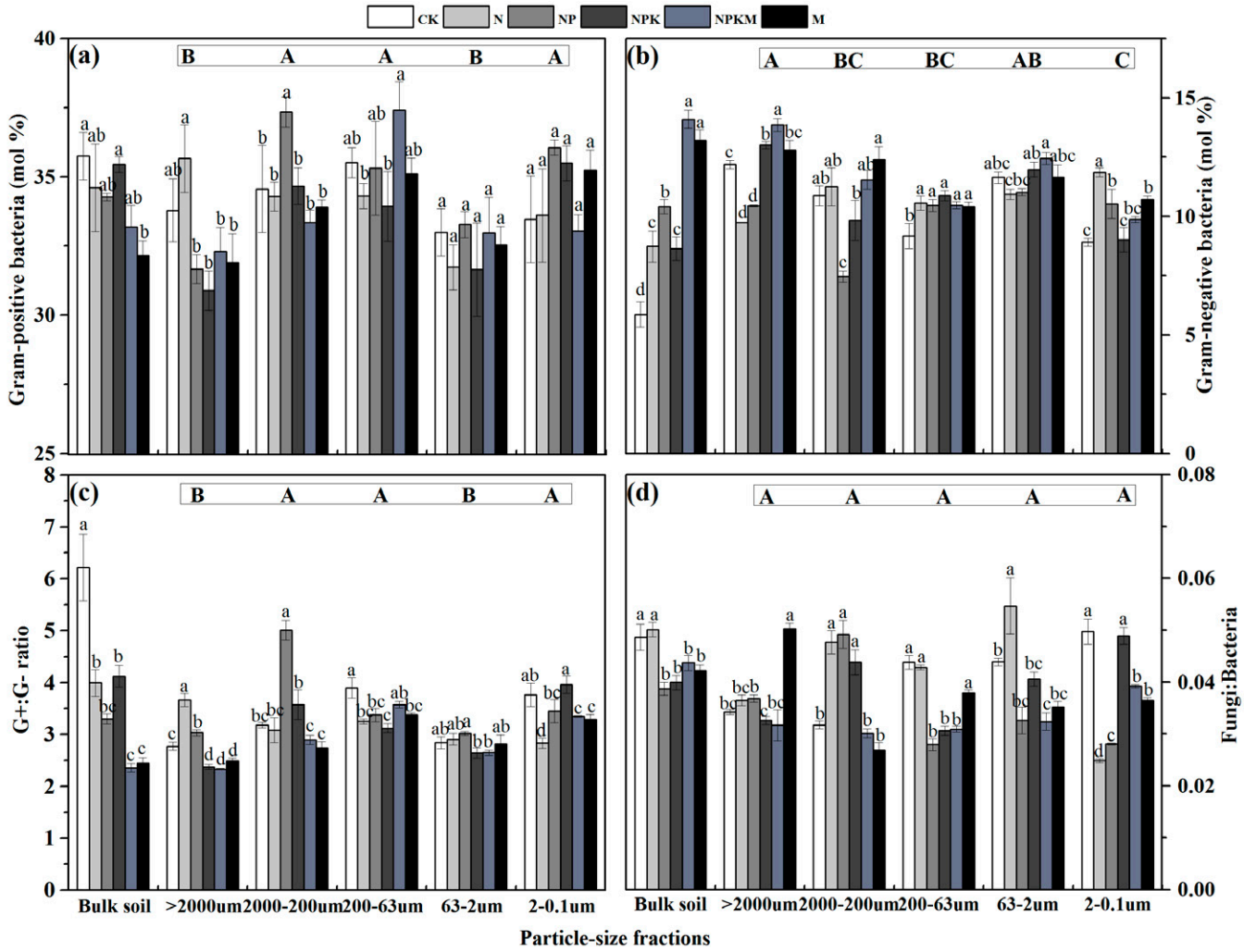


Fig. 3. Comparisons of (a) gram-positive bacteria, (b) gram-negative bacteria, (c) G+/G– ratio, and (d) fungi/ bacteria ratio in bulk soil and the five fractions of the six fertilizer treatments. Vertical bars represent the S.E. (n = 3) and lowercase letters indicate significant differences among fertilizer treatments (P < 0.05); capital letters indicate significant differences (P < 0.05) among the five fractions. G+/G- = Gram-positive bacteria/ Gram-negative bacteria ratio.

DISCUSSION

Fertilization Effects on the Distribution of Particle-Size Fractions and Soil Organic Carbon, Total Nitrogen

Addition of easily decomposable substrates to soil usually stimulates soil microflora and determines the proportion of different particle-size fractions, leading to a significant improvement in soil structure (De Gryze et al., 2005). In this study, compared with NPK, manure application significantly increased SOC and TN concentration in soils and improved the mass proportion of coarse sand fraction at the expense of silt and clay fraction (Table 1). Similar results were observed by Yu et al. (2012) in an intensively cultivated fluvo-aquic soil of North China and this was probably because input of organic substrates resulted in the binding of organic matter and soil particles into larger aggregates (Oades, 1984; Six et al., 1998). In contrast, though the 33-yr application of fertilizer NPK significantly increased SOC and TN concentrations of all fractions except fine sand compared with CK treatment in our study, there was no evidence of a major increase of larger fraction which is the same case with the Chinese red soil reported by Huang et al. (2010).

The increase of SOC concentration in particle-size fractions with addition of manure which was observed as expected in this experiment was supported by the studies of Kandeler et al. (1999a) in Germany and Gerzabek et al. (2001) in central Sweden. In the current study, the larger size fractions (>63 μm) showed higher SOC concentrations than silt and clay fractions (Fig. 1) with highest concentrations present in fine sand fraction. This result generally agrees with previous findings in Hydragric Anthrosol paddy soil (Jiang et al., 2011). In contrast, the highest TN concentrations were found in the fine sand, followed by clay fraction, which resulted in a significantly lower C/N ratio in clay fraction than the other fractions. The C/N ratios in different particle-size fractions followed the general pattern that they were significantly higher in larger fractions (>63 μm), especially in the fine sand fraction, suggesting that the organic matter associated with these fractions, originated from organic matter input such as manure or crop residues, was easier to be decomposed (Gerzabek et al., 2001). In comparison, silt and clay fractions

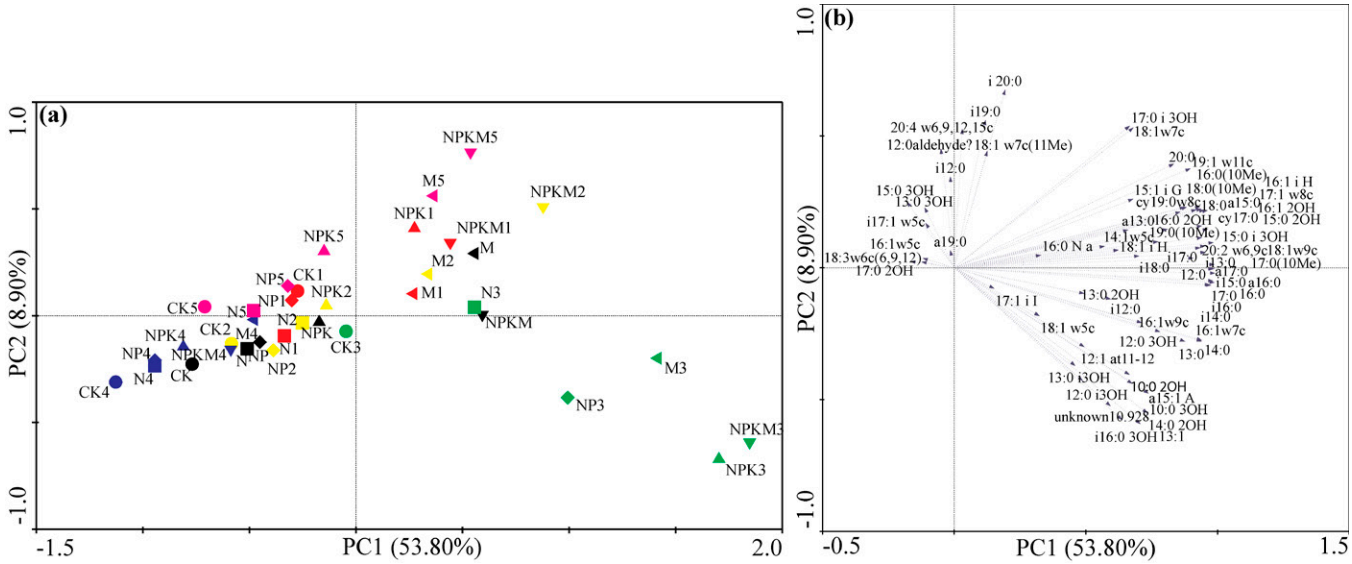


Fig. 4. Plot of the first two principle components (PC1 and PC2) grouped by (a) six fertilizer treatments and five particle-size fractions and (b) plot of two principle components among PLFA individuals (mol%) from bulk soil and the five fractions. Symbols: black, bulk soil; red, >2000 μm; yellow, 2000–200 μm; green, 200–63 μm; purple, 63–2 μm; pink, 2–0.1 μm; circle, control (CK); square, fertilizer N (N); diamond, fertilizer N and P (NP); up-triangle, fertilizer N, P, and K (NPK); down-triangle; fertilizer N, P, and K, plus manure (NPKM); left triangle, manure (M).

with relatively lower C/N ratios indicated a high degree of organic matter humification in these fractions (Christensen, 2001; Chen et al., 2014).

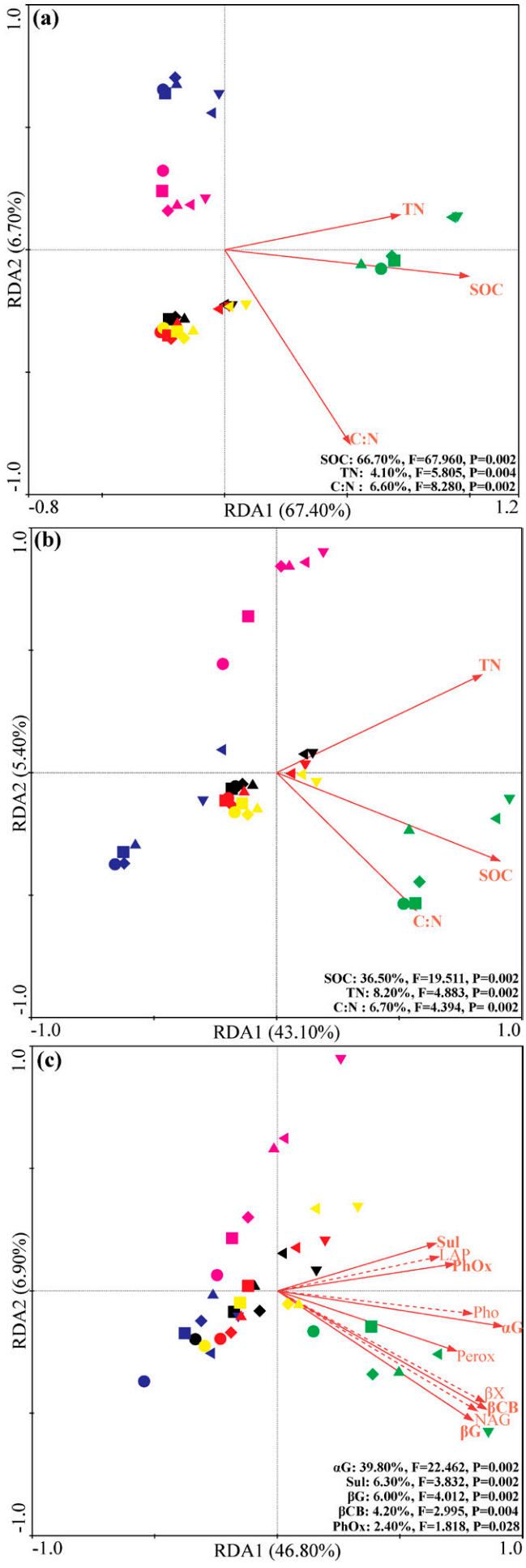
The accumulation of SOC and TN were largest in the coarse sand fraction, followed by the silt fraction and they were lowest in the fine sand fraction (Table 2). This might be caused by the high contribution of finer minerals (i.e., particulate organic matter) and clay to the coarse sand fraction during the formation of the larger-sized fraction (Six et al., 2000). Several publications have demonstrated that labile C and N originating predominantly from organic substrate are first incorporated into the coarse and fine sand fractions during the initial decomposition period and subsequently accumulated and become stable in the silt and clay fractions (Elliott, 1986; Six et al., 2000). However, other studies have shown that SOC in the silt and clay fraction was not always as stable as expected, and was greatly affected by management practices (Yu et al., 2012). This was also supported by our study, since manure application significantly accelerated SOC and TN accumulation in coarse sand fraction and, in contrast, reduced that in clay fraction (Table 2). This suggested that both coarse sand and clay fractions were sensitive to manure application.

Fertilization Effects on Enzyme Activities in Bulk Soil and 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). 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). Our findings affirmed that the majority of the variation in potential enzyme activities could be explained by soil nutrient availability (Fig. 5a), which are well known to be strongly influenced by fertilizer managements. The relationship is also reasonable for fractions with abundant labile C and N, such as the >200-μm particle size class showed by Marx et al. (2005) and the sand fraction of Allison and Jastrow (2006).

Manure delivers large amounts of organic materials into the soil, thereby stimulating the enzyme activities in these fractions (Liang et al., 2014). Our results are in agreement with this conclusion. Continuous application of manure significantly increased Pho, βG, βCB, NAG, βX, PhOx, and Perox activities compared with mineral fertilizer treatments in both bulk soil and the five particle-size fractions (Table S1), indicating an acceleration of C, N, P, and S cycling by manure addition. The effect of manure in increasing enzyme activities in particle-size fractions was also found by Kandeler et al. (1999a), who reported that application of organic fertilizer over 95 yr increased bX activity in all particle-size fractions in a long-term field experiment in a Haplic Phaeocem in Germany. Liang et al. (2014) also reported enhanced activities of invertase, βG, urease, Pho, and dehydrogenase in organic treatments.

Not only fertilizer treatment but also the particle-size fraction significantly affected enzyme activities (Fig. 2; Table S1). We observed that all enzyme activities (except Sul) were highest in the fine sand fraction. Enzymes involved in C cycling, including βG, βCB, NAG, and βX, but excluding αG, showed relatively higher activities in the fine and coarse sand fractions and followed the distribution pattern of SOC. This is probably because the large amount of labile C present in the sand fraction could be easily used by microorganisms for growth and enzyme production, for example, abundant polymeric material and cellulose which are important substrates of βG and βCB (Allison and Jastrow, 2006; Marx et al., 2005). Higher NAG activity might stem from enzyme production by microbes that degrade the chitin-rich cell walls of dead fungi, especially mycorrhizal



fungi (Guggenberger et al., 1999). Furthermore, Pho activity was higher in larger fractions and lower in finer fractions. This result supports that of Rojo et al. (1990), who found that Pho activity was mainly associated with large soil fractions (2000–100 μm) containing plant debris and less humified organic matter. Lagomarsino et al. (2009) also found the highest acid Pho activity in sand of a Calcaric–Gleyic Cambisol after a 13-yr tillage and fertilizer experiment in central Italy. Contrasting results were reported by Marx et al. (2005) who showed that the Pho activity was dominant in the clay-sized fractions under grassland in a silty clay loam at North Wyke, UK. These discrepancies suggested that the distribution of Pho might be influenced by various soil characteristics (Kandeler et al., 1999a).

The high recovery rates of enzyme activities validated the extraction method of Stemmer et al. (1998). We also observed the recovery of rates of several enzymes exceeded 100%, probably because of the liberation and activation of enzymes from soil fractions by sonication and wet-sieving (Liang et al., 2014). The increased accumulation of enzymes in manure treatment was mainly attributed to the significantly improved accumulation in coarse sand fraction (Table 2). The unequal distribution of enzyme activities among soil particle-size fractions has been reported previously (Saviozzi et al., 2007). In the current experiment, similar to SOC and TN, enzyme activities were mainly located in coarse sand and silt fractions. In general, the activities of carbohydrases (βG, βCB, NAG, and bX) are greatest in coarse sand and lowest accumulated in clay fraction (Stemmer et al., 1998; Kandeler et al., 1999b; Marx et al., 2005). Meanwhile, the enzymes involved in N (LAP), P (Pho), and S (Sul) transformations were dominant in both the silt and clay fractions (Kandeler et al., 1999a; Marx et al., 2005; Saviozzi et al., 2007) and in this study we found they were lowest in fine sand fraction.

Fertilization Effects on Microbial Community Composition in Bulk Soil and Particle-Size Fractions

Phospholipid fatty acid analysis is often used to fingerprint the composition and structure of soil microbial communities and measure their biomass (Williams and Hedlund, 2013). Our study showed that the composition of soil microbial communities was greatly affected by different particle-size fractions and fertilizer managements (Table 3). We found that total PLFAs and absolute abundance of microbial groups were heterogeneously distributed among the fractions, which were higher in larger fractions (>63 μm) and lower in smaller fractions; in

Fig. 5. Redundancy analyses (RDA) of the correlations between (a) soil enzyme activities to soil properties , (b) the correlations between soil properties and microbial community composition indicated by individual PLFAs (mol%), and (c) the correlations between soil enzyme activities and microbial community composition indicated by individual PLFAs (mol%). The red arrows indicate the soil parameters that had strong and significant impact on enzyme activities ($P < 0.05$), and corresponding explained proportion of variability was shown in the lower right corner. Symbols: black, bulk soil; red, > 2000 μm; yellow, 2000–200 μm; green, 200–63 μm; purple, 63–2 μm; pink, 2–0.1 μm; control (CK); square, fertilizer N (N); diamond, fertilizer N and P (NP); up-triangle, fertilizer N, P, and K (NPK); down-triangle; fertilizer N, P, and K plus manure (NPKM); left triangle, manure (M).

particular, the lowest abundance was obtained in the silt-size fraction (Tables 3 and S2). This may be because larger soil fractions had greater concentrations of SOC, and higher SOC provides more substrates for microbial proliferation, thus leading to higher microbial biomass (Mondini et al., 2006). This was confirmed by the results of RDA analysis which indicated the strong relationship between SOC, TN availability with the microbial community (Fig. 5b). Jiang et al. (2013) also found that SOC had a strong influence on total PLFAs and the biomass of different microbial functional groups within aggregates of an acid soil, and Briar et al. (2011) reported a positive correlation between total PLFAs and C concentrations in soil fractions of four management systems.

Bacterial enrichment is often reported for the smallest size fractions because of the favorable bacterial microclimate (Ranjard and Richaume, 2001). In this study, the relative abundance of bacteria was similarly affected by particle-size fraction as total PLFAs, which were highest in the fine sand fraction and lowest in the silt fraction (Table 3). According to van Gestel et al. (1996), the vicinity between bacteria, organic matter, and clay is required for the survival of bacteria, as both organic matter and clay particles offer substrates and nutrients to bacteria. This may explain why our clay-sized fraction had a relatively high proportion of bacteria. Generally, gram-negative bacteria preferentially use fresh organic inputs as C sources, whereas gram-positive bacteria are thought to favor older and more microbially processed organic matter (Fierer et al., 2003; Kramer and Gleixner, 2006). This was partially shown in our study as the relative abundance of gram-positive bacteria was higher in sand and clay fractions, whereas the distribution of gram-negative bacteria showed the opposite tendency. The greater G+/G− ratios in the clay-sized fractions compared with the >2000-μm fractions suggested that the clay fraction is depleted of easily decomposable substrate (Kramer and Gleixner, 2006). Reduced fungal abundance with decreasing soil particle size has been documented across soil and vegetation types (Kandeler et al., 1999b, 2000) and has been attributed to decreased bioavailability of C substrates in the smaller size fractions (Briar et al., 2011; Chiu et al., 2006). Fierer et al. (2003) reported that the fungi/bacteria ratio generally increases with increasing C/N ratio in soil because fungi have a lower N demand and use C more efficiently than bacteria. However, the differences in the relative abundance of fungi among the five fractions were not significant and consequently resulted in similar fungi/bacteria ratios in the five fractions which may suggest that fungi/bacteria was not an important contributing factor to the observed patterns in microbial composition in this study (Smith et al., 2014). Another reason for this was dominant bacteria in paddy soil may compete with fungi for the substrates thus resulting in the significant lower abundance of fungi and similar fungi/bacteria ratios among soil fractions (Qin et al., 2010).

Numerous studies have demonstrated that shifts in soil microbial community composition might occur as a result of changes to fertilizer management (Kennedy et al., 2004), which was shown to be the case in our experiment. The abundances of total PLFAs, bacteria, fungi and actinomycetes under organic treatments (NPKM and M) were significantly higher than those under CK and inorganic treatments in bulk soil (Tables 3 and S2). Manure plus fertilizer N, P, and K treatment also significantly enhanced the abundance of total PLFAs and specific microbial groups in the five fractions, except for fungal PLFAs in the large macroaggregate and silt fractions (Table S2). In addition, manure application reduced the proportion of gram-negative bacteria and increased the proportion of gram-positive bacteria in bulk soil, leading to the significantly low G+/G− ratio in organic treatments which is indicative of improved soil nutrition (Rajendran et al., 1997).

Principal component analysis conducted with relative abundance of individual PLFAs confirmed the key effects of soil nutrient availability on microbial community structure (Fig. 4a and b). Soil fertilizer treatments in the fine sand fraction and organic treatments of bulk soil and the five fractions, except for the silt fraction, were well separated along PC1. Different distributions of SOC and TN in soil could, in turn, drive microbial activity and community structure since all the samples mentioned above hold better improved nutrient conditions (Gude et al., 2012; Lagomarsino et al., 2009). Furthermore, the microorganisms that are naturally present in the manure also contribute to the enhanced microbial biomass in soil (Chu et al., 2007). Therefore, the strong relationships that were showed between SOC, TN, C/N ratio, αG, Sul, βG, βCB, and PhOx activities and the microbial community confirmed by RDA analyses (Fig. 5b and c) would not be surprised. The different size fractions, except fine sand fraction, did not separate well along PC1 and PC2, which might be because long-term flooded conditions during the rice season might blur the differences between each particle-size fraction.

The changes of biochemical and biological parameters in different particle-size fractions are often shadowed by other factors, possibly because of unknown land use history and soil management practices that strongly influence substrate availability and soil structure (Jiang et al., 2013). Most studies on the relationship between soil physical fractions and microbial communities have been conducted in temperate, agricultural soils, so their applicability to other systems is limited. Thus, we recommend soil fractionation as a promising approach that offers potential for understanding the relationship between soil structure and microbial abundance, composition and, ultimately, function across environmental gradients and a range of soil types.

CONCLUSION

Over the 33-yr period, long-term fertilization had a great impact on the soil particle distribution, and on the SOC, TN, soil enzyme activities and microbial community of different particle-size fractions. The application of manure significantly contributed to the improvement in SOC, TN, and most enzyme activities, which subsequently influenced the microbial community composition. This was supported by the strong relationships between SOC, TN, C/N ratio, αG, Sul, βG, βCB, and PhOx with PLFA profiles. Larger fractions (>63 μm), especially

the fine sand fraction, showed relatively higher C, N concentrations, C/N ratios, Pho, βG, βCB, βX, NAG activities, and total PLFA abundance than the smaller fractions (63–0.1 μm). The coarse sand fraction contained the greatest contents of C, N, and enzyme activities, followed by the silt fraction and the large macroaggregate fraction. In summary, long-term application of organic fertilizers enhanced soil fertility and microbial activity. The consistent differences in biological characteristics and soil chemistry among soil fractions across fertilizer managements suggested that the different particle-size fractions represented different C and N pools with potentially different turnover rates, which consequently led to changes in soil biological processes.

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