Science of the Total Environment 624 (2018) 1131–1139

Contents lists available at ScienceDirect

Science of the Total Environment

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

Long-term effects of nitrogen fertilization on aggregation and localization of carbon, nitrogen and microbial activities in soil

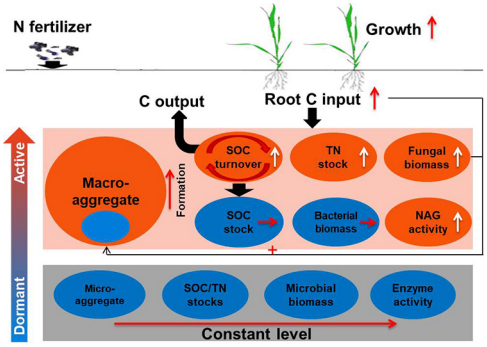
Yidong Wang [a,b], Zhong-Liang Wang [a,b,*], Qingzhong Zhang [c], Ning Hu [d], Zhongfang Li [d], Yilai Lou [c,**], Yong Li [a,b], Dongmei Xue [a,b], Yi Chen [e], Chunyan Wu [e], Chris B. Zou [a,f], Yakov Kuzyakov [a,g]

[a] Tianjin Key Laboratory of Water Resources and Environment, Tianjin Normal University, Tianjin 300387, China
[b] School of Geographic and Environmental Sciences, Tianjin Normal University, Tianjin 300387, China
[c] Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[d] Institute for Environmental Genomics, Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA
[e] Institute of Environmental Resources and Soil Fertilizer, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China
[f] Department of Natural Resource Ecology and Management, Oklahoma State University, Stillwater, OK 74078, USA
[g] Department of Soil Science of Temperate Ecosystems, Department of Agricultural Soil Science, University of Göttingen, Göttingen 37077, Germany

HIGHLIGHTS

- N fertilization improved soil structure by forming large macroaggregates (>2 mm).
- N addition increased TN, fungi and NAG activity in macro- but not in micro-aggregates.
- However, SOC in all aggregates remained constant, suggesting accelerated C turnovers.
- Macroaggregates are more sensitive to N fertilization than microaggregates.
- N fertilization shifted localization of microorganisms to the macroaggregates.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 August 2017
Received in revised form 10 December 2017
Accepted 11 December 2017
Available online xxxx

Editor: Charlotte Poschenrieder

Keywords:
Aggregate formation
Enzyme activity
PLFAs
Mineral fertilizer
Soil organic carbon
Soil structure

ABSTRACT

Long-term nitrogen (N) fertilization affects soil aggregation and localizations of soil organic carbon (SOC), N and microbial parameters within aggregates. The mechanisms of these N effects are poorly understood. We studied these processes in a loamy soil from a 23-year repeated N addition field experiment under a rice–barley rotation. Nitrogen fertilization increased plant productivity and the portion of large macroaggregates (>2 mm). However, SOC contents in macro- and micro-aggregates remained constant despite an N-induced increase of 27% in root C input into soil. Therefore, N fertilization accelerated SOC turnover. Nitrogen addition increased total N (TN) content in bulk soil and two macroaggregates (>2, and 1–2 mm), but not in microaggregates (<0.25 mm). Also, N fertilization increased the phospholipid fatty acids (PLFAs) contents of fungi in the large macroaggregates, but not in the microaggregates. In contrast, the effect of N addition on contents of bacterial and total microbial PLFAs was not apparent. Nitrogen fertilization increased *N*-acetyl-β-D-glucosaminidase (NAG) activities in the two larger macroaggregate size classes (>2, and 1–2 mm), but not in the aggregates (<1 mm). In both control and N fertilization, the large macroaggregates localized more TN, microbial PLFAs, and NAG activities than the microaggregates. In conclusion, long-term N fertilization not only directly promotes soil N resource but also

* Correspondence to: Z.-L. Wang, Tianjin Key Laboratory of Water Resources and Environment, Tianjin Normal University, Tianjin 300387, China.
** Corresponding author.
E-mail addresses: wangzhongliang@vip.skleg.cn (Z.-L. Wang), louyilai@caas.cn (Y. Lou).

https://doi.org/10.1016/j.scitotenv.2017.12.113
0048-9697/

indirectly improves soil structure by forming large macroaggregates, accelerates SOC turnover, and shiftes localization of microorganisms to the macroaggregates.

1. Introduction

Soil structure plays an important role in agroecosystem productivity and sustainability (Bronick and Lal, 2005). Aggregation, the formation of soil structure, rearranges the distribution and localization of organic matter and nutrients (Six et al., 2004; Jastrow et al., 2007). Changes in aggregation and aggregate-associated soil organic matter (SOM) and total nitrogen (TN) affect the microbial community and enzyme activities (Dorodnikov et al., 2009; Helgason et al., 2010).

Aggregation, resulting from rearrangement, flocculation, and cementation of primary particles, is mediated by physicochemical mechanisms (i.e., ligand exchange and polyvalent cation bridging) and biotic binding agents (e.g., organic matter, plant and microbial debris, polysaccharides, and roots and hyphae) (Bronick and Lal, 2005; Jastrow et al., 2007). Two aggregate size classes are commonly differentiated: microaggregates (<0.25 mm) and macroaggregates (>0.25 mm) (Tisdall and Oades, 1982; Miller and Jastrow, 1990) and fractions of those size classes are often used. Abiotic mechanisms primarily control the formation of microaggregates, and biotic mechanisms play crucial roles in the formation of macroaggregates (Six et al., 2004; Blankinship et al., 2016). SOM is partly embedded and bound within hierarchical aggregates and is more inaccessible by decomposers within microaggregates rather than within macroaggregates (Bird et al., 2002). Therefore, the turnover (formation and destruction) of macroaggregates is a critical process influencing SOM dynamics (Six et al., 2004).

Nutrient replenishment by fertilization is a conventional practice to maintain and increase crop productivity. Returning crop residues to fields along with the fertilizer application serves to promote soil organic carbon (SOC) sequestration (He et al., 2015) and aggregation in the form of macroaggregates (Sall et al., 2016). In contrast, microaggregates, as preliminary aggregates, generally exhibit very little response to the input of crop residues (Mikha and Rice, 2004). Most N fertilizations do not affect SOC sequestration when straw residues are not returned to the soil (Alvarez, 2005; He et al., 2015; Xu et al., 2016). One reason for this is that despite higher C input by plant residues into the soil, the N fertilization favors microbial activity and stimulates SOM mineralization (Balesdent et al., 1998). Various effects of N fertilization on the TN content in soils have been reported, including positive (Chen et al., 2015), negative (Dai et al., 2017) and no effects (Huang et al., 2010; He et al., 2015).

Microbial biomass and community structure are related to aggregate structure and substrate availability (Wang et al., 2017; Dai et al., 2017). Microorganisms and roots engineer aggregates with biotic glues and hyphae, and regulate the turnover and stabilization of SOM and N (Jastrow et al., 2007). In general, fungi and bacteria differ in aggregates formation and C and N cycles (Jastrow et al., 2007). Compared with bacteria, fungi play a more important role in macroaggregate formation because their mycelium (Denef et al., 2001; Ding and Han, 2014) and release of glycoproteins glue soil particles more effectively (Rillig and Mummey, 2006). Fungi predominantly proliferate in larger pores among macro- and micro-aggregates; whereas bacteria reside mostly in smaller pores within microaggregates (Tisdall and Oades, 1982; Ding and Han, 2014). Compared with bacteria, fungi have a lower metabolic nutrient demand (Danger et al., 2016) and wider enzymatic capabilities, which allow them to mineralize low-quality substrates like coarse particulate root residues (Güsewell and Gessner, 2009). Microbial biomass generally increases from micro- to macro-aggregates (Kanazawa and Filip, 1986; Helgason et al., 2010) due to the increase in substrate availability (Chen et al., 2015) and higher particulate organic matter content in macroaggregates. This leads to the redistribution of microbial groups within macro- vs. micro-aggregates. At the same time, the turnover of SOM in macroaggregates is faster, especially under N fertilization, leading to accelerated changes in the microbial communities. Therefore, the overall effect of long-term N fertilization on the formation of macro- and micro-aggregates and the redistribution of microbial groups and activities within aggregate size classes remains unknown.

Enzyme activity is highly sensitive to the availabilities of C and N (Aon et al., 2001). However, contradictory effects of N addition on enzyme activities have been reported, including positive (Nivelle et al., 2016), negative (Wang et al., 2015a) and no effects (Sall et al., 2016). Even though there are some studies investigating SOM turnover in aggregates, the effect of aggregate size classes (macro- vs. micro-aggregates) on enzyme activities have received much less attention (Dorodnikov et al., 2009). Both positive (Kanazawa and Filip, 1986; Wang et al., 2015a) and negative relationships (Allison and Jastrow, 2006; Dorodnikov et al., 2009) between aggregate size and enzyme activities have been reported. Given that macroaggregates have faster turnover rates for SOM and N (Six et al., 2004; Wang et al., 2017), we hypothesize that under long-term N fertilization enzyme activities will have greater responses in macroaggregates than in microaggregates.

The lower reach of the Yangtze River is one of the most important grain-producing regions in China (Wang et al., 2015b). The croplands of this area have been under long-term intensive N fertilization to ensure high and stable grain yields. However, it is unclear how N amendment affects the aggregate formation and the distribution of SOM, N, and microbial activities in the resultant size classes of aggregates. The objectives of this study were to investigate changes in SOM, TN, and microbial parameters within micro- vs. macro-aggregates in response to 23-years of N addition in a rice–barley rotation system, common for the most upland soils in the middle of China. We hypothesize that long-term N fertilization (1) increases soil aggregation, especially the formation of large macroaggregates; and (2) increases contents of SOC and TN, microbial biomass, and enzyme activities in macroaggregates especially for the large macroaggregates; and (3) has little effect on those same parameters in microaggregates because of their long-term stability, slow turnover, and their formation being driven by physicochemical processes.

2. Materials and methods

2.1. Study site

The study site was a rice–barley rotation field (30°26′04″ N, 120°25′01″ E, elevation 4 m a.s.l.) at the National Monitoring Station for Soil Fertility and Fertilizer Efficiency in Hangzhou of Zhejiang Province, China. The 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 precipitation of 1500 − 1600 mm, an annual evapotranspiration of 1000 − 1100 mm. The annual frost-free period is 240–250 d and an annual sunshine duration is 1900–2000 h (Wang et al., 2015b). The soil, classified as a Semihydromorphic Soil (Chinese Soil Taxonomy) and an Inceptisol (US Soil Taxonomy), had a loam texture with 42% sand, 38% silt, and 20% clay (Chen et al., 2010).

Prior to the experiment, the study field had been intensively cultivated with mineral fertilizer additions for over 30 years. From autumn 1988 to autumn 1990, the soil in the field was homogenized by growing barley, early rice and late rice in rotation over 2 years without fertilization. In autumn 1990, the soil properties at 0 − 20 cm depth were as

follows: bulk density, 1.24 g cm^{-3}; porosity, 53.2%; SOC, 16.6 g kg^{-1}; TN, 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 (Wang et al., 2017).

2.2. Experimental design

The long-term field experiment was launched in autumn 1990. Two fertilization regimes, no fertilization (Control) and N fertilization, were established using a random design with three field replicates. Each replicated plot had an area of 100 m^2. The N fertilization was applied as urea (46% N) at a rate of 315 kg N ha^{-1} $year^{-1}$. During the first 10 years (1990 − 2000), a rotation of barley–early rice–late rice was arranged for annual cropping. The annual N fertilization quotas were 20% for barley, 40% for early rice, and 40% for late rice. Afterwards, fields were converted to barley–rice rotation, and annual N fertilizer quotas were 32% for barley and 68% for rice. In every growing season, 70% N was applied as base fertilizer; while the remaining 30% N was used as top dressing. In the three-crop 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-crop rotation, the rice was seeded in middle June and harvested in early November. The barley was always seeded in late November and harvested in early May of the next year throughout the entire experimental period. The barley and rice were both harvested by hand. All other management practices (e.g. tillage and planting) were the same for both treatments. The aboveground biomass was removed except for 3-cm stubbles (Wang et al., 2015b).

2.3. Soil sampling and aggregate size fractionation

A composite soil sample (homogenized from three subsamples) in each replicated plot was collected from 0 − 20 cm depth using a soil auger after the rice harvest in November 2013. This sampling time was chosen to minimize the growing season influences such as plant growth and N addition. Fresh soil samples were stored on ice until they were transported to the laboratory. The gravimetric soil moisture of approximately 15% was the best condition for aggregates fractionation using field moisture sieving method (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 by field moisture sieving of fresh soil using the combination of sieving method described by Kristiansen et al. (2006) and Helgason et al. (2010). Briefly, 400 g fresh soil was sieved four times in turn 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 aggregate size classes while minimizing aggregate abrasion during the sieving. Finally, four aggregates size classes were obtained: >2 mm, large macroaggregates; 1–2 mm, medium-size macroaggregates; 0.25–1 mm, small macroaggregates; and <0.25 mm, microaggregates which included fine sand and silt particles.

2.4. Measurements of bulk density, soil moisture, SOC and TN

Bulk density (BD) was measured using a conventional core method with a volume of 100 cm^3. Gravimetric soil water content was determined by measuring water loss from fresh soil after drying at 105 °C for 8 h. Air-dried soil from each aggregate size class was analyzed for SOC and TN using an elemental analyzer (Elementar, Germany). Soil C stock at 0–20-cm depth per hectare (Mg C ha^{-1}) was calculated using measured SOC content (g C kg^{-1}) and bulk density (g cm^{-3}) values (Eq. (1)).

$$\text{Soil C stock} = \text{SOC} \times \text{BD} \times 2 \quad (1)$$

2.5. Phospholipid fatty acids analysis

Phospholipid fatty acids (PLFAs) were extracted using the method described by Helgason et al. (2010). Briefly, fatty acids were extracted from 8 g freeze-dried soil (0–20 cm depth) using a single-phase mixture of chloroform:methanol:phosphate buffer (1:2:0.5). The isolated fatty acid methyl esters (FAMEs) were analyzed using a gas-chromatography mass-spectroscopy system (TRACE GC Ultra ISQ, Thermo Fisher Scientific) with helium as the carrier gas and a DB-5 column (30 m length × 0.25 mm I.D. × 0.25 μm film thickness) as the separation tube (Xu et al., 2015). The temperature program started at 150 °C for 4 min, after which the temperature was ramped to 250 °C at a rate of 4 °C min^{-1} and held at 250 °C for 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). Concentrations of PLFAs (nmol g^{-1} dry weight soil) were quantified based on the internal standard (nonadecanoic acid methyl ester 19:0).

Summing the concentrations of eleven PLFAs (i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7t, i17:0, a17:0, 17:0, cy17:0 and cy19:0) constituted the bacterial biomass component (Frostegård and Bååth, 1996; Zogg et al., 1997), and fatty acids 18:2ω6 and the isomer 18:1ω9c were used as indicators for fungal biomass component (Frostegård et al., 1993). The sum of the bacterial biomass component and the fungal biomass component was considered as the total microbial biomass (nmol g^{-1}).

2.6. Analyses of enzyme activities

Based on the methods of Dorodnikov et al. (2009), we used fluorogenically labelled substrates to measure activities of four enzymes: (1) β-glucosidase (BG), which catalyzes one of the final steps in cellulose breakdown, (2) *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; Enzyme commission number = EC 3.2.1.21) for the determination of BG activity, MU-*N*-acetyl-β-D-glucosaminidase dehydrate (MU-NAG; EC 3.2.1.52) 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 correct contents. All chemicals were obtained from Sigma-Aldrich (Germany).

The fresh soil samples (1 g 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 of either MU-NAG, or MU-phosphate, or MU-sulfatase) which had already been pipetted into deep-well plates (24-wells × 10 mL, HJ-Bioanalytik GmbH, Germany), and was incubated at 25 °C for 4 h. 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. A microplate reader (Bio-Tek FLx800, USA) was used to measure fluorescence at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Calibration curves were included with every series of enzyme measurements. Enzyme activities were expressed as MU release in micromole per gram bulk soil/aggregates in dry weight per hour.

2.7. Measurements of grain yield, straw biomass, and total shoot biomass

Every year, grain and straw from each crop were carefully harvested and air-dried. The grain yield and straw biomass of each crop were calculated separately and then summed to obtain the total shoot biomass of each crop.

2.8. Estimates of root C input into soil

The 23-year cumulative root C input of each plot was calculated using Eqs. (2)–(4).

$$Root\ C\ input = \Sigma Barley_{Root} + \Sigma Rice_{Root} \quad (2)$$

where *Root C input* is the 23-year cumulative root C input (Mg C ha^{-1}), $\Sigma Barley_{Root}$ refers to the 23-year cumulative barley root C input (Mg C ha^{-1}), $\Sigma Rice_{Root}$ is the 23-year cumulative rice root C input (Mg C ha^{-1}).

$$\Sigma Barley_{Root} = B_S \times (1-W_B) \times R_{B-R/S} \times C_{BR} \times 23 \quad (3)$$

where B_S represents annual barley shoot (grain + straw) biomass (Mg C ha^{-1}), W_B refers to mean water content of barley shoot (12%), $R_{B-R/S}$, the average ratio of root to shoot biomass of barley (0.179) (Wang et al., 2015b), C_{BR} is the C concentration of dried barley root (42%).

$$\Sigma Rice_{Root} = [(ER_S + LR_S) \times 10 + SR_S \times 13] \times (1-W_R) \times R_{R-R/S} \times C_{RR} \quad (4)$$

where ER_S and LR_S are annual shoot (grain + straw) biomass of early rice and late rice (Mg C ha^{-1}) during the first 10 years, respectively, SR_S is the annual shoot (grain + straw) biomass of single cropping rice (Mg C ha^{-1}) during 1991 − 2013, W_R refers to water content of rice shoot (14%), $R_{R-R/S}$, the average ratio of root to shoot biomass of rice (0.087) (Wang et al., 2015b), and C_{BR} is C concentration of dry barley root (41%).

2.9. Recovery calculation and statistical analysis

The recoveries of SOC, TN, PLFAs, and enzyme activity in aggregate size classes were calculated as the ratios of the weighted sum of four aggregate size classes to the bulk soil. Two-way analysis of variance (ANOVA) was used to test the main effects of fertilization and aggregate size, and their interactions 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) was based on the CANOCO 5.0. Figures were prepared in ORIGIN 9.0 (OriginLab Corporation, Northampton, MA).

3. Results

3.1. Cumulative crop biomass and C input into soil

Twenty-three years of mineral N fertilization increased the cumulative grain yield, straws biomass, and shoot biomass compared with the unfertilized control (Table 1). Similarly, N addition led to a 4.1 Mg C ha^{-1} increase in root C inputs into the soils when compared with the control (Table 2).

3.2. Soil aggregates size distribution

Mineral N fertilization increased the portion of large macroaggregates (>2 mm) by +7.2% compared with the control ($p < 0.05$) (Fig. 1a). In contrast, effect of N addition on the portion of the other two macroaggregate size classes (1–2, and 0.25–1 mm) and the microaggregates (<0.25 mm) was not apparent (Fig. 1a). For both the control and the N addition soils, the dominant aggregate size class was the large macroaggregates (>2 mm) (Fig. 1a).

3.3. SOC and TN in bulk soil and aggregate size classes

Despite N fertilization, SOC content remained constant in the bulk soil and across the four aggregate fractions (Fig. 1b). For both the control and the N fertilization soils, the four aggregate size classes had similar SOC contents (Fig. 1b). The SOC stocks at 0–20-cm depth of the control and N fertilization soils were each about 38 Mg C ha^{-1}. In contrast, N fertilization did increase the TN content of the bulk soil, and this increase was exhibited in the two larger macroaggregates (>2, and 1–2 mm), but not for the microaggregates (<0.25 mm) and the macroaggregates (<1 mm) (Fig. 1c). Under the control, the four aggregates size classes had similar TN contents. However, under the N addition, the large macroaggregates (>2 mm) had higher TN than the microaggregates (Fig. 1c). The C/N ratio of the four aggregates remained constant under both the control and the N fertilization soils (Fig. 1d). The recoveries of SOC and TN were 95–104% in isolated aggregate size classes.

3.4. Microbial PLFAs

The effect of N fertilization on total microbial and bacterial PLFAs contents was not apparent (Fig. 2a, b). In contrast, N addition increased the fungal PLFAs contents of the bulk soil, and this was exhibited in the large macroaggregate fractions (>2 mm) ($p < 0.05$) but the effect was not apparent in the other three aggregate size classes (Fig. 2c). Likewise, N fertilization increased the fungal/bacterial-PLFAs ratio (F/B) of the large macroaggregate (>2 mm) ($p < 0.05$) (Fig. 2d).

In the unfertilized soil, the large macroaggregates (>2 mm) had higher microbial and bacterial PLFAs content than the other three size classes ($p < 0.05$) (Fig. 2a, b). However, in soil with N fertilization, there was no significant difference among the aggregate size classes (Fig. 2a, b). In contrast, under both the control and N addition, the large macroaggregates (>2 mm) had higher fungal PLFAs contents and F/B-PLFAs ratios than the microaggregates (<0.25 mm) ($p < 0.05$) (Fig. 2c, d). N fertilization and aggregate size had interacting effects on fungal ($p = 0.010$), but not on the total microbial ($p = 0.32$) and bacterial PLFAs ($p = 0.07$). The PLFA recoveries were 97–123% in isolated aggregate size classes.

3.5. Enzyme activities

Compared with the control, the N fertilization did not affect the activities of BG, phosphatase, and sulfatase in the bulk soil and the four aggregate size classes (Fig. 3a, c, d). In contrast, N fertilization increased NAG activities in the two macroaggregates (>2 mm, 1–2 mm) and the bulk soil compared with the unfertilized soil (Fig. 3b). Under both the

Table 1
23-year cumulative grain yield, straw biomass, and total shoot biomass.

Treatments	Grain yield (Mg ha^{-1})		Straw biomass (Mg ha^{-1})		Total shoot biomass (Mg ha^{-1})		
	Barley	Rice	Barley	Rice	Barley	Rice	Barley + Rice
Unfertilized control	50 ± 9	147 ± 5	54 ± 7	123 ± 6	105 ± 16	269 ± 11	374 ± 27
N fertilization	68 ± 2*	176 ± 10*	74 ± 3*	148 ± 8*	142 ± 5*	323 ± 16*	465 ± 21*

Each value refers to mean ± SD ($n = 3$).
* Denotes significant differences between the unfertilized control and N fertilization soils at $p < 0.05$ level.

control and N fertilization, BG and sulfatase activities were similar for all aggregate size classes (Fig. 3a, d). In contrast, the large macroaggregates (>2 mm) had higher NAG activities compared with the microaggregates under both the control and N addition (Fig. 3b). However, microaggregates had elevated phosphatase activities compared to the macroaggregates (>0.25 mm) under both the control and N addition soils (Fig. 3c). N addition and aggregate size interacted to affect NAG activity ($p = 0.007$), but not the activities of the other three enzymes ($p = 0.74$–0.93).

3.6. Principal components analysis

The 1st and 2nd principal components (PC1 and PC2) explained 72.4% and 20.8% of total variance, respectively (Fig. 4). Compared with the microaggregates (<0.25 mm), the large macroaggregates (>2 mm) localized more TN, microbial (bacterial + fungal) PLFAs, ratios of F/B-PLFAs and NAG activities in both the control and the N fertilization soils (Fig. 4). However, aggregates size classes (macro- vs. microaggregates) had similar SOC (Fig. 1b), C/N ratio (Fig. 1d), BG activity (Fig. 3a), and sulfatase activity (Fig. 3d).

4. Discussion

4.1. Effects of N fertilization on soil aggregation and its associated SOC and TN

Under the control and the N fertilization soils, the portions of large macroaggregates (>2 mm) were higher than the other aggregate size classes based on the field moisture sieving method (Fig. 1a). The outcome of this aggregate distribution is in agreement with other studies using the same method (e.g. Dorodnikov et al., 2009; Chen et al., 2015; Wang et al., 2017) and the air-dried soil sieving method (Blankinship et al., 2016), but is different from studies based on the wet sieving procedure (e.g. Denef et al., 2001; Huang et al., 2010). Mineral N fertilization increased the portion of large macroaggregates (>2 mm) by 7% compared with the control (Fig. 1a), which is consistent with previous studies (Guo et al., 2012; Hua et al., 2014). This result is explained by the N-induced increase in root biomass (Table 2) and fungal biomass in the large macroaggregates (>2 mm) (Fig. 2c). Root and fungal biomass are two main biotic parameters crucial for the formation of macroaggregates (Bronick and Lal, 2005; Jastrow et al., 2007). In contrast, the portions of the other two macroaggregate size classes (1–2, and 0.25–1 mm) and the microaggregates (<0.25 mm) responded little to N fertilization (Fig. 1a). The result is in agreement with previous studies (Huang et al., 2010; Chen et al., 2015). Besides root and fungi, SOC and bacteria also serve as main binding agents for aggregates formation (Denef et al., 2001; Ding and Han, 2014), but N fertilization had little effect on SOC content (Fig. 1b). Also, the bacterial and fungal biomass in the two macroaggregates (1–2, and 0.25–1 mm) and the microaggregates remained independent of N fertilization (Fig. 2b, c). Because of increased root and fungal biomasses, the dynamics (formation and destruction) of large macroaggregates (>2 mm) are more sensitive to N fertilization than those of the smaller aggregates. Furthermore, coarse root residues, commonly accumulated in the macroaggregates, are more attractive to fungi than bacteria (Güsewell and Gessner, 2009). Based on these, we conclude that compared with fungi, the increase of root biomass is the initial cause for the formation of large macroaggregates (>2 mm) under N addition.

SOC content responded little to N fertilization (Fig. 1b) despite the increase of C input by roots (Table 2). This result is consistent with many previous studies (Alvarez, 2005; Huang et al., 2010; He et al., 2015). One possible reason is that N fertilization improves the quality of root residues and thus accelerates the decomposition of residual roots (Balesdent et al., 1998). Based on the aggregate hierarchy concept (Tisdall and Oades, 1982; Six et al., 2004), the process of aggregation is generally from micro- to macro-aggregates. Root-derived C was preferentially localized in macroaggregates compared with microaggregates. Therefore, it is reasonable that microaggregate-associated SOC responded little to N addition (Fig. 1b). However, macroaggregate-linked SOC also remained constant despite N addition (Fig. 1b). In addition to N-accelerated residual roots decomposition, the relative low root C input rate (0.18 Mg C ha^{-1} year^{-1}) is another possible reason.

Mineral N fertilization increased the TN contents in the soil under rice–barley rotation (Fig. 1c) which is consistent with a paddy soil with 233 kg N ha^{-1} year^{-1} fertilization (Chen et al., 2015). In contrast, the lack of response of TN content to N addition was reported in upland soils with similar N application level to our study (Huang et al., 2010; He et al., 2015). The contradictory effects of N fertilization on the TN contents may be attributed to land use types: paddy field vs. dryland. Paddy fields can reduce N loss compared with drylands (Li et al., 2017; Dai et al., 2017). Furthermore, TN increased in macroaggregates (>2, and 1–2 mm) under N fertilization, but TN in microaggregates remained independent of N fertilization (Fig. 1c). And macroaggregates localized more total N compared with microaggregates (Fig. 4). These results justify the aggregate hierarchy concept with respect to the longer stability of the microaggregates and faster turnover of the macroaggregates. In addition, C/N ratio was independent of N fertilization (Fig. 1d) and aggregate size classes (Fig. 4). This results from the fact that despite N fertilization, SOC generally remained constant (Alvarez, 2005; He et al., 2015; Xu et al., 2016), and mineral N fertilizer was largely removed by plant uptake, and lost by nitrification, denitrification, and leaching.

Table 2
Estimated cumulative (23 years) root C inputs (Mg C ha^{-1}) into soil.

Treatments	Barley	Rice	Total
Unfertilized control	6.9 ± 1.0	8.3 ± 0.3	15.2 ± 1.4
N fertilization	9.4 ± 0.3*	9.9 ± 0.5*	19.3 ± 0.8*

Each value refers to mean ± SD ($n = 3$).
* Denotes significant differences between the unfertilized control and N fertilization plots at $p < 0.05$ level.

4.2. Responses of aggregate-associated microbial groups to N fertilization

The field moisture sieving of soil (Kristiansen et al., 2006; Helgason et al., 2010; Wang et al., 2017) was used to separate aggregates and to study indigenous aggregate-linked microbial parameters. The conventional wet sieving or air-dried soil sieving procedures were avoided

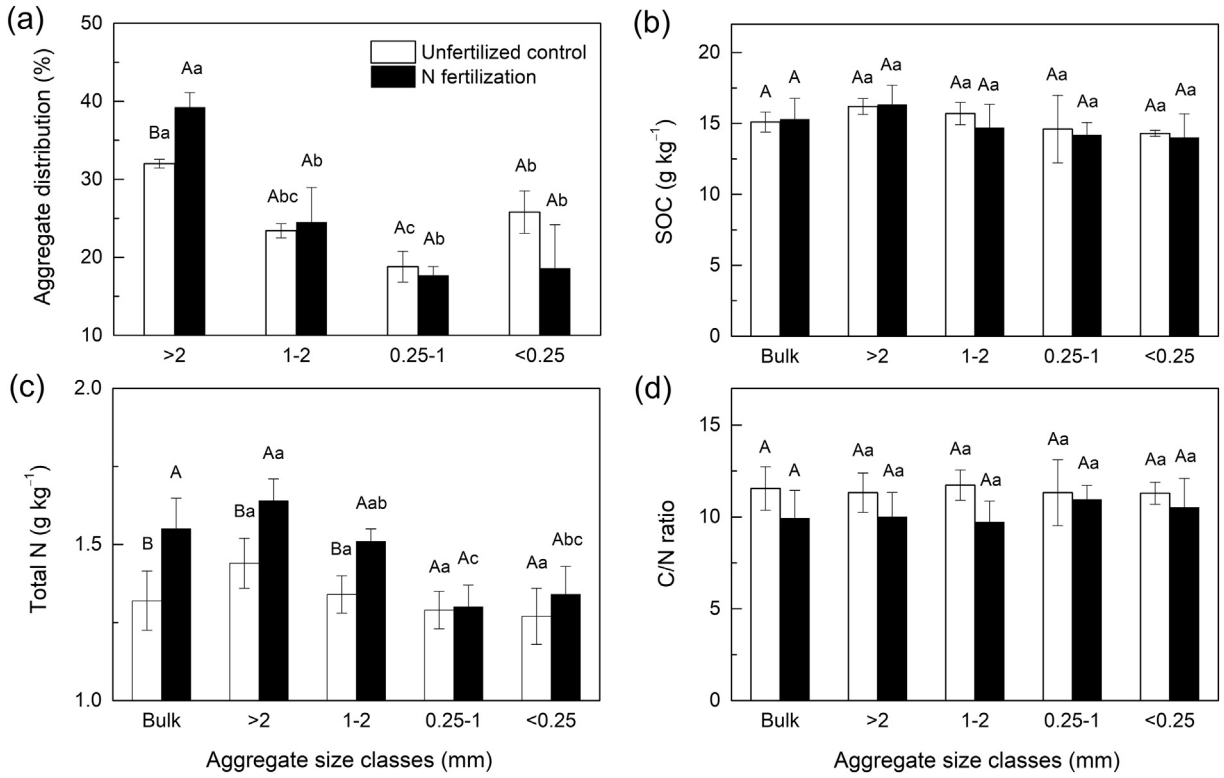


Fig. 1. Effects of long-term (23 years) N fertilization on soil aggregation and its associated SOC, total N, and C/N ratio (dry weight basis). Each value refers to mean ($n = 3$) ± standard deviation (SD). Different uppercase letters denote statistically significant differences between the unfertilized control and N fertilization plots at $p < 0.05$ level. Different lowercase letters indicate differences among the four aggregate size classes within the unfertilized control and N addition plots at $p < 0.05$ level.

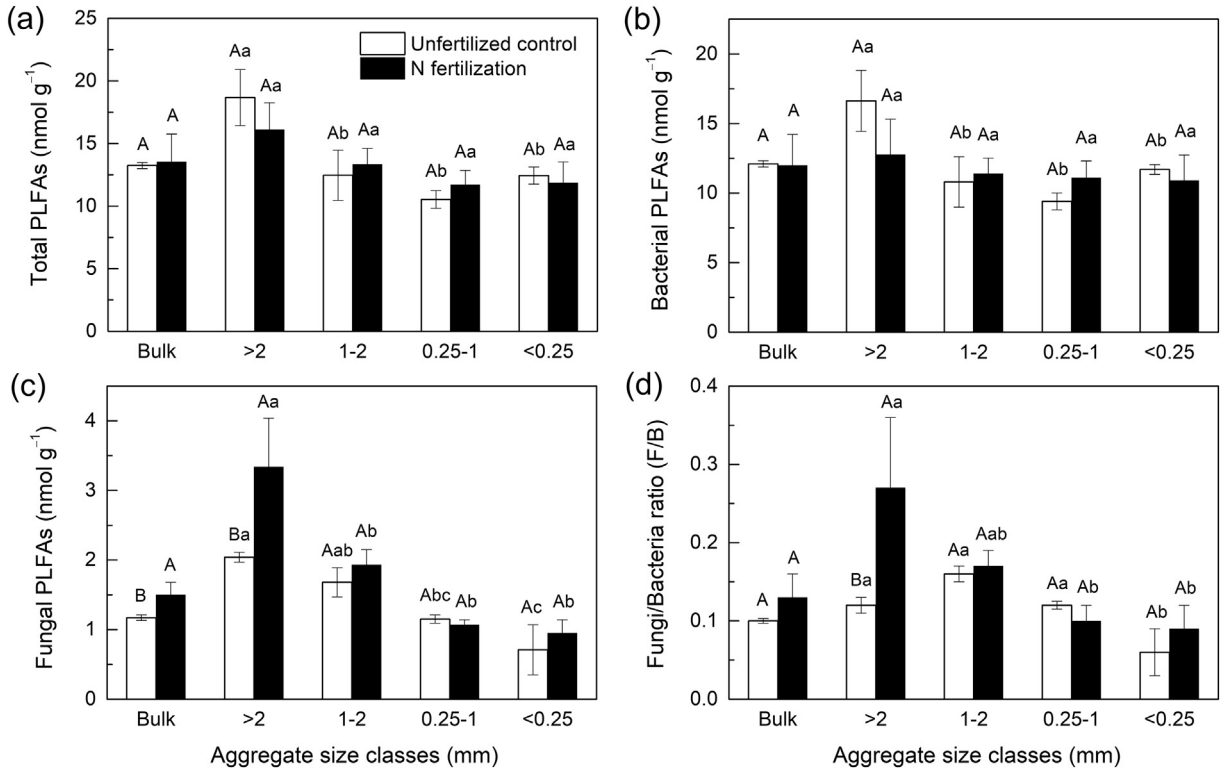


Fig. 2. Effects of long-term (23 years) N fertilization on total microbial, bacterial, fungal PLFAs, and fungal/bacterial-PLFAs ratio (F/B) ($n = 3$). The error bar represents SD. Different uppercase letters denote differences between the unfertilized control and N fertilization at $p < 0.05$. Different lowercase letters indicate differences among the four aggregate size classes within the unfertilized control and N addition plots at $p < 0.05$.

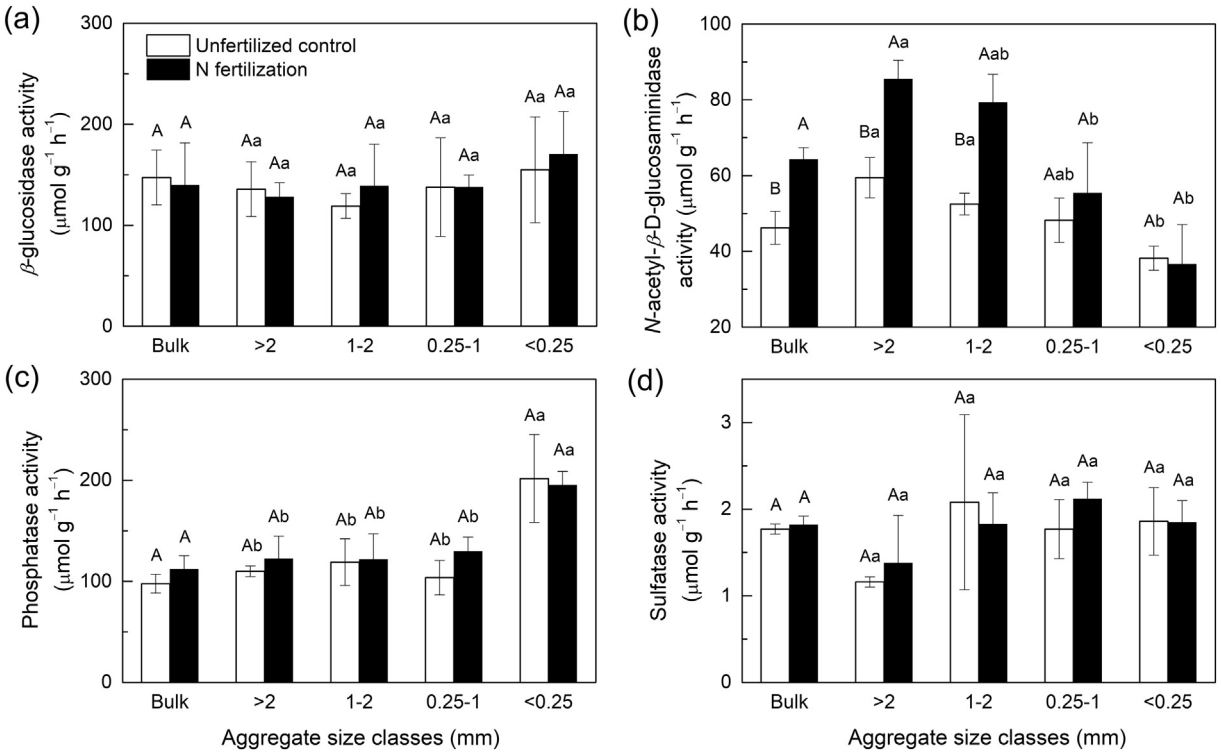


Fig. 3. Effects of long-term (23 years) N fertilization on activities of β-glucosidase, *N*-acetyl-β-D-glucosaminidase, phosphatase, and sulfatase ($n = 3$). The error bar represents SD. Different uppercase letters indicate differences between the unfertilized control and N fertilization at $p < 0.05$. Different lowercase letters indicate differences among the four aggregate size classes within the unfertilized control and N addition plots at $p < 0.05$.

because of their disturbance of indigenous microbial habitats (Dorodnikov et al., 2009; Helgason et al., 2010). In contrast to the procedure by Kristiansen et al. (2006), we did not isolate free fine sand and silt fractions from the microaggregates to reduce bias in microbial analyses.

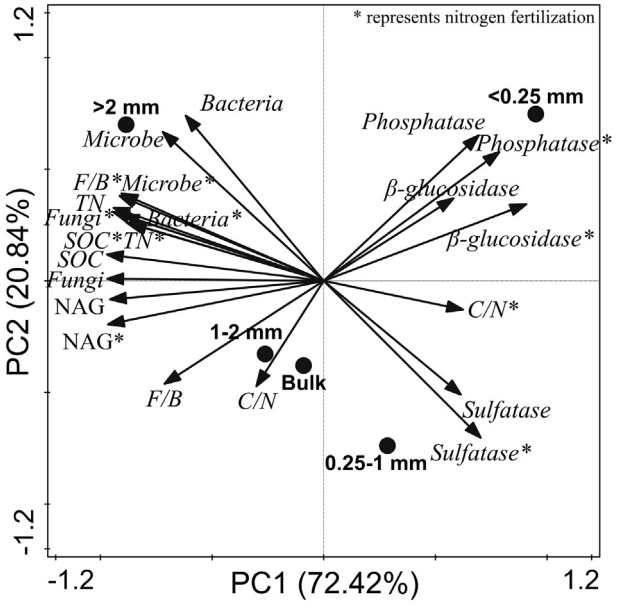


Fig. 4. Principal components analysis (PCA) of soil properties and microbial parameters in aggregate size classes under long-term (23 years) N fertilization. Black solid circles are aggregate size classes. Solid arrows are soil properties and microbial parameters. * represents soil properties and microbial parameters in plots with long-term N fertilization. NAG refers to *N*-acetyl-β-D-glucosaminidase. TN represents total nitrogen.

A recent meta-analysis revealed that N fertilization led to a 15% increase in microbial biomass (Geisseler and Scow, 2014). In our present study, we found no effect of N fertilization on bacterial and microbial biomass (based on PLFA) in bulk soil (Fig. 2a, b). This result is attributed to the unchanged SOC content (Fig. 1b) and bacteria dominated the microbial community. In contrast, fungal biomass increased in bulk soil and large macroaggregates (>2 mm) under N fertilization (Fig. 2c, e). Compared with bacteria, fungi predominantly proliferate in larger pores and favor coarse particulate root residues which were increased by N addition (Table 2). Also, the soil sampling time was only ten days after the rice harvest, and consequently the fresh root residues were abundant and contributed to fungal proliferation.

The localization of plant residues and living roots is a critical factor for microbial colonies and growth within the soil matrix (Kuzyakov and Blagodatskaya, 2015). Given that root residues accumulate in large-size fractions (Jastrow et al., 2007), large macroaggregate (>2 mm) has higher fungal biomasses compared with the microaggregate (Fig. 2c; Fig. 4). Similarly and consistent with previous studies (Chen et al., 2015; Wang et al., 2017), bacteria within the large macroaggregates (>2 mm) were also more abundant than within the microaggregates in both the N fertilized and unfertilized soils (Fig. 2b; Fig. 4).

4.3. Responses of aggregate-linked enzyme activity to N fertilization

Enzyme activities depend mainly on active microorganisms (Blagodatskaya and Kuzyakov, 2013) and growing roots. Enzymes have various ecological functions: BG is for cellulose degradation and NAG is for chitin breakdown (Allison and Jastrow, 2006). Phosphatase and sulfatase are two nutrient-acquiring enzymes for stoichiometric microbial growth (Sinsabaugh et al., 1993; Štursová and Baldrian, 2011).

Positive responses of BG activities to N fertilization had been widely reported (Geisseler and Scow, 2014; Jian et al., 2016). However, BG

activities in bulk soil and even in the macroaggregate fractions remained independent of N addition (Fig. 3a). The activities of phosphatase and sulfatase were also independent of N fertilization (Fig. 3b, c) because of unchanged SOM contents under N fertilization (Fig. 1b). Contrary to these results, N fertilization increased the NAG activities in the macroaggregates but not in the microaggregates (Fig. 3b). NAG activity reflects the fungal activity for chitin breakdown (Miller et al., 1998), and fungi predominantly reside in macroaggregates (Ding and Han, 2014). Therefore, the patterns of NAG activities (Fig. 3b) were consistent with that of fungal biomass, especially in the macroaggregates (Fig. 2c; Fig. 4). Compared with the macroaggregates, the microaggregates have lower NAG activities because of the lower TN N content (Allison and Jastrow, 2006; Wang et al., 2015a). This result is further confirmed in the present study (Fig. 3b).

5. Conclusions

In the rice–barley cropping system, the 23-year N fertilization not only promotes plant growth but also improves soil structure by increasing the number of large macroaggregates (>2 mm) because of the increases in root and fungal biomasses. However, N fertilization maintains SOC contents in macro- and micro-aggregates by accelerating SOC turnover. In contrast, N addition increases total N, fungal biomass, ratios of F/B and *N*-acetyl-β-D-glucosaminidase activities in the large macro- but not in the micro-aggregates. Furthermore, the large macroaggregates localize more N resource, microbial biomass and activity than the microaggregates. Consequently, the macroaggregates and their-associated total N, fungal biomass and chitinase activities are more sensitive to long-term N fertilization than the microaggregates.

Acknowledgments

The authors appreciate Elaine Stebler (Oklahoma State University) for English editing. This research was supported by the National Natural Science Foundation of China (31300381; 31570523; 41501334), the S&T Innovation Program of Chinese Academy of Agricultural Sciences, and the Innovation Team in Key Areas of the Tianjin Municipal Science and Technology Commission (TD12-5037).

References

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.
Alvarez, R., 2005. A review of nitrogen fertilizer and conservation tillage effects on soil organic carbon storage. Soil Use Manag. 21, 38–52.
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.
Balesdent, J., Besnard, E., Arrouays, D., Chenu, C., 1998. The dynamics of carbon in particle-size fractions of soil in a forest-cultivation sequence. Plant Soil 201, 49–57.
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.
Blankinship, J.C., Fonte, S.J., Six, J., Schimel, J.P., 2016. Plant versus microbial controls on soil aggregate stability in a seasonally dry ecosystem. Geoderma 272, 39–50.
Bronick, C.J., Lal, R., 2005. Soil structure and management: a review. Geoderma 124, 3–22.
Chen, Y., Wu, C., Tang, X., Yang, S., Wang, J., 2010. 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. Soils Sediments 15, 292–301.
Dai, X., Wang, H., Fu, X., 2017. Soil microbial community composition and its role in carbon mineralization in long-term fertilization paddy soils. Sci. Total Environ. 580, 556–563.
Danger, M., Gessner, M.O., Bärlocher, F., 2016. Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. Fungal Ecol. 19, 100–111.
Denef, K., Six, J., Bossuyt, H., Frey, S.D., Elliott, E.T., Merckx, R., Paustian, K., 2001. Influence of dry-wet cycles on the interrelationship between aggregate, particulate organic matter, and microbial community dynamics. Soil Biol. Biochem. 33, 1599–1611.
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. Chang. Biol. 15, 1603–1614.
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.
Geisseler, D., Scow, K.M., 2014. Long-term effects of mineral fertilizers on soil microorganisms—a review. Soil Biol. Biochem. 75, 54–63.
Guo, S., Wu, J., Coleman, K., Zhu, H., Li, Y., Liu, W., 2012. Soil organic carbon dynamics in a dryland cereal cropping system of the Loess Plateau under long-term nitrogen fertilizer applications. Plant Soil 353, 321–332.
Güsewell, S., Gessner, M.O., 2009. N:P ratios influence litter decomposition and colonization by fungi and bacteria in microcosms. Funct. Ecol. 23, 211–219.
He, Y.T., Zhang, W.J., MG, Xu, Tong, X.G., Sun, F.X., Wang, J.Z., Huang, S.M., Zhu, P., He, X.H., 2015. Long-term combined chemical and manure fertilizations increase soil organic carbon and total nitrogen in aggregate fractions at three typical cropland soils in China. Sci. Total Environ. 532, 635–644.
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.
Hua, K.K., Zhu, B., Wang, X.G., Guo, X.S., Wang, D.Z., Guo, Z.B., 2014. Effect of long-term fertilization on soil aggregate-associated dissolved organic nitrogen on sloping cropland of purple soil. Plant Soil Environ. 60, 51–56.
Huang, S., Peng, X., Huang, Q., Zhang, W., 2010. Soil aggregation and organic carbon fractions affected by long-term fertilization in a red soil of subtropical China. Geoderma 154, 364–369.
Jastrow, J.D., Amonette, J.E., Bailey, V.L., 2007. Mechanisms controlling soil carbon turnover and their potential application for enhancing carbon sequestration. Clim. Chang. 80, 5–23.
Jian, S., Li, J., Chen, J., Wang, G., Mayes, M.A., Dzantor, K.E., Hui, D., Luo, Y., 2016. Soil extracellular enzyme activities, soil carbon and nitrogen storage under nitrogen fertilization: a meta-analysis. Soil Biol. Biochem. 101, 32–43.
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., Blagodatskaya, E., 2015. Microbial hotspots and hot moments in soil: concept & review. Soil Biol. Biochem. 83, 184–199.
Li, Z., Jin, Z., Li, Q., 2017. Changes in land use and their effects on soil properties in Huixian karst wetland system. Pol. J. Environ. Stud. 26, 699–707.
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., Palojärvi, A., Rangger, A., Reeslev, M., Kjøller, A., 1998. The use of fluorogenic substrates to measure fungal presence and activity in soil. Appl. Environ. Microbiol. 64, 613–617.
Nivelle, E., Verzeaux, J., Habbib, H., Kuzyakov, Y., Decocq, G., Roger, D., Lacoux, J., Duclercq, J., Spicher, F., Nava-Saucedo, J.E., Catterou, M., Dubois, F., Tetu, T., 2016. Functional response of soil microbial communities to tillage, cover crops and nitrogen fertilization. Appl. Soil Ecol. 108, 147–155.
Rillig, M.C., Mummey, D.L., 2006. Mycorrhizas and soil structure. New Phytol. 171, 41–53.
Sall, S.N., Masse, D., Diallo, N.H., Sow, T.M.B., Hien, E., Guisse, A., 2016. Effects of residue quality and soil mineral N on microbial activities and soil aggregation in a tropical sandy soil in Senegal. Eur. J. Soil Biol. 75, 62–69.
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.
Šťursová, 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.
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.

Wang, Y., Hu, N., Ge, T., Kuzyakov, Y., Wang, Z.-L., Li, Z., Tang, Z., Chen, Y., Wu, C., Lou, Y., 2017. Soil aggregation regulates distributions of carbon, microbial community and enzyme activities after 23-year manure amendment. Appl. Soil Ecol. 111, 65–72.

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.

Xu, X., Zhang, W., Xu, M., Li, S., An, T., Pei, J., Xiao, J., Xie, H., Wang, J., 2016. Characteristics of differently stabilised soil organic carbon fractions in relation to long-term fertilisation in Brown Earth of Northeast China. Sci. Total Environ. 572, 1101–1110.

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.