Applied Soil Ecology 111 (2017) 65–72

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Soil aggregation regulates distributions of carbon, microbial community and enzyme activities after 23-year manure amendment

Yidong Wang[a], Ning Hu[b], Tida Ge[c], Yakov Kuzyakov[d], Zhong-Liang Wang[a], Zhongfang Li[e,*], Zheng Tang[e], Yi Chen[f], Chunyan Wu[f], Yilai Lou[b,*]

[a] Tianjin Key Laboratory of Water Resources and Environment, Tianjin Normal University, Tianjin 300387, China
[b] Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[c] Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410215, China
[d] Department of Soil Science of Temperate Ecosystems, Department of Agricultural Soil Science, University of Göttingen, Göttingen 37077, Germany
[e] Chemistry and Bioengineering College, Hezhou University, Hezhou 542899, China
[f] Institute of Environmental Resources and Soil Fertilizer, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China

ARTICLE INFO

Article history:
Received 15 March 2016
Received in revised form 14 November 2016
Accepted 26 November 2016

Keywords:
Manure addition
Aggregate formation
Soil organic matter
PLFAs
Microbial community
Enzyme activity

ABSTRACT

Manure amendment affects soil organic carbon (SOC) sequestration, microbial biomass and activity, and aggregate formation. However, how soil aggregation regulates SOC sequestration and microbial activity after manuring has received less attention. We studied the distribution of SOC, microbial community composition and activity in four aggregate classes (>2, 1–2, 0.25–1, and <0.25 mm) using field moisture sieving of soil from a 23-year manure addition field experiment under a rice–barley rotation. Long-term manuring increased the portion of large macroaggregates (>2 mm) by 2.4% ($p < 0.05$), and reduced the portion of microaggregates (<0.25 mm, including sand and silt) by 5.9% ($p < 0.05$) compared with soil without manure (control). Manuring increased SOC and total nitrogen contents of the large macroaggregates by 9.1% and 7.1%, respectively, but not of the microaggregates. Also, manuring increased the phospholipid fatty acids (PLFAs) contents of bacteria, fungi, arbuscular mycorrhizal fungi, and total microbes of the macroaggregates (>2, 1–2, and 0.25–1 mm) but not of the microaggregates. The fungal/bacterial PLFA ratio remained unchanged in all aggregates. Manuring increased β-glucosidase and chitinase activities in two macroaggregate classes (>2, and 1–2 mm), but not in the microaggregates. In conclusion, SOC, microbial biomasses and enzyme activities in the macroaggregates are more sensitive to manuring than in the microaggregates. Soil aggregation regulates the distributions of SOC and microbial parameters after 23-year manure amendment.

1. Introduction

Agroecosystem productivity and sustainability are dependent on soil structure and fertility. Aggregation is the formation of soil structure, physically protecting organic matter (OM) from microbial decomposition, regulating water, gas and nutrient dynamics, and reducing erosion (Jastrow, 1996; Six et al., 2004; Jastrow et al., 2007). Increasing aggregate-protected soil organic carbon (SOC) has potential to mitigate climate change because SOC decomposition is governed by accessibility by decomposers (Dungait et al., 2012).

Soil aggregates, composed of primary particles and binding agents, are the basic units of soil structure (Bronick and Lal, 2005). Two functional aggregate classes are commonly differentiated. Microaggregates (<0.25 mm) comprise primary mineral granules and organic debris, and macroaggregates (>0.25 mm) comprise microaggregates and particulate OM (Tisdall and Oades, 1982; Gupta and Germida, 1988; Miller and Jastrow, 1990). SOC is embedded and bound within hierarchical aggregates, and SOC is likely inaccessible by decomposers within microaggregates rather than within macroaggregates (Bird et al., 2002; Tian et al., 2015). The formation and turnover of macroaggregates are critical processes influencing SOC dynamics (Six et al., 2004).

Manure amendment firstly promotes aggregates formation and its-associated carbon (C) incorporation due to direct and indirect microbial-derived binding agents (Aoyama et al., 1999a; Mikha and Rice, 2004; Six et al., 2004; Ding and Han, 2014). The

* Corresponding authors.
E-mail addresses: lizhongfang08@126.com (Z. Li), louyilai@caas.cn (Y. Lou).

http://dx.doi.org/10.1016/j.apsoil.2016.11.015
0929-1393/

microaggregates, as preliminary aggregates, generally respond little to manuring (Mikha and Rice, 2004). In contrast, the macroaggregates occlude more manure-derived SOC due to the physical entrapment of particulate OM (Aoyama et al., 1999b; Bhattacharyya et al., 2009; Yu et al., 2012). Furthermore, manuring affects crop growth and further influences aggregate-associated SOC. Finally, increase in crop-derived SOC resulted from manure amendment stimulates microbial activities (Fontaine et al., 2007; Kuzyakov, 2010). Therefore, it is important to differentiate how manuring affects SOC between micro- and macro-aggregates with different stability and turnover rates.

Microbial biomass and community are primarily influenced by soil structure and substrate availability (Elliott and Coleman, 1988; Garcia-Franco et al., 2015; Li et al., 2015). Microbial biomass generally increases with aggregate size (from micro- to macro-aggregates) (Kanazawa and Filip, 1986; Helgason et al., 2010) due to the increasing OM amount (Chen et al., 2015). Therefore, manuring was reported to increase microbial biomass and change microbial community composition through forming aggregates and bringing surplus of OM (Zhang et al., 2015). In turn, the shift in microbial community further affects the aggregation (Gupta and Germida, 1988) and the turnover of encapsulated SOC. In general, fungi and bacteria have partly different functions in SOC turnover and stabilization (Jastrow et al., 2007). For example, fungi are more important for macroaggregate formation (Ding and Han, 2014) because of their hyphae as compared with bacteria (De Gryze et al., 2005). Fungi predominantly proliferate in larger pores among macro- and micro-aggregates; whereas bacteria reside in smaller pores within microaggregates (Tisdall and Oades, 1982; Ding and Han, 2014). However, the redistribution of microbial groups within macro- vs. micro-aggregates after long-term manuring has received little attention in the literature.

Enzyme activity is highly sensitive and related to C turnover (Aon et al., 2001; Wang et al., 2015a). Even though the C turnover in aggregates has received much attention, there are only few studies investigating the impacts of soil structure (macro- vs. micro-aggregates) on enzyme activities (Dorodnikov et al., 2009). The relationships between aggregate size and enzyme activities were found to be complex, and both positive (Kanazawa and Filip, 1986; Gupta and Germida, 1988) and negative relationships (Allison and Jastrow, 2006; Dorodnikov et al., 2009; Wang et al., 2015a) were reported. Because of the higher C accumulation level of macro-aggregates compared with microaggregates, we hypothesize higher increase of enzyme activities in macroaggregates than that in microaggregates in response to manuring.

The lower plain of the Yangtze River is one of the most important agricultural regions in China (Wang et al., 2015b). The croplands in this region have been under intensive management with the use of conventional chemical fertilizers or additional manure to ensure high grain yields for a long period. However, it is not clear how additional manure amendment affects the distributions of SOC, microbial community composition and enzyme activities within macro- vs. micro-aggregates.

The objectives of this study were to investigate changes of SOC and microbial characteristics within micro- vs. macro-aggregates in response to a 23-year manure amendment in a rice–barley rotation system. We hypothesize that: (1) manuring increases macroaggregate-associated SOC but has little effect on microaggregate-associated SOC; consequently, (2) manuring increases microbial biomass and enzyme activities in macro-aggregates but has little effect on those in microaggregates; and (3) higher fungi/bacteria biomass increase in macro-aggregates compared to in microaggregates when manured because fungi are more important than bacteria in macroaggregate formation.

2. Materials and methods

2.1. Study site

This study was carried out in a rice–barley rotation field (30°26′04″ N, 120°25′01″ E, elevation 3–4 m a.s.l.) at the National Monitoring Station for Soil Fertility and Fertilizer Efficiency in Hangzhou (Zhejiang Province, China). The study site is flat and well-drained. The region is characterized by a subtropical humid monsoon climate, with a mean air temperature of 16–17 °C, an annual rainfall of 1500–1600 mm, an annual evapotranspiration of 1000–1100 mm, an annual frost-free period of 240–250 d and an annual sunshine duration of 1900–2000 h (Wang et al., 2015b). The soil, classified as an Inceptisol (US Soil Taxonomy), has a loam texture with 42% sand, 38% silt, and 20% clay (Chen et al., 2010a, 2010b).

The long-term field experiment was launched in autumn 1990. Prior to the formal experiment, the field had been intensively cultivated for more than 30 years with conventional chemical fertilizers, and afterward, the soil was homogenized by growing barley, early rice and late rice in rotation for 2 years (from autumn 1988 to autumn 1990) without fertilization. The main characteristics of the initial soil (0–20 cm) in 1990 were as follows: bulk density, 1.24 g cm^{-3}; porosity, 53.2%; SOC, 16.6 g kg^{-1}; total N, 1.67 g kg^{-1}; available N, 94.1 mg kg^{-1}; total phosphorus (P), 2.53 g kg^{-1}; available P, 37.4 mg kg^{-1}; available potassium (K), 67.5 mg kg^{-1}; cation exchange capacity (CEC), 14.6 cmol kg^{-1}; and pH, 6.4.

2.2. Experimental design

Two fertilization regimes, conventional chemical NPK fertilization (CK), and the NPK fertilization combined with composted pig manure amendment (manuring) were established using a random design. The manuring treatment had same amounts of N, P, and K fertilization with same chemical fertilizers as the CK treatment. Each fertilization regime has three field replicates. The N, P and K fertilizations were applied as urea (46% N), calcium superphosphate ($CaP_2H_4O_8$, 7.0% P) and potassium chloride (KCl, 49.8% K), at rates of 315 kg N ha^{-1} $year^{-1}$, 68.7 kg P ha^{-1} $year^{-1}$ and 130.7 kg K ha^{-1} $year^{-1}$, respectively. The fresh composted pig manure, with 68.9% gravimetric water content, was applied at a rate of 22.5 Mg ha^{-1} $year^{-1}$. The dry matter of pig manure had a C content of 197 g kg^{-1}, N content of 14.5 g kg^{-1}, P content of 14.2 g kg^{-1}, and K content of 13.1 g kg^{-1}. Thus, the application rates of C, N, P and K of pig manure are equivalent to 1.4 Mg C ha^{-1} $year^{-1}$, 101 kg N ha^{-1} $year^{-1}$, 99.5 kg P ha^{-1} $year^{-1}$ and 91.8 kg K ha^{-1} $year^{-1}$, respectively.

Each replicated plot has an area of 100 m^2. During the first 10 years (1990 – 2000), a rotation of barley–early rice–late rice was arranged for annual cropping. The annual fertilization quotas were 20% for barley, 40% for early rice, and 40% for late rice. After 10 years, soils were converted to barley–rice rotation, and the annual fertilization quotas were 32% for barley and 68% for rice. In each single growing season, the pig manure, P, and K and 70% N were applied as base fertilizer; while the remaining 30% N was used as top dressing. All other management practices (e.g. tillage and planting) were the same for both treatments. The barley was seeded in late November and harvested in early May of the next year in both the two and three crops of annual rotations over the 23-years. In the three crops rotation, the early rice was seeded in mid-May and harvested in late July, and the late rice was seeded in late July and harvested in early November. In the two crops rotation, the rice was seeded in middle June and harvested in the early November. The barley and rice were both harvested by hand. The aboveground biomass was removed leaving less than 3-cm stubble (Wang et al., 2015b).

2.3. Soil sampling and aggregate size fractionation

Soil was sampled from 0 to 20 cm depth in each replicated plot using a soil auger after the rice harvest in November 2013. This sampling time was chosen to minimize the in-season influences such as plant growth and fertilization. Fresh soil samples were stored on ice during transport back to the laboratory for processing. The gravimetric soil water content, approximately 15%, was suitable for aggregates fractionation according to previous studies (Dexter and Bird, 2001; Kristiansen et al., 2006; Helgason et al., 2010).

Four aggregates size classes (>2, 1–2, 0.25–1, and <0.25 mm) were separated through field moisture sieving of fresh soils using the combination of sieving procedure described by Kristiansen et al. (2006) and Helgason et al. (2010). Briefly, 400 g fresh soils were sieved using the 2-mm sieve for 15 s on a Retsch AS200 sieving machine (Retsch GmbH., Haan, Germany) and the retained macroaggregates (>2 mm) were collected. The passed soil was then transferred to 1-mm sieve for a further 20 s sieving and the residual aggregates (1–2 mm) were isolated. The soil passing 1-mm sieve was sieved using 0.25-mm sieve for 45 s and the other two aggregate fractions (0.25–1 mm and <0.25 mm) were finally separated. All visible plant residues, fauna and stones were removed before the sieving procedure. Preliminary tests showed that the sieving durations were sufficient to quantitatively isolate the four aggregates size classes while minimizing aggregate abrasion during the sieving. In this way, four aggregates size classes were obtained: >2 mm (large macroaggregates), 1–2 mm (moderate macroaggregates), 0.25–1 mm (small macroaggregates), and <0.25 mm (microaggregates, also including sand and silt).

2.4. Bulk density, soil moisture, SOC and total N

Bulk density was measured using a conventional core method with a volume of 100 cm^3 after removing stones. Gravimetric soil water content was determined by measuring water loss from fresh soil after drying at 105 °C for 8 h. SOC and total N (TN) of each aggregate class was analyzed using the air-dried soil by an elemental analyzer (Elementar, Germany).

2.5. Phospholipid fatty acids analysis

Phospholipid fatty acids (PLFAs) analysis was performed using the method described by Helgason et al. (2010). Briefly, fatty acids were extracted from 4 g freeze-dried soils using a single phase chloroform, methanol, phosphate buffer solution. The isolated fatty acid methyl esters (FAMEs) were analyzed by a gas-chromatography mass-spectroscopy system (TRACE GC Ultra ISQ, Thermo Fisher Scientific) (carrier gas: helium) using a DB-5 column with 30 m length, 0.25 mm I.D., and 0.25 μm film thickness. The temperature program started at 150 °C for 4 min, thereafter the temperature was ramped to 250 °C at a rate of 4 °C min^{-1} and held 5 min. The PLFAs were identified by a comparison of retention times to known standards (FAME 37 47885-U, Supelco, Inc.) and a standard bacterial acid methyl ester mixture (BAME 26 47080-U, Supelco, Inc.) (Xu et al., 2015). PLFAs contents (nmol g^{-1} dry weight soil) were quantified based on the internal standard (nonadecanoic acid methyl ester 19:0).

The sum of thirteen PLFAs (i15:0, a15:0, 15:0, i16:0, 16:1w9, 16:1w7t, i17:0, a17:0, 17:0, cy17:0, cy19:0, 18:2w6 and 18:1w9c) serves as a measure of total microbial biomass (nmol g^{-1}). PLFAs i15:0, a15:0, 15:0, i16:0, 16:1w9, 16:1w7t, i17:0, a17:0, 17:0, cy17:0 and cy19:0 refer to bacterial biomass (Frostegård and Bååth, 1996; Zogg et al., 1997). Fatty acids 18:2w6 and the isomer 18:1w9c are used as indicators for fungal biomass (Frostegård et al., 1993). The 16:1w5c is the biomarker of arbuscular mycorrhizal fungi (AMF) (Olsson, 1999).

2.6. Measurements of enzyme activities

Using fluorogenically labelled substrates according to the procedures of Dorodnikov et al. (2009), we measured activities of four enzymes: (1) β-glucosidase (BG) which catalyzes one of the final steps in cellulose breakdown, (2) chitinase (*N*-acetyl-β-D-glucosaminidase, NAG) which is involved in chitin degradation, (3) phosphatase which aids the decomposition of deoxyribonucleotide/ribonucleotide triphosphates and proteins, and (4) sulfatase which catalyzes the hydrolysis of sulfate esters of a range of substrates, including steroids, carbohydrates and proteins. Briefly, four 4-methylumbelliferone (MU)-labelled fluorogenic substrates were used: MU-β-D-glucopyranoside (MU-BG; EC 3.2.1.21) for the determination of BG activity, MU-*N*-acetyl-β-D-glucosaminidase dehydrate (MU-NAG; EC 3.2.1.14) for NAG activity, MU-phosphate disodium salt (MU-phosphate; EC 3.1.3.2) for phosphatase activity, and MU-sulfate potassium salt (MU-sulfatase; EC 3.1.6) for sulfatase activity. 2 mL of 2-methoxyethanol (Hoppe, 1983) was used to dissolve the MU-substrates. Predissolved MU-substrates were further diluted with sterile distilled water to give the desired contents. All chemicals were derived from Sigma-Aldrich (Germany).

The fresh soil samples (1 g in dry weight) were suspended in 20 mL sterile distilled water. The suspensions were shaken forcefully for 15 min to ensure thorough mixing. 1 mL of the suspension was added to 3 mL MU-substrate solution (containing either 400 μmol MU-BG, or 200 μmol MU-NAG, or MU-phosphate, or MU-sulfatase) which already pipetted in deep-well plates (24-wells × 10 mL, HJ-Bioanalytik GmbH, Germany), incubating at 25 °C for 4 h. The calibration solutions were prepared using 1 mL soil suspension and 3 mL MU of different concentrations (0–100 μmol). The deep-well plates with soil-MU-substrate and soil-MU-calibration were centrifuged at 20*g* for 5 min. Subsequently, 0.5 mL of the supernatant was pipetted to the 24-well microplates (Becton Dickinson, Franklin Lakes, NJ, USA) which already contained 1.25 mL sterile distilled water and 0.25 mL of 20 mmol glycine–NaOH buffer solution (pH 11) to stop enzyme reactions. Fluorescence was measured in microplates at an excitation wavelength of 365 nm and an emission wavelength of 460 nm using a microplate reader (Bio-Tek FLx800, USA). Calibration curves were included in every series of enzyme measurements. Enzyme activities were expressed as MU release in micromolar per gram bulk soil/aggregates in dry weight and per hour.

2.7. Recovery calculation and statistical analysis

The recoveries of SOC, TN, PLFAs, and enzyme activity were calculated as the ratios of the weighted sum of four aggregate classes to the entire soil. Two-way analysis of variance (ANOVA) was used to test the main effects of manuring, aggregate sizes and their interacting effects on a SOC, TN, PLFAs, and enzyme activities. Multiple comparisons were performed using a one-way ANOVA with a Tukey's HSD post hoc test of SPSS 17.0 for Windows (SPSS Inc., Chicago, Illinois). Principal components analysis (PCA) of soil and microbial properties in aggregate size classes was based on the CANOCO software 4.5. The data were log(x + 1)-transformed during PCA. Figures were prepared in ORIGIN 9.0 (OriginLab Corporation, Northampton, MA).

3. Results

3.1. Soil aggregates size distribution

Compared with the CK, the manuring significantly increased the portion of large macroaggregates (>2 mm) by 2.4% ($p < 0.05$), and reduced the portion of microaggregates (<0.25 mm) by 5.9% ($p < 0.05$). In contrast, the manuring had no significant effect on the percent of other two macroaggregate classes (1–2, and 0.25–1 mm) (Fig. 1a). Under both the CK and manuring treatments, the percentage of different aggregate classes decreased in following order: large macroaggregates (> 2 mm) > moderate macroaggregates (1–2 mm) > small macroaggregates (0.25–1 mm) > microaggregates (<0.25 mm) (Fig. 1 a).

3.2. Soil organic C, and total N in bulk soil and aggregate classes

Compared with the CK, the manuring significantly increased the SOC contents of two macroaggregate classes (9.1% for >2 mm, and 12.4% for 0.25–1 mm) and of the bulk soil (15.2%) ($p < 0.05$), but not of the microaggregates (Fig. 1b). Under both the CK and manuring treatments, all macroaggregates had higher SOC contents than the microaggregates (Fig. 1). Therefore, manuring and aggregate size both had significant effects on SOC contents ($p < 0.01$), however, there were no interactions ($p = 0.29$). Manuring significantly increased the TN contents of the large macroaggregates (7.1% for >2 mm) and the bulk soil (10.3%), but not for the microaggregates (Fig. 1c). The C/N ratio was not affected by the manure addition (Fig. 1d). The recoveries of SOC and TN were 99.1 ± 4.6% and 101.1 ± 2.4%, respectively, in isolated aggregates.

3.3. Microbial PLFAs

Manuring significantly increased the total microbial, bacterial, fungal, and AMF PLFAs of all macroaggregate classes and the bulk soil ($p < 0.05$), but not of the microaggregates (Fig. 2a–c and e). Manure amendment did increase F/B ratio numerically in all size classes except microaggregates but had no significantly influence on the ratio of F/B-PLFAs ($p > 0.05$) (Fig. 2d). Under the CK groups, the macroaggregates had similar total microbial and bacterial PLFAs and F/B-PLFAs ratios, but had higher fungal (> 2 mm) and AMF (1–2 mm) PLFAs compared with the microaggregates. However, under the manuring groups, the macroaggregates (>2 and 1–2 mm) had higher contents of the total microbial, bacterial, fungal, and AMF PLFAs and F/B-PLFAs ratio than the microaggregates (Fig. 2). Manuring and aggregate size had significant effects on microbial PLFAs ($p < 0.01$). The recoveries of total, bacterial, fungal, and AMF PLFAs were 103.6 ± 10.7%, 103.4 ± 12.1%, 96.1 ± 6.4%, and 106.6 ± 7.1%, respectively, in isolating aggregate classes.

3.4. Enzyme activities

Compared with the CK, manuring increased the activities of β-glucosidase and chitinase (*N*-acetyl-β-D-glucosaminidase) in the macroaggregates (>1 mm) as well as in the bulk soil, but not in the microaggregates (Fig. 3a and b). However, the manuring had no significant effect on the activities of the phosphatase (except phosphatase in >2 mm macroaggregates) and sulfatase in all aggregates (Fig. 3c and d). Under both the CK and manuring treatments, the macroaggregates usually had lower activities in

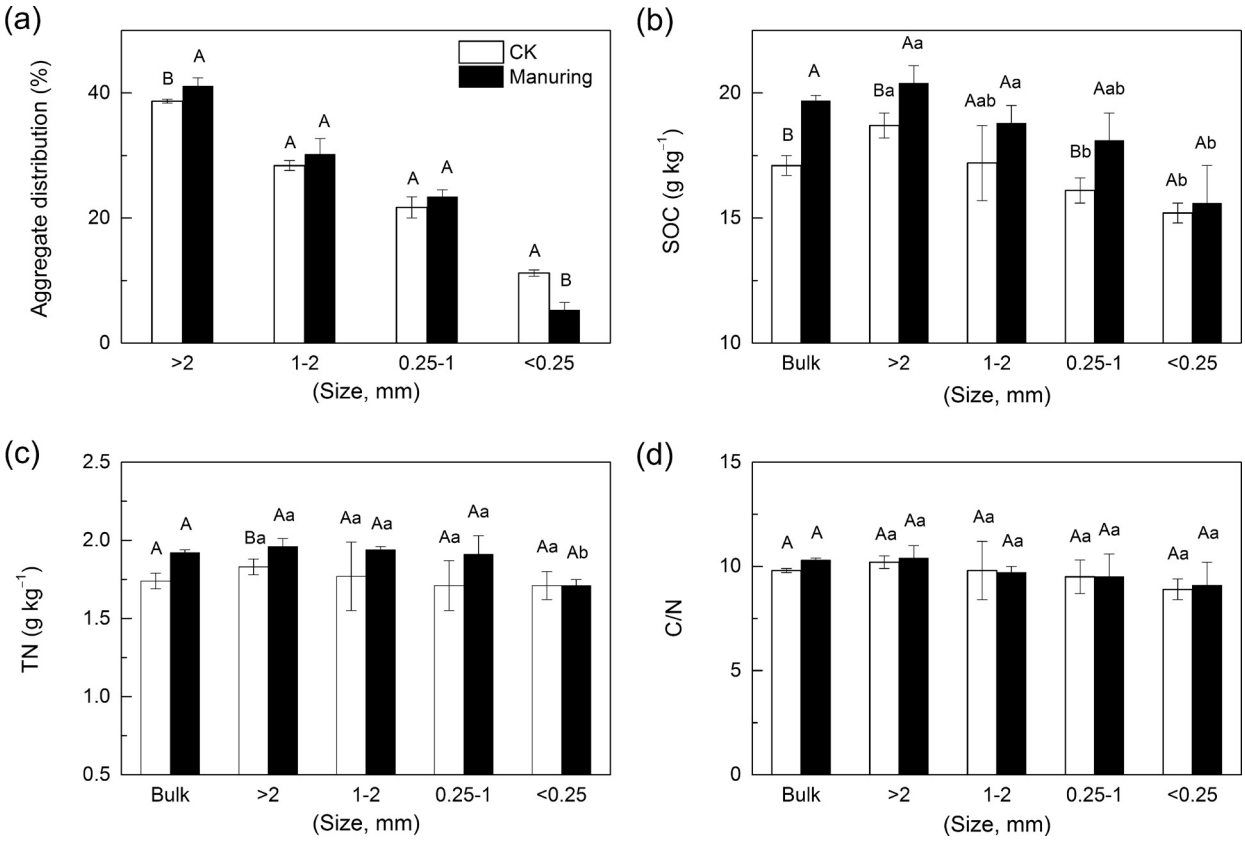

Fig. 1. Soil aggregate classes and their characteristics in SOC, TN and C/N (dry weight basis) of the control (CK) and manuring treatments ($n = 3$). Each value refers to mean ± standard deviation (SD). Uppercase letters denote differences between the CK and manuring at $p < 0.05$ level. Lowercase letters indicate differences among the four isolated aggregate classes at $p < 0.05$ level.

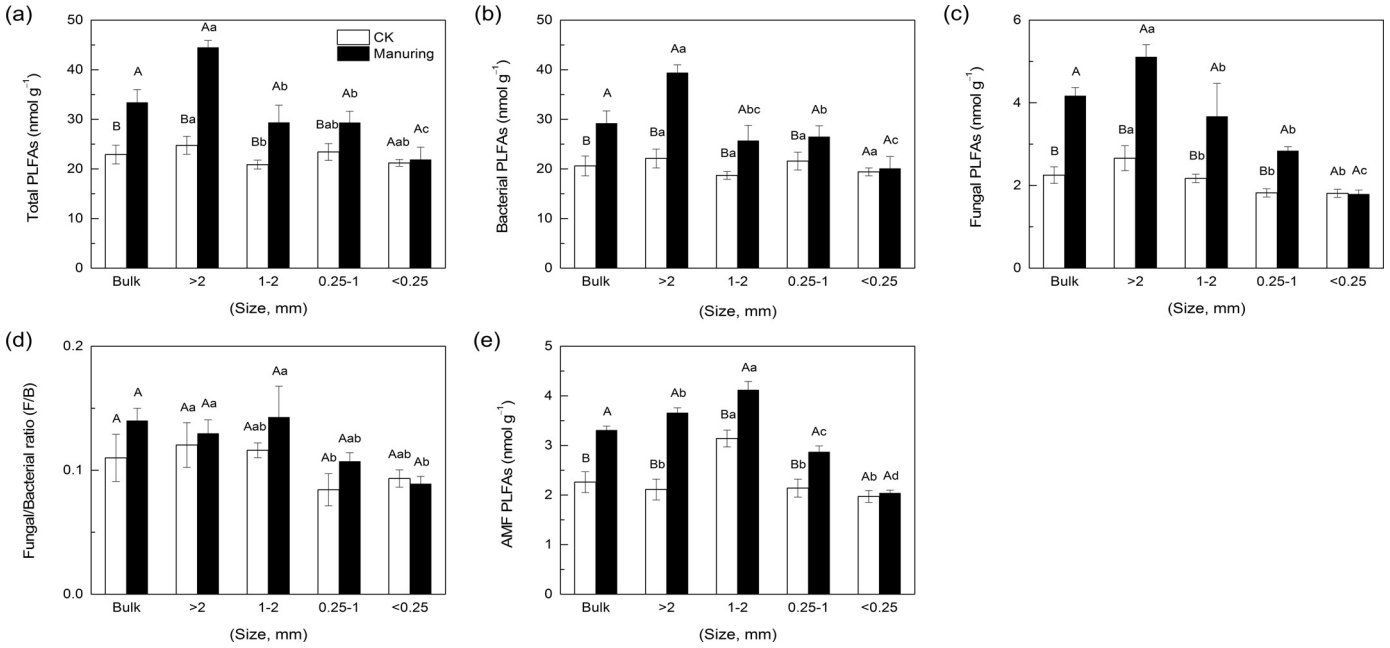

Fig. 2. Microbial total, bacterial, fungal, arbuscular mycorrhizal fungi (AMF) PLFAs, fungal/bacterial-PLFAs ratio (F/B) ($n = 3$). The error bar represents SD. Uppercase letters refer to differences between the CK and manuring at $p < 0.05$. Lowercase letters indicate differences among the four isolated aggregate classes at $p < 0.05$.

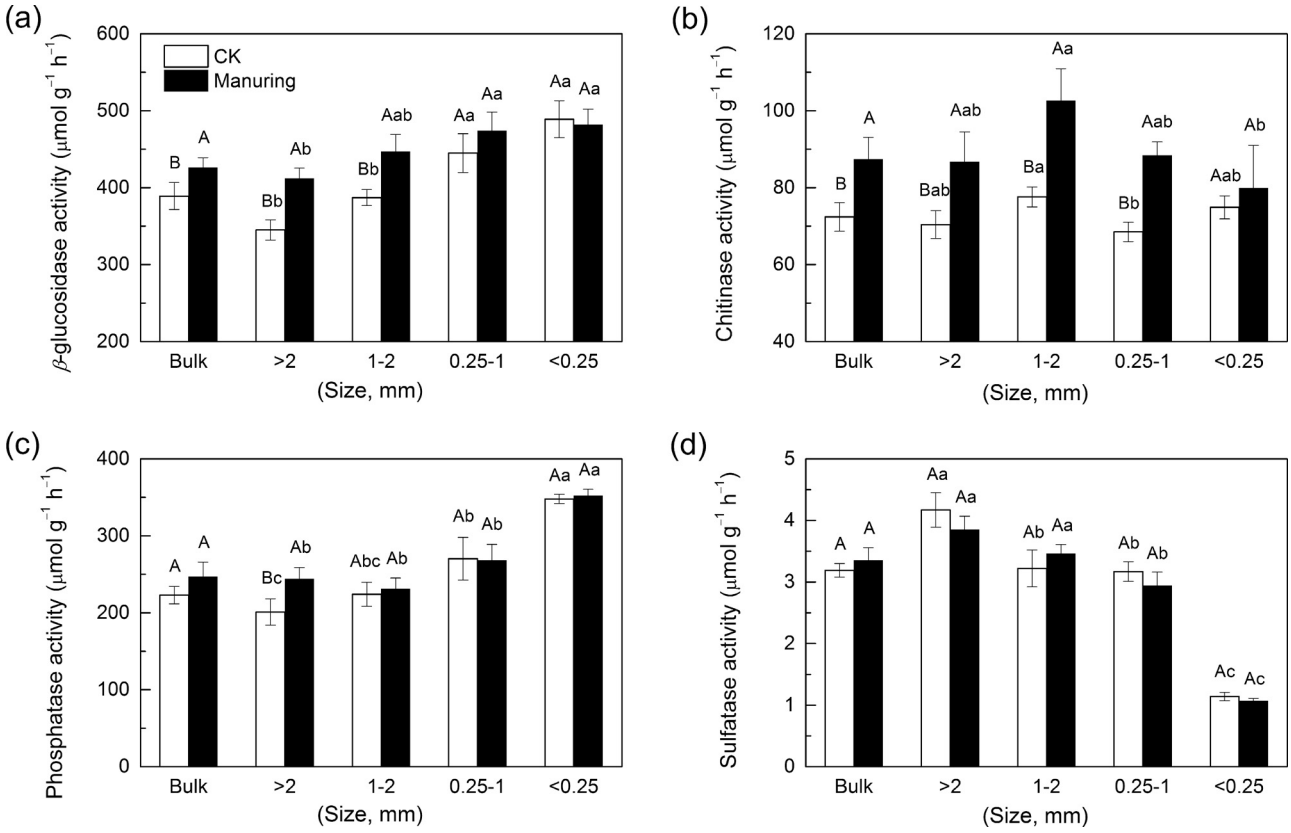

Fig. 3. The activities of β-glucosidase, chitinase (*N*-acetyl-β-D-glucosaminidase), phosphatase, and sulfatase ($n = 3$). The error bar represents SD. Uppercase letters refer to differences between the CK and manuring at $p < 0.05$. Lowercase letters represent differences among the four isolated aggregate classes at $p < 0.05$.

β-glucosidase and phosphatase, similar activity in chitinase, but higher activity in sulfatase as compared with the microaggregates (Fig. 3). This result indicated that microbial biomass, enzyme activities and occluded SOC are not always linked. In addition, manuring and aggregate size had interacting effects on β-glucosidase ($p = 0.024$), but not on the other three enzyme activities ($p = 0.09$–0.16). The recoveries of the four enzymes activities varied from 100.9 ± 9.5% to 106.1 ± 7.2% in separating aggregates.

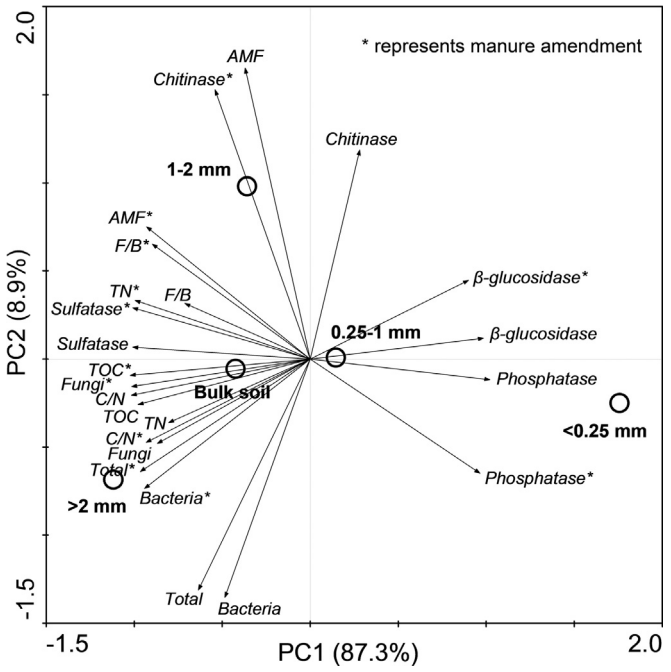

Fig. 4. Principal components analysis (PCA) of soil properties and microbial parameters in aggregate size classes. Black circles are aggregate size classes. Solid arrows are soil properties and microbial parameters. * represents manure amendment.

With manure amendment, most chemical properties and microbial parameters changed in macroaggregates, especially in >2 mm macroaggregates, but not in microaggregates (Fig. 4). The 1st and 2nd principal components (PC1 and PC2) explained 87.3% and 8.9% of total variance, respectively (Fig. 4). The result suggests that SOC, microbial biomass and enzyme activities in the macroaggregates are more sensitive to manure amendment than those in the microaggregates.

4. Discussion

4.1. Effect of manuring on aggregation and aggregate-associated SOC

Manuring increased binding agents thus stimulating soil aggregation, especially the formation of macroaggregates. This result supports our hypothesis that manuring increases macroaggregate-associated SOC. The improvement of soil structure is likely directly attributed to the 23-year manuring because there was no change in crop biomass reported by our previous study (Wang et al., 2015b). The result suggested that long-term manuring greatly improved the soil structure and most likely the soil aeration and water holding capacity, which is consistent with previous studies (Aoyama et al., 1999a; Mikha and Rice, 2004; Six et al., 2004; Chen et al., 2015). Therefore, long-term manure amendment is recommended for improving soil structure and fertility.

Based on the aggregate hierarchy concept (Tisdall and Oades, 1982; Oades, 1984; Six et al., 2004), the process of aggregate formation is from micro- to macro-aggregates. Manuring-brought SOC were likely fixed, adsorbed or immobilized during macroaggregate formation. Compared with microaggregates, macroaggregates occlude more particulate organic C and have higher SOC saturation level (Aoyama et al., 1999b; Bhattacharyya et al., 2009; Yu et al., 2012). Our experimental fields had been intensively cultivated over 30 years prior to the experiment and further 23 years across the experiment. This long-term cultivation without manuring allowed the fields reaching a relatively stable but low SOC level (Wang et al., 2015b). Therefore, it is reasonable to suggest that manuring increased the SOC accumulation in the macroaggregates, which further supports the above findings (Fig. 1). However, long-term manuring did not change the SOC level in the microaggregates, which also supported our hypothesis that manuring has little effects on microaggregate-associated SOC. This could be explained by the following three lines of reasoning. Firstly, manure-derived C at current input level is unlikely to reach microaggregates because of the priority of C localization in macroaggregates. The second possibility is that the turnover of microaggregates (destruction + formation) was too slow to incorporate significant amounts of C from manuring. Thirdly, the SOC in microaggregates was at a relatively stable level prior to the present experiment even with long-term cultivation history. Therefore, manure amendment is recommended for improving SOC sequestration.

4.2. Responses of aggregate-associated microbial groups to manuring

The field moisture sieving of soil (Kristiansen et al., 2006; Dorodnikov et al., 2009; Helgason et al., 2010) was used to separate aggregates into four size classes to study indigenous aggregate-associated microbial characteristics. The conventional wet sieving or air-dried soil sieving procedures were avoided because both methods disturbed the original indigenous microbial inhabitants within and on the aggregates and changed the microbial activities (Dorodnikov et al., 2009; Helgason et al., 2010). The high recoveries of SOC and TN further proved the advantage of the field moisture sieving of soil method. In contrast to the procedure by Kristiansen et al. (2006), however, we did not separate free sand and silt fractions (<53 μm) from the microaggregates because this procedure required stronger drying of field moisture soil leading to bias in microbial analysis. Therefore, the current field moisture sieving method may have noise on the microbial parameters of the microaggregates (>53 μm and <250 μm).

In the CK groups, we found that the macroaggregates, compared with the microaggregates, had higher fungal and AMF PLFAs, but similar bacterial PLFAs. The localization of microbial colonies within the soil matrix is a critical factor for their growth (Chenu et al., 2001). Higher inclusions of fungi and AMF in the macroaggregates are reasonable because these two groups predominantly proliferate in larger pores (Tisdall and Oades, 1982) and so, promote the formation of the macroaggregates (De Gryze et al., 2005; Wilson et al., 2009; Ding and Han, 2014). In contrast, three reasons may explain the similar contents of bacterial PLFAs between the microaggregates and macroaggregates. Firstly, bacteria in the microaggregates have equal (or even better) access to water, nutrients and oxygen, compared with those in the macroaggregates. Secondly, bacteria predominantly reside in smaller pores within microaggregates (Tisdall and Oades, 1982; Ding and Han, 2014). Finally, the microaggregates and macroaggregates had similar TN content. In the manuring groups, macroaggregates had higher bacterial PLFAs compared with the microaggregates. This difference may be resulted from the higher C availability in macroaggregates (Kanazawa and Filip, 1986; Helgason et al., 2010; Chen et al., 2015).

Manuring increases microbial biomass (Chen et al., 2015; Zhang et al., 2015), especially in the macroaggregates (Gupta and Germida, 1988). We also found increase in microbial biomass in the macroaggregates, but not in the microaggregates with the manuring treatment. It is widely reported that manuring increases the formation of macroaggregates and availability of OM to microbial activities within the macroaggregates (Chen et al., 2015; Li et al., 2015; Zhang et al., 2015). In contrast, there was no change in SOC content in the microaggregates under manuring, which

limited microbial growth. However, the F/B ratios of different aggregate classes did not change under the 23-year manure amendment, which rejected the third hypothesis. Fungi generally have higher C/N ratio compared with bacteria (10 vs. 4) (Sterner and Elser, 2002). The F/B ratio was reported to be positively correlated with the C/N ratio (Deng et al., 2016). In this study, the low C/N ratio (13.6) of the composted pig manure may be responsible for the absence of F/B ratio change after manuring.

4.3. Responses of aggregate-linked enzyme activity to manuring

Microorganisms secrete enzymes to decompose OM. Among them, β-glucosidase is for cellulose breakdown and *N*-acetyl-β-D-glucosaminidase is for chitin degradation (Allison and Jastrow, 2006). Meanwhile, phosphatase and sulfatase are two nutrient-acquiring enzymes for stoichiometric growth and enzyme synthesis (Sinsabaugh et al., 1993; Štursová and Baldrian, 2011). As related to active microorganisms (Blagodatskaya and Kuzyakov, 2013), enzyme activities are used to investigate the microbial response to manuring. In the present study, manuring enhanced the activities of β-glucosidase and *N*-acetyl-β-D-glucosaminidase in the macroaggregates but not in the microaggregates. This is because (1) the β-glucosidase and *N*-acetyl-β-D-glucosaminidase are both involved in SOC degradation (Allison and Jastrow, 2006; Dorodnikov et al., 2009), (2) the macroaggregates accumulated more SOC under manuring, but not for the microaggregates, and (3) the increment of fungal biomass is thought to drive the *N*-acetyl-β-D-glucosaminidase activity and fungi predominantly reside in the macroaggregates (Miller et al., 1998; Chung et al., 2007).

Aggregates, especially microaggregates, physicochemically protect OM and so potentially impede the access of enzymes (Six et al., 2004; Dungait et al., 2012). Compared with the macroaggregates, the microaggregates were reported to have lower enzymes activities because of the lower content of available OM (Kanazawa and Filip, 1986; Gupta and Germida, 1988; Wang et al., 2015a). In support of these results, the microaggregates had lower sulfatase activity compared with the macroaggregates in the present study. Contrary to these results, the microaggregates within both the CK and manuring treatments had higher activities in β-glucosidase and phosphatase compared with the macroaggregates. One possible explanation is that more enzymes are needed to metabolize the relatively recalcitrant C substrates within the microaggregates than within the macroaggregates (Allison and Jastrow, 2006).

5. Conclusions

The 23-year manure amendment changed soil structure of the rice–barley cropped soil: the large macroaggregates increased and the microaggregates decreased. Consequently, manuring improved soil structure for aeration and water holding capacity. Manuring increased SOC contents and microbial biomasses in the macroaggregates but not in the microaggregates. There were no effects on the F/B ratio in any aggregate classes. Manuring stimulated the β-glucosidase and chitinase activities in the macroaggregates but not in the microaggregates. In conclusion, SOC, microbial biomasses and enzyme activities in the macroaggregates are more sensitive to manure amendment than in the microaggregates. Manure amendment benefited soil structure, increased C sequestration, microbial activities, and most likely soil fertility.

Acknowledgments

The authors thank Prof. Shiping Deng (Oklahoma State University) and four anonymous reviewers for valuable comments which help to improve this paper. This research was supported by the National Natural Science Foundation of China (31570523, 41361068, 31300381, 41661073), and the S&T Innovation Program of Chinese Academy of Agricultural Sciences.

References

Štursová, M., Baldrian, P., 2011. Effects of soil properties and management on the activity of soil organic matter transforming enzymes and the quantification of soil-bound and free activity. Plant Soil 338, 99–110.
Allison, S.D., Jastrow, J.D., 2006. Activities of extracellular enzymes in physically isolated fractions of restored grassland soils. Soil Biol. Biochem. 38, 3245–3256.
Aon, M.A., Cabello, M.N., Sarena, D.E., Colaneri, A.C., Franco, M.G., Burgos, J.L., Cortassa, S., 2001. I. Spatio-temporal patterns of soil microbial and enzymatic activities in agricultural soils. Appl. Soil Ecol. 18, 239–254.
Aoyama, M., Angers, D.A., N'Dayegamiye, A., 1999a. Particulate and mineral–associated organic matter in water-stable aggregates as affected by mineral fertilizer and manure applications. Can. J. Soil Sci. 79, 295–302.
Aoyama, M., Angers, D.A., N'Dayegamiye, A., Bissonnette, N., 1999b. Protected organic matter in water-stable aggregates as affected by mineral fertilizer and manure applications. Can. J. Soil Sci. 79, 419–425.
Bhattacharyya, R., Prakash, V., Kundu, S., Srivastva, A.K., Gupta, H.S., Mitra, S., 2009. Long-term effects of fertilization on carbon and nitrogen sequestration and aggregate associated carbon and nitrogen in the Indian sub-Himalayas. Nutr. Cycl. Agroecosyst. 86, 1–16.
Bird, M., Santrùcková, H., Lloyd, J., Lawson, E., 2002. The isotopic composition of soil organic carbon on a north–south transect in western Canada. Eur. J. Soil. Sci. 53, 393–403.
Blagodatskaya, E., Kuzyakov, Y., 2013. Active microorganisms in soil: critical review of estimation criteria and approaches. Soil Biol. Biochem. 67, 192–211.
Bronick, C.J., Lal, R., 2005. Soil structure and management: a review. Geoderma 124, 3–22.
Chen, Y., Tang, X., Yang, S., Wu, C., Wang, J., 2010a. Contributions of different N sources to crop N nutrition in a Chinese rice field. Pedosphere 20, 198–208.
Chen, Y., Wu, C., Tang, X., Yang, S., Wang, J., 2010b. Fate of nitrogen from organic and inorganic sources in rice–wheat rotation cropping system. Agric. Sci. China 9, 1017–1025.
Chen, X., Li, Z., Liu, M., Jiang, C., Che, Y., 2015. Microbial community and functional diversity associated with different aggregate fractions of a paddy soil fertilized with organic manure and/or NPK fertilizer for 20 years. J. Soil Sediments 15, 292–301.
Chenu, C., Hassink, J., Bloem, J., 2001. Short-term changes in the spatial distribution of microorganisms in soil aggregates as affected by glucose addition. Biol. Fertil. Soils 34, 349–356.
Chung, H., Zak, D.R., Reich, P.B., Ellsworth, D.S., 2007. Plant species richness, elevated CO_2, and atmospheric nitrogen deposition alter soil microbial community composition and function. Glob. Change Biol. 13, 980–989.
De Gryze, S., Six, J., Brits, C., Merckx, R., 2005. A quantification of short-term macroaggregate dynamics: influences of wheat residue input and texture. Soil Biol. Biochem. 37, 55–66.
Deng, Q., Cheng, X., Hui, D., Zhang, Q., Li, M., Zhang, Q., 2016. Soil microbial community and its interaction with soil carbon and nitrogen dynamics following afforestation in central China. Sci. Total Environ. 541, 230–237.
Dexter, A.R., Bird, N.R.A., 2001. Methods for predicting the optimum and the range of soil water contents for tillage based on the water retention curve. Soil Tillage Res. 57, 203–212.
Ding, X., Han, X., 2014. Effects of long-term fertilization on contents and distribution of microbial residues within aggregate structures of a clay soil. Biol. Fertil. Soils 50, 549–554.
Dorodnikov, M., Blagodatskaya, E., Blagodatsky, S., Marhan, S., Fangmeier, A., Kuzyakov, Y., 2009. Stimulation of microbial extracellular enzyme activities by elevated CO_2 depends on soil aggregate size. Glob. Change Biol. 15, 1603–1614.
Dungait, J.A.J., Hopkins, D.W., Gregory, A.S., Whitmore, A.P., 2012. Soil organic matter turnover is governed by accessibility not recalcitrance. Glob. Change Biol. 18, 1781–1796.
Elliott, E.T., Coleman, D.C., 1988. Let the soil work for us. Ecol. Bull. 39, 23–32.
Fontaine, S., Barot, S., Barré, P., Bdioui, N., Mary, B., Rumpel, C., 2007. Stability of organic carbon in deep soil layers controlled by fresh carbon supply. Nature 450, 277–280.
Frostegård, Å., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol. Fertil. Soils 22, 59–65.
Frostegård, Å., Bååth, E., Tunlid, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol. Biochem. 25, 723–730.
Garcia-Franco, N., Martínez-Mena, M., Goberna, M., Albaladejo, J., 2015. Changes in soil aggregation and microbial community structure control carbon sequestration after afforestation of semiarid shrublands. Soil Biol. Biochem. 87, 110–121.
Gupta, V.V.S.R., Germida, J.J., 1988. Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation. Soil Biol. Biochem. 20, 777–786.
Helgason, B.L., Walley, F.L., Germida, J.J., 2010. No-till soil management increases microbial biomass and alters community profiles in soil aggregates. Appl. Soil Ecol. 46, 390–397.

Hoppe, H.G., 1983. Significance of exoenzymatic activities in the ecology of Brackish water-measurements by means of methylumbelliferyl-substrates. Mar. Ecol. Prog. Ser. 11, 299–308.
Jastrow, J.D., Amonette, J.E., Bailey, V.L., 2007. Mechanisms controlling soil carbon turnover and their potential application for enhancing carbon sequestration. Clim. Change 80, 5–23.
Jastrow, J.D., 1996. Soil aggregate formation and the accrual of particulate and mineral-associated organic matter. Soil Biol. Biochem. 28, 665–676.
Kanazawa, S., Filip, Z., 1986. Distribution of microorganisms, total biomass, and enzyme-activities in different particles of brown soil. Microb. Ecol. 12, 205–215.
Kristiansen, S.M., Schjønning, P., Thomsen, I.K., Olesen, J.E., Kristensen, K., Christensen, B.T., 2006. Similarity of differently sized macro-aggregates in arable soils of different texture. Geoderma 137, 147–154.
Kuzyakov, Y., 2010. Priming effects: interactions between living and dead organic matter. Soil Biol. Biochem. 42, 1363–1371.
Li, N., Yao, S.-H., Qiao, Y.-F., Zou, W.-X., You, M.-Y., Han, X.-Z., Zhang, B., 2015. Separation of soil microbial community structure by aggregate size to a large extent under agricultural practices during early pedogenesis of a Mollisol. Appl. Soil Ecol. 88, 9–20.
Mikha, M.M., Rice, C.W., 2004. Tillage and manure effects on soil and aggregate-associated carbon and nitrogen. Soil Sci. Soc. Am. J. 68, 809–816.
Miller, R.M., Jastrow, J.D., 1990. Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. Soil Biol. Biochem. 22, 579–584.
Miller, M., Paloj€arvi, A., Rangger, A., Reeslev, M., Kjøller, A., 1998. The use of fluorogenic substrates to measure fungal presence and activity in soil. Appl. Environ. Microb. 64, 613–617.
Oades, J.M., 1984. Soil organic matter and structural stability: mechanisms and implications for management. Plant Soil 76, 319–337.
Olsson, P.A., 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiol. Ecol. 29, 303–310.
Sinsabaugh, R.L., Antibus, R.K., Linkins, A.E., McClaugherty, C.A., Rayburn, L., Repert, D., Weiland, T., 1993. Wood decomposition: nitrogen and phosphorus dynamics in relation to extracellular enzyme activity. Ecology 74, 1586–1593.
Six, J., Bossuyt, H., Degryze, S., Denef, K., 2004. A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil Tillage Res. 79, 7–31.
Sterner, R.W., Elser, J.J., 2002. Ecological Stoichiometry: the Biology of Elements from Molecules to the Biosphere. Princeton University Press, New Jersey.
Tian, J., Pausch, J., Yu, G., Blagodatskaya, E., Gao, Y., Kuzyakov, Y., 2015. Aggregate size and their disruption affect ^{14}C-labeled glucose mineralization and priming effect. Appl. Soil Ecol. 90, 1–10.
Tisdall, J.M., Oades, J.M., 1982. Organic matter and water-stable aggregates in soils. J. Soil Sci. 62, 141–163.
Wang, R., Dorodnikov, M., Yang, S., Zhang, Y., Filley, T.R., Turco, R.F., Zhang, Y., Xu, Z., Li, H., Jiang, Y., 2015a. Responses of enzymatic activities within soil aggregates to 9-year nitrogen and water addition in a semi-arid grassland. Soil Biol. Biochem. 81, 159–167.
Wang, Y., Hu, N., Xu, M., Li, Z., Lou, Y., Chen, Y., Wu, C., Wang, Z.-L., 2015b. 23-year manure and fertilizer application increases soil organic carbon sequestration of a rice–barley cropping system. Biol. Fertil. Soils 51, 583–591.
Wilson, G.W.T., Rice, C.W., Rillig, M.C., Springer, A., Hartnett, D.C., 2009. Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. Ecol. Lett. 12, 452–461.
Xu, Z., Yu, G., Zhang, X., Ge, J., He, N., Wang, Q., Wang, D., 2015. The variations in soil microbial communities, enzyme activities and their relationships with soil organic matter decomposition along the northern slope of Changbai Mountain. Appl. Soil Ecol. 86, 19–29.
Yu, H., Ding, W., Luo, J., Geng, R., Cai, Z., 2012. Long-term application of organic manure and mineral fertilizers on aggregation and aggregate-associated carbon in a sandy loam soil. Soil Tillage Res. 124, 170–177.
Zhang, H., Ding, W., Yu, H., He, X., 2015. Linking organic carbon accumulation to microbial community dynamics in a sandy loam soil: result of 20 years compost and inorganic fertilizers repeated application experiment. Biol. Fertil. Soils 51, 137–150.
Zogg, G.P., Zak, D.R., Ringelberg, D.B., MacDonald, N.W., Pregitzer, K.S., White, D.C., 1997. Compositional and functional shifts in microbial communities due to soil warming. Soil Sci. Soc. Am. J. 61, 475–481.