The Contribution of Microorganisms to Soil Organic Carbon Stock Under Fertilization Varies among Aggregate Size Classes

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The contribution of microorganisms to soil organic carbon stock under fertilization varies among aggregate size classes

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Abstract

Long term fertilization alters soil microbiological properties and then affects soil organic carbon (SOC) stocks. However, the interrelations of SOC with biological drivers and their relative importance are rarely analyzed quantitatively at aggregate scale. We investigated the contribution of soil microbial biomass, diversity and enzyme activity to C stock in soil aggregate fractions (> 5 mm, 2−5 mm, 1−2 mm, 0.25−1 mm and < 0.25 mm) at topsoil (0–15 cm) from 27-year long term fertilization regime. Compared to CK (no fertilization management), NPS and NPM (inorganic fertilization plus the incorporation of maize straw or composted cow manure) significantly reduced the impact of NP (inorganic fertilizers application alone) on the growth of microbial community, and increased the microbial contribution to C stock. The results showed that microbial variables were significantly correlated with SOC content in > 0.25 mm aggregates rather than in < 0.25 mm aggregates. Fungal variables (fungal, AM biomass, and F/B ratio) and enzyme activities (BXYL and LAP) in > 0.25 mm aggregates explained 21% and 2% on C, respectively. Overall, organic matter (OM) addition could contribute to higher C storage by boosting fungal community and enzyme activity rather than by changing microbial community diversity in macro-aggregates.

Keywords

Fertilization; Soil aggregates; Microbial properties; Enzyme activity; SOC

Terrestrial soils contain approximately three times the stock of carbon (C) of the atmosphere, hence small changes in soil organic carbon (SOC) have a significant impact on climate change[1]. Among the numerous drivers that regulate the SOC pool, microorganisms are essential for SOC turnover[2]. Microorganisms have been reported to promote the formation of macro-aggregates to physically protect C[3], and their residues are also considered to be an important source of stable C[4]. Simultaneously,

38 microbe-driven soil C decomposition plays acritical role in C cycling[5]. It is reported that over half of
39 the cumulative CO_2-C emitted from soil was induced by microbial community[6]. In addition, soil
40 organic matter (SOM) could be synthesized or degraded by soil enzyme activity[7], which were linked to
41 CO_2 production[8]. As such, understanding the contribution of microorganisms and enzymes to the
42 accumulation or consumption of SOC in soil is of utmost importance for regulating soil C stock and
43 reducing the impact of CO_2 on the climate system.

44 Despite the direct microbe-driven in soil C cycling, the contribution of microorganism to C
45 turnover is often overlooked in C cycle prediction[9]. Maintaining high richness and diversity of soil
46 microorganisms is critical to mediate C cycling. However, there is less consistency on the research
47 regarding soil microbiological properties, such as soil microbial diversity, microbial community and
48 enzyme activity. With the most abundance in soil system[10], microorganisms (e.g., bacteria and fungi)
49 have been reported to facilitate the C cycling, through increasing metabolic actions[11] and bonding
50 organic particles together or stimulating root secretion of OM[12]. Some studies found that bacteria
51 contribute to the SOC storage more greatly than fungi in the rice and wheat system[13]. Differently,
52 arbuscular mycorrhizal (AM) fungi have been thought not to be very important in C decomposition[14].
53 Both microbial biomass C and diversity are suspected to play a crucial role in influencing SOC pool[15,16].
54 Another result showed that microbial biomass had a significant influence on soil C cycling rather than
55 its community composition under manure application[17]. Additionally, soil enzymes, produced by soil
56 microorganisms, are reported to regulate the overall processing of SOC through degrading different
57 molecules[18] or depolymerizing macromolecular substrates[19]. Some enzymes show a strong relationship
58 with SOC content and are generally reported to be good indicators of soil biological change[20,21]. It is
59 remarquable that, even if the complexity of SOC-related mechanisms is widely recognized, most of

60 studies focused on a single factor, less on multiple factors regulating SOC. Moreover, the potential
61 mechanisms by which microbiological properties are linked to C regulation are ignored.

62 As the basic unit of soil, aggregate plays a key role in C cycling[22]. Containing more than 90%
63 sequestered SOC, aggregate can be divided into macro- (> 0.25 mm) and micro- (< 0.25 mm)
64 aggregates[23,24]. Previous studies have shown that macro-aggregate (> 0.25 mm) contains more SOC
65 content than micro-aggregate (0.053–0.25 mm), the same as labile SOC[25]. As the basic elements in soil
66 structure, aggregate provides spatially heterogeneous microenvironments for soil microorganisms[26].
67 Large variance in environmental conditions in different sizes of aggregate, including water potential,
68 oxygen concentration and resource availability[27], could result in diverse biomass and community
69 diversity for microorganisms[28–30] and affect their functions on C turnover[31]. It was reported that soil
70 spatial heterogeneity could stimulate biodiversity by limiting these specific or individualized microbial
71 communities[29,30,32]. The distributions of microbial biomass and enzyme activity in aggregate fractions
72 were reported to be primarily governed by the aggregate sizes[33], due to different availability of organic
73 substances[34]. Furthermore, fungi were found to contribute to the C turnover more greatly and rapidly in
74 macro-aggregates than in micro-aggregates[35]. There are many evidences that the aggregate sizes affect
75 microbial community composition and enzyme activity. However, to the best of our knowledge, few
76 studies have investigated the mechanisms linking aggregate size and multiple microbial properties with
77 SOC turnover.

78 Regarding food demands, the application of inorganic and organic fertilizers in agricultural
79 systems is necessary to increase crop productivity in the world[36,37]. As one of the most common organic
80 amendments in fields, crop straw and manure application could increase the unstable C contents (e.g.,
81 dissolved organic carbon (DOC) and readily oxidizable organic carbon (ROC) contents[38]) that are the

82 main C sources for microorganisms. Several studies revealed that the alterations in microbial activity
83 could cause priming effects due to the addition of the substrate, which might simulate the turnover of
84 nature SOM in soil[39]. These practices have exhibited high impacts on soil microorganisms community
85 structure and diversity[40]. It also affected the enzyme activity through altering the habitat conditions for
86 soil microorganisms. Some researchers also have reported that soil C cycling is stimulated by changing
87 microbial biomass rather than its community composition under manure application[17]. During this
88 process, soil enzyme activityis influenced and related to C cycling[7]. As the microhabitats for
89 microorganisms, the aggregates are greatly changed in their physical conditions under fertilization[41].
90 For example, soil moisture within aggregate was directly altered by feitilizer application, which plays a
91 key role in the survival of soil microorganisms[42]. The application of manure increased the microbial
92 biomass in the form of phospholipid fatty acids (PLFA) in macro-aggregates, e.g., bacterial, fungal, and
93 AM fungal biomass, while not significantly in micro-aggregates[13]. Additionally, soil enzymes are
94 reported to react quickly to changes in most of soil managements[20]. Thus, the aggregate size plays a
95 significant role in the relationships between microbial properties and C cycling. Hence, a better
96 understanding of the influence of microbial properties on C cycling at aggregate scale under long term
97 fertilization regime is important, which aid to develop suitable management practices to better increase
98 C stock, simultaneously maintain a healthier soil microbial environment.

99 The objective of this study was to investigate the effects of 27-year fertilizations (CK: no
100 fertilization management; NP: inorganic fertilizers application alone; NPS: inorganic fertilization plus
101 the incorporation of maize straw; NPM: inorganic fertilization plus the incorporation of composted cow
102 manure) on soil microbial community and enzyme activity and their roles in influencing C stock at
103 aggregate scale in the Loess Plateau of China. Here are three hypotheses: (1) In comparison with the

CK and NP, NPS and NPM will positively improve microbial community properties and enzyme activity in aggregates; (2) the relationship between soil microorganisms and enzymes, and their contributions to C stock vary with aggregate size; (3) NPS and NPM could increase the contribution of microorganism to C stock through influencing the population of microorganism community at aggregate scale.

Materials and Methods

Site Description and Soil Sampling. The study site is located in the Dryland Farming Experimental Station in Shanxi province (112–113◦E, 37–38◦N) in northern China and was initiated in 1993. The site is characterized by a continental monsoon climate with an elevation of approximately 1,100 m above sea level, annual rainfall of 520 mm and temperature of 7–8°C. Spring maize is the main crop grown under the one-crop-per-year cropping system. Soils belong to a sandy clay loam cinnamon soil series which are characterized as Calcaric-Fluvic Cambisols (ISS-CAS, 2003; IUSS, 2006). At the start of the project, soil pH was on average 7.9, and SOC and soil organic N concentrations were 15.0 g kg^{-1} and 1.0 g kg^{-1}, respectively.

The long-term experiment had a randomized block design with three replicates, each plot was 6 × 6 m. The four treatments included in this study were as follows: no fertilization management (CK), inorganic fertilizers application alone (NP), inorganic fertilization plus the incorporation of 3,000 kg ha^{-1} maize straw (NPS), and inorganic fertilization in combination with 1,500 kg ha^{-1} composted cow manure (NPM). Each plot of the treatments NP, NPS and NPM had nitrogen 105 kg ha^{-1} and phosphorus 105 kg ha^{-1} applied once a year, respectively, using urea (46% N) and calcium superphosphate (7% P) in a ratio of N to P of 1:0.44. The mean proportions of SOM, total nitrogen, total phosphorus and total potassium were 75%, 0.63%, 0.039% and 0.72% in maize straw, and 36%,

0.96%, 0.17% and 0.74% in cattle manure, respectively. All of the fertilizers and OM were applied with conventional tillage (plowing once each year at a depth of 20 cm) after harvesting. Seeding was done at the end of April without any tillage and harvesting at the beginning of October, with twice weeding during growth seasons every year.

Soil samples were obtained after harvesting in the middle of October, 2018. For each treatment, five soil cores (10 × 10 cm in diameter) were collected randomly at a depth of 0–15 cm in each plot and pooled together, and thereafter taken to laboratory immediately. The fresh soil was separated manually along the natural cracks of fracture to obtain aggregate sizes of < 6 mm. After removing stones, plant material and visible soil fauna, 100 g fresh soil samples were air-dried for the analyses of aggregates distribution using dry sieving method, and the rest were sieved (5 mm, 2 mm, 1 mm and 0.25 mm mesh sieves) immediately. Then, the classified aggregates (20 groups of aggregate samples in total) were divided in half: one half was immediately stored at −80 °C for biochemical analysis, and the other half was kept at 4 °C for the analysis of organic carbon contents.

Aggregate fractionation. The fractionation of soil aggregates was measured using the dry sieving method[25]. After removing the visible impurities, 200-gram samples (dried soil) were passed through a series of four sieves (5 mm, 2 mm, 1 mm and 0.25 mm) and divided into five aggregate sizes.

Determination of organic C concentrationof soil samples. The SOC (from dried soil) was estimated with an element analyzer (C/N Flash EA 112 Series-LecoTruspec). Dissolved organic carbon (DOC) content was measured through detecting soil extracted solution made by distilled water (1:5 w:v) using a C analyzer (Multi N/C 3100, Analytic Jena, Germany). Readily oxidizable organic carbon (ROC) content was analyzed by $KMnO_4$ oxidation. Briefly, after passing through 0.15 mm sieve, the air-dried soil (containing approximately 15–30 mg C) was added in 25 mL of 333 mmol L^{-1} $KMnO_4$,

148 then shook and centrifuged. The supernatants were diluted with deionized water (1:250) and detected
149 by a UV spectrophotometer at 565 nm. Microbial biomass carbon (MBC) content was measured using
150 the fumigation-incubation method[43]. In detail, four aliquots offreeze-dried soil samples (25 g each
151 aliquot) were prepared, two aliquots being fumigated with ethanol-free $CHCl_3$ for 24 h in the dark at
152 room temperature, while the other two was kept untreated ascontrol. Then, these samples were mixed
153 into 100 mL of 0.5 mol L^{-1} K_2SO_4 solution, respectively, and shaken for 30 min at 200 r min^{-1}. The
154 supernatants were diluted with deionized water and then detected by a total organic C (TOC) analyzer.
155 **Analysis of enzyme activity.** In this study, the four soil enzymes activities of β-Glucosidase (BG),
156 β-Xylosidase (BX), N-acetyl-glucosaminidase (NAG) and leucine aminopeptidase (LAP) were
157 estimated following the method of previous studies[44,45]. 4-Methyllumbelliferyl (MUB) and
158 7-amino-4-methylcoumarin (AMC) were used as substrate to determine the activities of all enzymes
159 (MUB for BG, BXYL and NAG; AMC for LAP). Firstly, 1 g of fresh soil was mixed in 125 mL of
160 $NaHCO_3$ buffer (pH = 8) and stirred at 800 rpm for 2.5 min. Secondly, the slurry was transferred into
161 96-well microplate using an eight channel pipet, and substrates were quickly added. Thirdly, all the
162 microplates were incubated in a dark for 3 h at 25°C. Finally, the fluorescence of the supernatants was
163 detected using a multilabel fluorescence reader (Tecan Infinite F200/M200).
164 **PLFA extraction and analysis.** Phospholipid fatty acids (PLFA) were measured to calculate the soil
165 microbial biomass and diversity. The method details were described in previous research[46]. In brief,
166 aliquots of 3 g (freeze-dried) aggregate samples were extracted twice in 7.6 mL
167 chloroform/methanol/citrate buffer (1:2:0.8 v/v/v) system. After that, phospholipids were separated
168 from neutral and glycolipids with silica acid columns (Supelco Inc., Bellefonte, PA). After methylation
169 of the polar lipids, the fatty acid methyl esters (FAME) were identified by Gas Chromatograph Agilent

Series (GC 6890, Agilent Technologies, Wilmington, DE) and calculated by MIDI microbial identification system (MIDI, Inc., Newark, DE). Nonadecanoic acid (19:0) was used as an internal standard.

PLFA were assigned to general bacteria (16:0, 17:0, 18:0, 20:0), gram-negative bacteria (G-), gram-positive bacteria (G+), general fungi (18:2ω6c), arbuscular mycorrhizae (AM, 16:1ω5c) and actinomycetes (16:1ω5c)[47,48]. Cyclopropyl and monounsaturated fatty acids were indicators for G-bacteria, whereas iso- and anteiso-branched fatty acids were biomarkers for G+ bacteria.

Data calculation and statistical analysis. The proportions of graded aggregates with different particle sizes were calculated as following equation 1:

$$Dry - pi = (Wi * 100\%)/200 \quad (1)$$

Where, Wi was the mass of i-th graded aggregates with different particle sizes, and Dry-pi was the proportion of i-th graded aggregates in total soil. MBC concentration was obtained by calculating the difference in OC between fumigated and non-fumigated samples with 0.45 (the proportion of soil biomass C extracted by K_2SO_4 after chloroform fumigation) using the equation 2[49]:

$$MBC = (Fumigated - Unfumigated)/0.45 \quad (2)$$

Where, Fumigated and Unfumigated were the OC extracted in K_2SO_4 from fumigated and non-fumigated soil samples per gram of soil. Microbial community diversities were evaluated using Shannon–Wiener diversity index (H'), Simpson evenness index (D) and Margalef richness index (M). They were generally calculated as follow:

$$H' = -\sum pi * ln\, pi \quad (3)$$

$$D = 1 - \sum (pi)\text{^}2 \quad (4)$$

$$M = (S - 1)/lnN \quad (5)$$

where H′, D and M were Shannon–Wiener, Simpson and Margalef indexes, respectively; p_i was the percentage of the peak area of i-th FAME to the total area in each sample; S was the total number of FAME in each sample; and N was the amount of total microbial PLFA.

Statistically, all data were carried out by SAS 9.4 in Windows 10. One-way ANOVA with Duncan's test was used to test the effects of fertilization regime andaggregate size on the soil biological properties, soil organic C contents, proportions and moisture of aggregates, respectively. Two-way analysis of ANOVA was used to examine fertilization treatment and aggregate size on all of the soil physicochemical and microbial indicators. Correlations with p-value lower than 0.05, 0.01 and 0.001 were considered. Spearman correlation was computed between microbial parameters and SOC under each aggregate size, and between soil physicochemical and microbial parameters in > 0.25 mm and < 0.25 mm aggregates. Additionally, principal component analysis (PCA) was performed to divide the microbial factors correlated with SOC into different groups in > 0.25 mm aggregates using Vegan package in R and variation partitioning analysis (VPA) was further applied to quantify how much variation in SOC was explained by fungal community, microbial diversity and enzyme activity in the Vegan package of R.

Results

Soil organic C and moisture in aggregate and aggregate proportions under different fertilizations. From Table 1, SOC and DOC contents varied with fertilization and aggregate size, both of which were higher under NP, NPS and NPM than under CK in almost all aggregates ($p < 0.05$; Fig. 1A and B). MBC and ROC contents were significantly affected by fertilization and aggregate size, respectively ($p < 0.001$; Table 1). The four treatments had no significant differences in ROC and MBC contents

(Fig. 1C and D) in micro-aggregates (< 0.25 mm), and a similar observation was also found in the ratio of MBC to SOC content (Fig. 2).

Fertilization, aggregate size and their interaction significantly affected the moisture in aggregate (p < 0.001; Table 1). The moisture was lower in NP than in CK almost in all aggregates (p < 0.05; Fig. 3A). Compared to NP, NPS and NPM significantly increased moisture in all aggregates (p < 0.05). The moisture was higher in macro-aggregates (> 0.25 mm) than in micro-aggregates (< 0.25 mm) under the four treatments.

There was an extremely significant difference in aggregate mass proportions among aggregate sizes (p < 0.001; Table 1). Compared to CK, NP significantly (p < 0.05; Fig. 3B) reduced the mass proportions of macro-aggregates (> 2 mm) but increased the mass proportions of micro-aggregates (< 0.25 mm), while the results under NPS and NPM were opposite.

Soil microbial biomass and diversity and enzyme activity. Soil microbial biomass was significantly impacted by fertilization and aggregate size (p < 0.01; Table 1). Compared to CK (Fig. 4A), NP (Fig. 4C) decreased all of microbial indices, while NPS and NPM increased those (except fungi indices) in different sized aggregates (Fig. 4B and D). The increases in fungal biomass (including AM) and F/B ratio were observed only in micro-aggregates (< 0.25 mm) under NPS and NPM relative to CK. In micro-aggregates (< 0.25 mm), the bacterial and fungal biomass were higher under NPS treatment than under CK. Most of the microbial groups biomass increased with the decreasing of aggregate size under NPS and NP, while there was no significant difference in microbial biomass (except bacterial biomass) among all aggregates under NPM. Additionally, the ratio of fungi:bacteria (F/B) and G+: G- (G+/G-) is also affected by fertilization (Table 2), and there were no significant differences in the two ratios among all aggregate sizes under NPM. The biomass of different microbial groups was lower in

macro-aggregates (> 0.25 mm) than in micro-aggregates (< 0.25 mm) under fertilization. The total PLFA was significantly associated with moisture in macro-aggregates (> 0.25 mm).

As showed in Table 1, significant ($p < 0.05$) interactive effects between fertilization and aggregate size were observed in all indices of microbial diversity. The three indices were affected significantly by fertilization almost in 0.25–1 mm and 2–5 mm ($p < 0.05$; Table 2), but not in micro-aggregates. Unlike CK and NP treatments, these three indices had no significant difference among variably sized aggregates under NPS and NPM. From Fig. 5B, the microbial diversities of Shannon-wiener (H′) and Simpson (D) were respectively correlated with bacteria (including G+ and G-) and actinomycetes in macro-aggregates (> 0.25 mm).

All of the four soil enzymes activities varied with fertilization and aggregate size ($p < 0.05$; Table 1). In Fig. 6, the four soil enzymes activities were higher under NPM and NPS than under CK in all macro-aggregates (except 1–2 mm). NP increased those only in 2–5 mm and 0.25–1 mm while decreased in other aggregate sizes, compared to CK. There were no differences on LAP and NAG between NPS, NPM and CK in micro-aggregates (< 0.25 mm). The four soil enzymes activities were higher in > 0.25 mm than in < 0.25 mm aggregates under NP treatment. The activities of NAG, LAP and BXYL were higher under NPS and NPM in > 0.25 mm aggregates (except 2–5 mm) than in micro-aggregates (< 0.25 mm). Soil enzyme activity was positively correlated with bacterial biomass and moisture in > 0.25 mm aggregates ($p < 0.05$; Fig. 5B).

Contributions of microbial community and soil enzyme to SOC storage. The correlations between SOC storage and indicators of microbial community and enzyme activity in different sized aggregates showed that microbial and enzyme indicators, i.e., fungal and AM biomass, F/B ratio, BXYL, LAP, H' and M, were significantly related to SOC content in > 0.25 mm aggregates, while no significant

correlation in < 0.25 mm aggregates (Table 3). Soil microorganism and enzyme contributions to C storage under different fertilizations were analyzed by PCA and VPA, considering the differences in their compositions in > 0.25 mm aggregates (Fig. 7).

The PCA revealed that the predictors explained 63.9% of the variation in > 0.25 mm aggregates, and all of these indicators were clearly divided into 3 groups by the first two principal components among all samples (Fig. 7A). The Venn diagram (Fig. 7B) revealed that the fraction of C storage variation explained by the fungal indices was 21% (fraction [a]; $p = 0.005$). The soil enzyme component explained a lower proportion the variation of SOC content in > 0.25 mm aggregates ([b] = 2%, $p = 0.013$). Meanwhile, the explanation from microbial diversity was far less than fungal community and enzyme activity, and was not displayed in Fig. 7B with a value of less than zero. Most of the SOC variation remained unexplained by the model variables (Residuals = 77 %).

Discussion

Long term inorganic and organic fertilization was considered to affect soil microbial community because they change the microenvironmental conditions and nutrients for microbial survival[50,51]. In this study, there was a remarkable reduction of the soil microbial biomass in all aggregates under NP compared to CK (Fig. 4). Although inorganic fertilizer provided the nutrients such as nitrogen and phosphorus, it reduced the moisture values in aggregate (Fig. 3A), which affects the survival of microorganisms inside[52]. Microbial community is sensitive to the increasing of available nutrients under organic matter (OM) application[53]. In comparison with NP, the OM incorporation resulted in an increase of microbial biomass (i.e., PLFA, bacteria and actinomycetes) in almost all of the aggregates, which vary with the aggregate size and OM type (Fig. 4). It indicated that OM addition could alleviate this negative effect of NP on the microbial community, and the effect of fertilization on microbial

biomass is associated with the soil aggregate size and nature of the OM[54]. Notably, the amount of fungi was higher only in micro-aggregates under NPS and NPM than under CK, which indicates that fungi within macro-aggregate are was more sensitive to environmental perturbation than in micro-aggregates because of the vulnerability of microenvironment in macro-aggregate[55]. Especially, the amount of AM was slightly higher in NPS than in NPM, due to the high decomposition ability of AM in cellulose[56]. This can also explain why lower F/B was found in macro-aggregates (> 0.25 mm) than in micro-aggregates (< 0.25 mm) under the application of organic and inorganic fertilizers. Therefore, the results supported that OM incorporation can build a more suitable environment for microbial surviving.

Our results indicated that there were no significant influences of fertilization on H′, M and D indices (Table 1). Among the aggregate sizes, significant differences in microbial diversity were observed under NP and CK, while no difference under NPS and NPM. OM addition could promote higher bacterial richness or evenness among all fractions and enhance the microbial community resistance to disturbance relative to inorganic fertilization alone or no fertilization[57]. Based on these findings, the results suggested that the biomass and diversity of microbial community were changed with the application of fertilization, which were obviously ($p < 0.05$) associated with soil aggregate size[58]. Similar changes in soil enzyme activities corresponded with microbial biomass. Relative to CK, NPM and NPS increased soil enzyme activities in almost all macro-aggregate sizes (except for 1–2 mm), due to the increasing substrates for soil enzymes provided by the increasing of microbial population[59,60]. Soil enzyme activities were suggested to be suppressed by the inorganic fertilization[61], however, they were favored in 2–5 mm and 0.25–1 mm aggregates under NP, in order to provide C or nitrogen nutrients for the survival of microorganisms[62]. In all of the aggregates, soil enzyme activities were correlated to the distribution of microbial community and moisture[63]. Therefore, our results

301 indicated that the effects of fertilization regime on soil microbial community were associated with the
302 balance between microbial nutrient requirements and secretion of enzymes, and varied with soil
303 aggregate size.

304 It has been documented that microorganisms and enzymes affect C cycling[13,18]. As Table 1 showed,
305 almost all soil microbial community and enzyme indices were associated with aggregate size.
306 Meanwhile, it has subsequently been confirmed in the Table 3, that soil microbial and enzyme indices
307 were significantly related to SOC in > 0.25 mm but not in < 0.25 mm in response to the different
308 fertilization, supporting that micro-aggregates could protect SOC from being decomposed by
309 microorganisms[64], whereas macro-aggregates enhance SOC sequestration due to their greater stability
310 mainly caused by the adhesion of microorganisms and secretions[65].

311 As a primary elemental energy source for microorganism, ROC was positively correlated with
312 microbial biomass in < 0.25 mm aggregates (Fig. 5A), i.e., bacteria, fungi and actinomycetes. It
313 indicated that the microbial activity may be limited by ROC, supported by the research that limited
314 labile C is one of the main reasons restricting the growth of soil heterotrophic microorganisms[66]. The
315 same trends were found on MBC content and MBC/SOC ratio, that the fertilization had no effect on
316 them in < 0.25 mm aggregates. It indicated that microbial biomass C may have been saturated in
317 micro-aggregates, supporting the findings that higher quality and protection of SOC in
318 macro-aggregate are more conducive to the growth of microorganisms compared to
319 micro-aggregate[67,68]. However, our results that a significant increase of SOC occurred in
320 micro-aggregates (< 0.25 mm) under fertilizations, indicated that more contribution to C sequestration
321 comes from other pathways than microbial processes. For instance, the processes of chemical bonding

to minerals or physical protection contribute to the mineral-associated organic C formation, which is one of the main components of organic C[69,70].

In > 0.25 mm aggregates, the PCA analysis divided the indices that have a significant ($p < 0.05$) impact on SOC into three groups, and Venn models revealed that fungi-related factors were more important than microbial diversity and enzyme activities in affecting the SOC stock in macro-aggregates (Fig. 7). This is largely due to the structural heterogeneity of the macro-aggregate and the strong viability of fungal community[71,72]. Especially, as the dominant mycorrhizal type, AM fungi were reported to alter the C storage through enhancing litter decomposition[73] or reducing the rhizosphere priming effect to increase C retention[54]. Microbial diversity had no significant ($p > 0.05$) relationship with SOC in macro-aggregates (> 0.25 mm), the same trend as bacterial biomass (Fig. 5B). Some research reported that bacterial diversity increased more greatly than fungi in higher pH values[74], thus bacterial community plays a key role in microbial diversity. It was in line with our result that the microbial diversity of H′ was correlated ($p < 0.05$) with the bacterial biomass in > 0.25 mm aggregates. Inconsistent with our results, the abundance of bacteria was supported to contribute to the rapid decomposition of soil C[75]. Similarly, soil enzyme activities were significantly and positively correlated with bacterial and actinomycetic biomass, which supported that the diversity and composition of bacterial community could be partially reflected by the soil enzymes[76]. It also can explain why no significant contribution of enzyme activity to SOC was observed. The high unexplained residuals suggest that important aspects driving C dynamics were not included in this analysis, such as physical and chemical approaches. Our results confirmed that microbial variables had greater impact on SOC in > 0.25 mm macro-aggregates under different fertilizations, whereas no significant effect was found in < 0.25 mm micro-aggregates. The total effect of fungi-related indicators contribution was higher than

other microbial indices[77]. More in-depth studies are needed to detect fungal reaction and variation resulting from fertilization and should be incorporated in the main causes of microbial approach that affect C stock.

Soil C redistribution and microbial habitat condition were altered under manure and crop residues inputs[78]. Previous studies confirmed that different fertilization managements might affect the soil biological processes, through changing the soil environment, the nutrients and turnover of aggregate, which could directly or indirectly affect C storage[80-82]. This study showed that NP made a significant reduction of the microbial biomass (Fig. 4C), i.e., fungal and bacterial biomass, and led to a stronger decrease in C decomposition by microorganisms than NPS and NPM. The main reason may be that long-term inorganic fertilizer inhibited the growth of microorganisms through affecting the soil pH or moisture condition[83–85], which lead to the weakened microbial decomposition of C. It also might be one of the reasons for the higher ROC content in NP than in CK. The ratio of MBC/SOC and the mass proportions of macro-aggregates (> 2 mm) were lower under NP than CK, whereas the total SOC content was higher, indicating that the contribution of microbial process and physical protection to C reduced under inorganic fertilization[64]. Therefore, other important aspects should contribute more greatly to C stocks than microbial process under inorganic fertilization. For example, the chemical process of the interaction between C and soil minerals has been known as an important pathway of mechanisms of C sequestration[69]. Simultaneously, the combined application of OM significantly reduced the impact of inorganic fertilizers on the growth of microbial community, due to the improvement of water condition and agglomeration (Fig. 3). For dryland agriculture, periodic rainfall causes soil to be in a long-term alternating state of dry and wet, causing periodic fluctuations in soil water contents. In this study, the application of OM alleviated the fluctuation of moisture and

maintained it at a relatively high level, which is conducive to microbial activity[86,87]. This is consistent with the result that MBC content (Fig.1C) was higher under NPM and NPS than under NP in macro-aggregates (except 0.25–1 mm). The fungal biomass were positively ($p < 0.05$) related to macro-aggregates proportion (> 0.25 mm), which promotes the physical protection of C sequestration[64]. However, both of ROC and SOC contents have no significant differences between inorganic and organic fertilizations, which were negatively ($p < 0.05$) correlated with fungal biomass. This indicated that the C stock was in dynamic equilibrium under the inorganic and organic fertilizations during the process of C sequestration and decomposition in macro-aggregate, in which fungi are key regulators[88,89]. A similar observation was also found that fungi were affected more greatly by fertilization in macro-aggregates than in micro-aggregates[58], with the great ability to degrade complex C polymers[90]. The lower F/B under fertilization than no fertilization in macro-aggregates suggested that bacteria were more able to adapt to fertile environment. Both of actinomycetes and bacteria, including G+, G- and G+/G-, had no significant relationship with C among all treatments, which was supported by the study that low net accumulation of C was found in fertile soils that are dominated by bacterial community[75]. Therefore, it indicated that the effect of fertilization management on the contribution of fungal community to C is more pronounced in macro-aggregates, compared to other microbial community.

Conclusions

Microbiological properties play an important role in C reserves at aggregate scale under different fertilization regimes. We emphasized that the alteration of microorganism community (i.e., fungal and AM biomass and F/B ratio) and enzyme activities (BXYL and LAP), rather than microbial diversity, contributed greatly to C storage in macro-aggregates (> 0.25 mm). However, the influence of microbial

388 factors in C storage was not significant in micro-aggregates. Compared to inorganic fertilization,
389 combined application of organic and inorganic fertilizer increased the microbial contribution to C
390 storages. Our study indicated that the contribution of microbial processes to C stock not only depends
391 on the aggregate size, but also on the varietyof the microbial properties and their interrelationships
392 under different fertilization regimes. Future research is needed to reduce the negative impact of
393 inorganic fertilizers on soil flora by using OM application while maximizing the soil C stock in
394 agroecosystems.

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Figure captions:

Figure 1.

The distribution of SOC (A), DOC (B), MBC (C), ROC contents (D) in different sized aggregates under 4 fertilization managements (CK, NP, NPS and NPM).

Figure 2.

The ratio of MBC to SOC in different aggregates under 4 fertilization managements (CK, NP, NPS and NPM).

Figure 3.

The moisture (A) and mass proportions (B) of different aggregates under 4 fertilization managements (CK, NP, NPS and NPM).

Figure 4.

Microbial biomass and the phospholipid acid biomarkers in different sized aggregates under 4 fertilization managements, CK (A), NPS (B), NP (C), and NPM (D).

Figure 5.

Correlative coefficients among the properties of soil organic C, microbial indices and enzyme activity in < 0.25 mm (A) and > 0.25 mm (B) aggregates, respectively.

Figure 6.

Histogram of N-acetyl-glucosaminidase (A), leucine aminopeptidase (B), β-glucosidase (C) and β-xylosidase (D) activities in aggregates in different treatments.

Figure 7.

PCA analysis (A) of soil microbial properties and enzyme activity in macroaggregates (> 0.25mm) under 4 fertilization managements (CK, NP, NPS and NPM). Variation-partitioning Venn diagram (B) of SOC accumulation variance partitioning among fungal indices (a), soil enzyme activity (b), and microbial diversity (c) predictor matrices in macroaggregates (> 0.25mm).

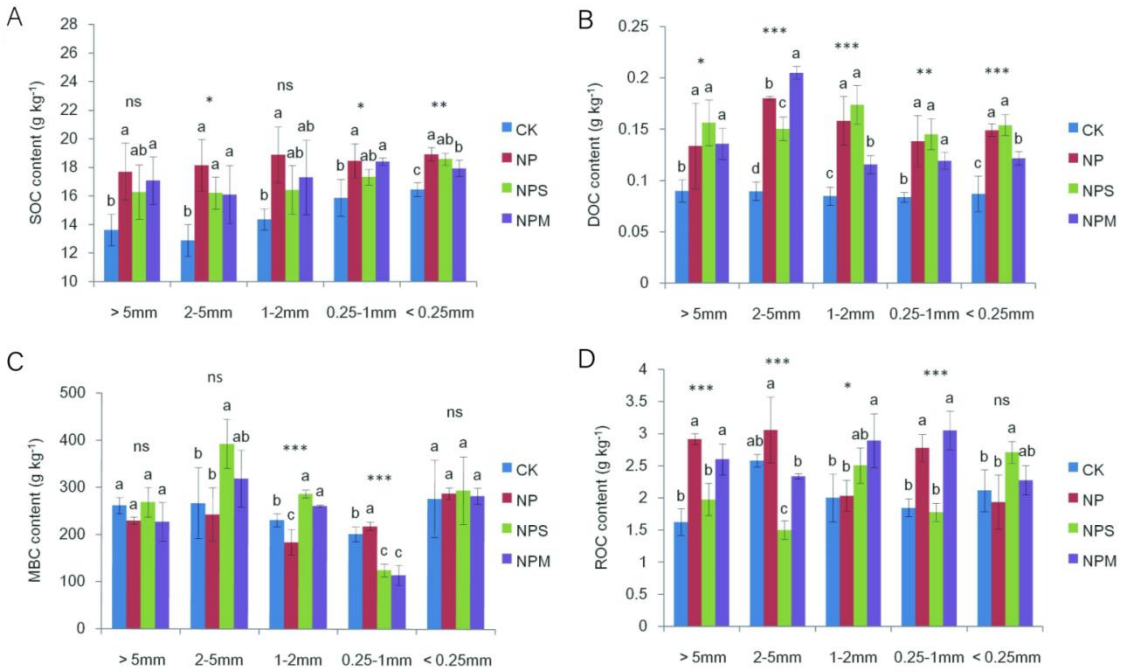
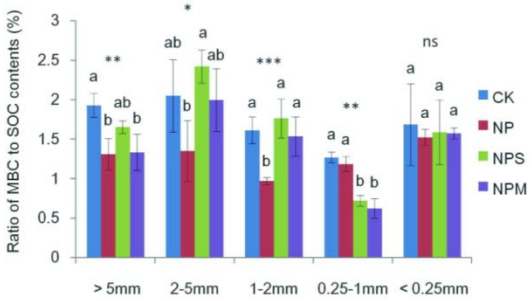


Figure 1. The distribution of SOC (A), DOC (B), MBC (C), ROC contents (D) in different aggregates under 4 fertilization managements (CK, NP, NPS and NPM). CK: the control treatment without fertilization management; NP: the treatment with only inorganic fertilizer; NPS: the treatment with inorganic fertilizer and maize straw addition; NPM: the treatment with inorganic fertilizer and cattle manure. Vertical bars mean the standard error of the mean (n = 3). Different lowercase letters indicated significant difference at $p < 0.05$ among the different fertilization managements. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant.



640

641 **Figure 2.** The ratio of MBC to SOC in different aggregates under 4 fertilization managements (CK, NP,
642 NPS and NPM). Values in the same aggregate size followed by the same lowercase letters are not
643 significantly different ($p < 0.05$) according to LSD test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns:
644 not significant.

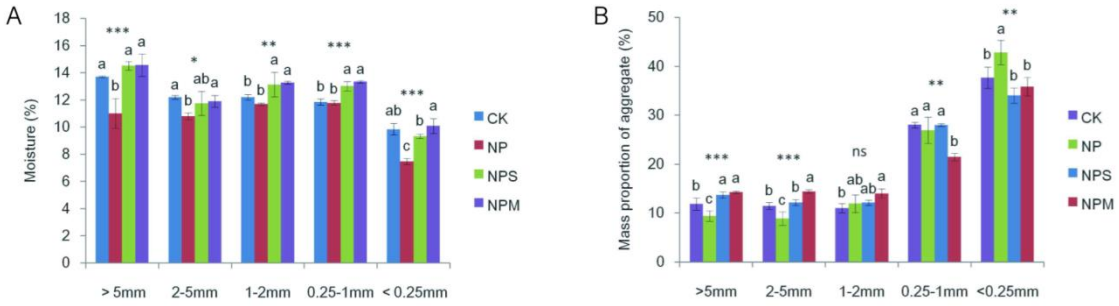


Figure 3. The moisture (A) and mass proportion (B) of different aggregates under 4 fertilization managements (CK, NP, NPS and NPM). Values in the same aggregate size followed by the same lowercase letters are not significantly different ($p < 0.05$). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant.

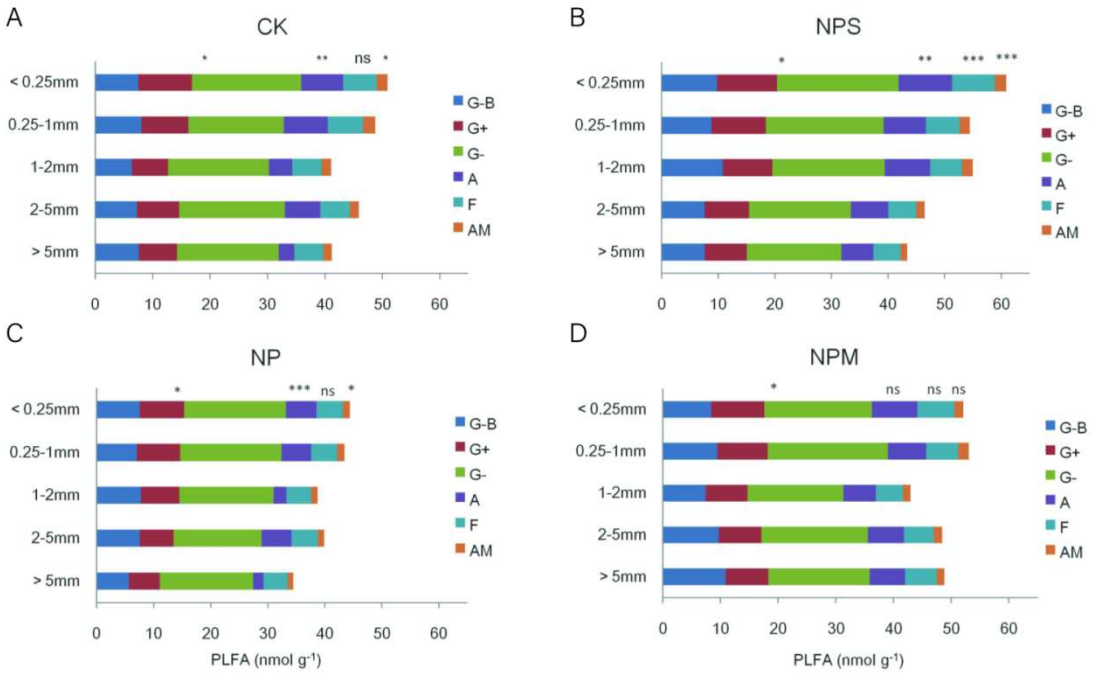


Figure 4. Microbial biomass and the phospholipid acid biomarkers in different sized aggregates under 4 fertilization managements, CK (A), NPS (B), NP (C), and NPM (D). G-B: general bacterial PLFA; G+: Gram-positive bacterial PLFA; G-: Gram-negative bacterial PLFA; A: actinomycetic PLFA; F: general fungal PLFA; AM: arbuscular mycorrhizal fungi. The differences in total bacteria (the sum of G-B, G+ and G- bacteria), actinomycetes, general fungi and AM PLFA among the different sized aggregates were showed at * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$ and ns: not significant.

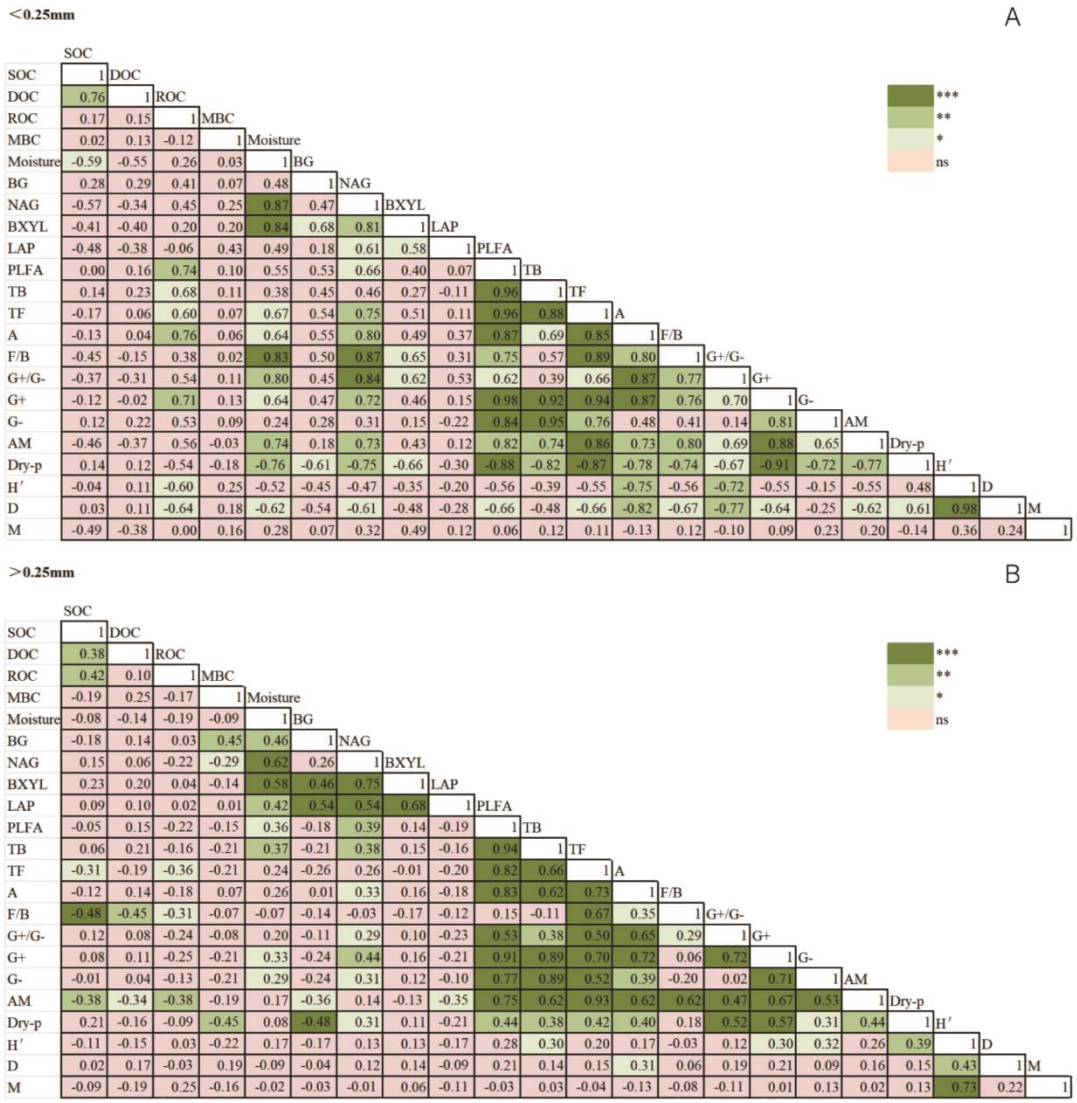


Figure 5. Correlative coefficients among the properties of soil organic C, microbial indices and enzyme activity in < 0.25 mm (A) and > 0.25 mm (B) aggregates, respectively. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

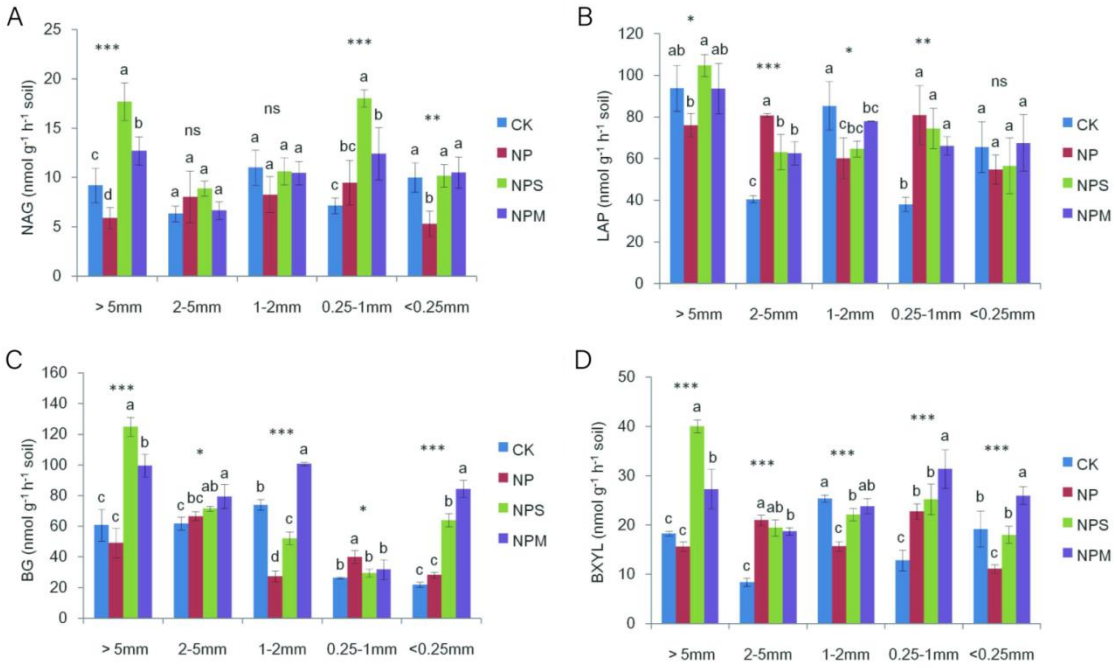


Figure 6. Histogram of N-acetyl-glucosaminidase (A), leucine aminopeptidase (B), β-glucosidase (C) and β-xylosidase (D) activities in aggregates in different treatments. NAG, N-acetyl-glucosaminidase; LAP, leucine aminopeptidase; BG, β-xylosidase; BXYL, β-xylosidase. Different lowercase letters mean signifcant differences among aggregate size fractions ($p < 0.05$). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant.

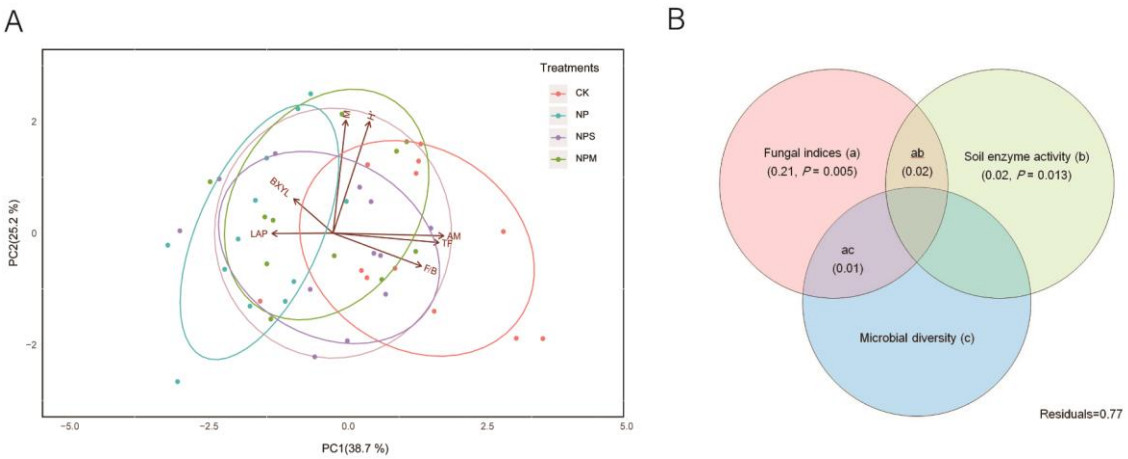


Figure 7. PCA analysis (A) of soil microbial properties and enzyme activity in macroaggregates (> 0.25 mm) under 4 fertilization managements (CK, NP, NPS and NPM). Variation-partitioning Venn diagram (B) of SOC accumulation variance partitioning among fungal indices (a), soil enzyme activity (b), and microbial diversity (c) predictor matrices in macroaggregates (> 0.25 mm). Fungal indices, soil enzyme activity, and microbial diversity contained three, two, and two factors, respectively.

Table captions:

Table 1.

Two-way ANOVA of fertilization treatment (T), aggregate size (A) and their interaction on soil C, enzyme activity and microbial variables and aggregate properties.

Table 2.

Microbial biomass and ratios of the phospholipid acid biomarkers and microbial diversity in different sized aggregates.

Table 3.

Correlative coefficients between SOC and soil biological properties for CK, NP, NPS and NPM in different sized aggregates.

Table 1. Two-way ANOVA of fertilization treatment (T), aggregate size (A) and their interaction (T*A) on soil C, enzyme activity and microbial variables and aggregate properties.

Indices	Treatment (T)		Aggregate size (A)		T*A	
	F	P	F	P	F	P
SOC	19.1***		4.81**		0.55	
DOC	57.15***		8.01***		4.33***	
ROC	14.82***		0.35		7.6***	
MBC	2.08		17.73***		4.51***	
BG	35.32***		5.54**		2.66*	
CBH	39.34***		14.74***		2.11*	
NAG	12.23***		17.37***		1.61	
LAP	4.08*		54.97***		3**	
Total PLFA	35.05***		17.59***		3.00**	
Bacteria	22.39***		9.91***		3.22**	
Fungi	23.08***		12.23***		2.44*	
AM	46.27***		15.89***		2.18*	
Actinomycetes	21.84***		12.38***		1.72	
G+	20.73***		22.59***		0.99	
G-	8.16***		5.17**		2.26*	
G+/G-	5.87**		10.33***		1.91	
F/B	10.46***		1.69		1.56	
H′	0.95		6***		3.48**	
D	0.41		5.2**		2.11*	
M	1.92		2.13		2.62*	
Moisture	54.91***		137.16***		4.85***	
Dry-p	0		879.68***		14.77***	

B/F: the ratio of bacterial and fungal PLFA; G+/G-: the ratio of G+ and G- bacterial PLFA; AM: arbuscular mycorrhizal fungi; H′: Shannon–Wiener diversity index; D: Simpson evenness index; M: Margalef richness index; Dry-p: the proportions of aggregates using dry sieving method. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant.

690 **Table 2.** Microbial PLFA biomass and ratios of the phospholipid acid biomarkers and microbial diversities in different
691 sized aggregates.

Microbial indices	Soil fraction (mm)	Fertilizer management				P value
		CK	NP	NPS	NPM	
F/B	> 5mm	21.71±2.19Ab	18.00±5.07Aa	18.75±0.52Ab	19.67±2.80Aa	ns
	2–5mm	20.42±0.18Ab	19.72±1.22Aa	19.22±0.20Ab	18.59±2.12Aa	ns
	1–2mm	22.53±0.75Aab	17.74±2.29Ba	19.09±1.48Bb	19.21±1.02Ba	*
	0.25–1mm	25.17±1.22Aa	18.19±0.46Ba	19.78±0.61Bb	19.05±0.99Ba	***
	< 0.25mm	21.68±1.00Ab	17.69±1.11Ba	22.86±0.86Aa	22.03±1.36Aa	**
	P value	*	ns	***	ns	
G+/G-	> 5mm	0.37±0.22ABb	0.34±0.04Bb	0.45±0.03Ab	0.42±0.06AB	ns
	2–5mm	0.40±0.02ABb	0.39±0.02Bab	0.43±0.01Ab	0.40±0.02AB	ns
	1–2mm	0.36±0.03Ab	0.41±0.05Aa	0.44±0.02Ab	0.44±0.05Aa	ns
	0.25–1mm	0.50±0.06Aa	0.42±0.01Aa	0.46±0.02Ab	0.42±0.02Aa	ns
	< 0.25mm	0.49±0.03Aa	0.44±0.02Ba	0.50±0.01Aa	0.50±0.03Aa	ns
	P value	**	*	*	ns	
Microbial diversity						
Shannon-wiener	> 5mm	3.21±0.09ABb	3.10±0.07Bc	3.26±0.05Aa	3.23±0.08AB	ns
	2–5mm	3.38±0.03Aa	3.16±0.04Bc	3.19±0.13Ba	3.25±0.03AB	*
	1–2mm	3.25±0.07Aab	3.28±0.05Ab	3.24±0.05Aa	3.25±0.12Aab	ns
	0.25–1mm	3.23±0.06Bb	3.41±0.02Aa	3.26±0.10Ba	3.40±0.05Aa	*
	< 0.25mm	3.32±0.09Aab	3.38±0.07Aa	3.26±0.05Aa	3.29±0.07Aab	ns
	P value	ns	***	ns	ns	
Simpson (D)	> 5mm	0.94±0.01Aa	0.93±0.006Bb	0.95±0.001Aa	0.94±0.006A	*
	2–5mm	0.95±0.001Aa	0.96±0.01Aa	0.96±0.01Aa	0.95±0.005Aa	ns
	1–2mm	0.95±0.005Aa	0.94±0.006Aa	0.95±0.004Aa	0.94±0.01Aa	ns
	0.25–1mm	0.94±0.004Ca	0.95±0.001Aa	0.95±0.005BCa	0.95±0.001A	*
	< 0.25mm	0.95±0.006ABa	0.95±0.004Aa	0.94±0.002Ba	0.95±0.005Ba	ns
	P value	ns	**	ns	ns	
Margal (M)	> 5mm	10.79±0.48Ab	11.25±1.57Ab	12.04±0.83Aa	11.83±0.80Aa	ns
	2–5mm	13.59±0.05Aa	10.94±0.43Bb	11.12±1.66Ba	11.17±0.92Ba	*
	1–2mm	11.93±1.26Aab	12.03±0.70Aa	10.73±0.52Aa	12.13±2.07Aa	ns
	0.25–1mm	11.17±1.29Bb	13.34±0.96Aa	10.85±1.42Ba	13.45±0.83Aa	*
	< 0.25mm	12.89±0.63Aa	12.13±0.22Aa	12.33±0.46Aa	12.82±0.90Aa	ns
	P value	*	ns	ns	ns	

692 CK: the control treatment without fertilization; NP: the treatment with only inorganic fertilizer; NPS: the treatment with
693 inorganic fertilizer and maize straw addition; NPM: the treatment with inorganic fertilizer and cattle manure. B/F: the
694 ratio of total bacterial and fungal PLFA; G+/G-: the ratio of G+ and G- bacterial PLFA. Capital and lowercase letters
695 indicate significant difference among fertilization and aggregate size, respectively, at $p < 0.05$. ***: $p < 0.001$; **: $p <$
696 0.01; *: $p < 0.05$; ns: not significant.

Table 3. Correlative coefficients between SOC and soil biological properties for CK, NP, NPS and NPM in different sized aggregates.

Index	> 5mm	2–5mm	1–2mm	0.25–1mm	< 0.25mm
Total PLFA	-0.14	-0.44	-0.22	0.01	0.003
Actinomycetes	-0.17	-0.21	-0.3	-0.23	-0.13
Bacteria	0.02	-0.41	-0.05	0.22	0.14
Fungi	-0.53	-0.43	-0.64*	-0.48	-0.17
AM	-0.67*	-0.64*	-0.68*	-0.56	-0.46
F/B	-0.76**	-0.07	-0.73**	-0.66*	-0.45
G+/G-	-0.17	0.04	0.33	-0.32	-0.37
H′	-0.27	-0.75**	-0.27	0.61*	-0.04
D	-0.43	0.47	-0.44	0.56	0.03
M	0.06	-0.74**	-0.14	0.50	-0.49
BG	0.12	0.17	-0.28	0.56	0.28
NAG	-0.03	0.54	-0.37	0.26	-0.57
BXYL	0.05	0.83***	-0.63*	0.65*	-0.41
LAP	-0.51	0.8**	-0.32	0.66*	-0.48

Note: See Table 1 for abbreviations of some soil biological properties.

* : $p < 0.05$.

** : $p < 0.01$.

*** : $p < 0.001$.

Figures

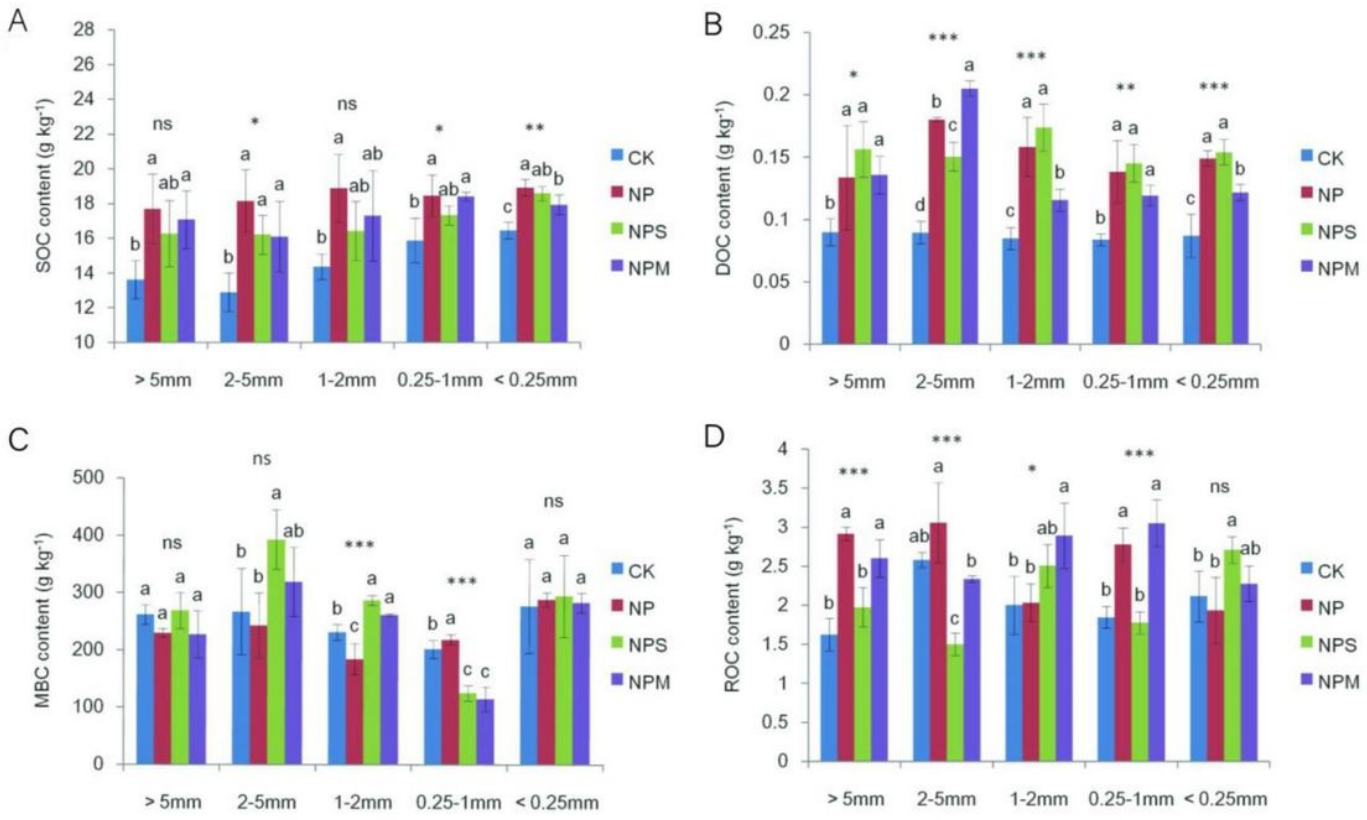


Figure 1

The distribution of SOC (A), DOC (B), MBC (C), ROC contents (D) in different aggregates under 4 fertilization managements (CK, NP, NPS and NPM). CK: the control treatment without fertilization management; NP: the treatment with only inorganic fertilizer; NPS: the treatment with inorganic fertilizer and maize straw addition; NPM: the treatment with inorganic fertilizer and cattle manure. Vertical bars mean the standard error of the mean (n = 3). Different lowercase letters indicated significant difference at $p < 0.05$ among the different fertilization managements. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant.

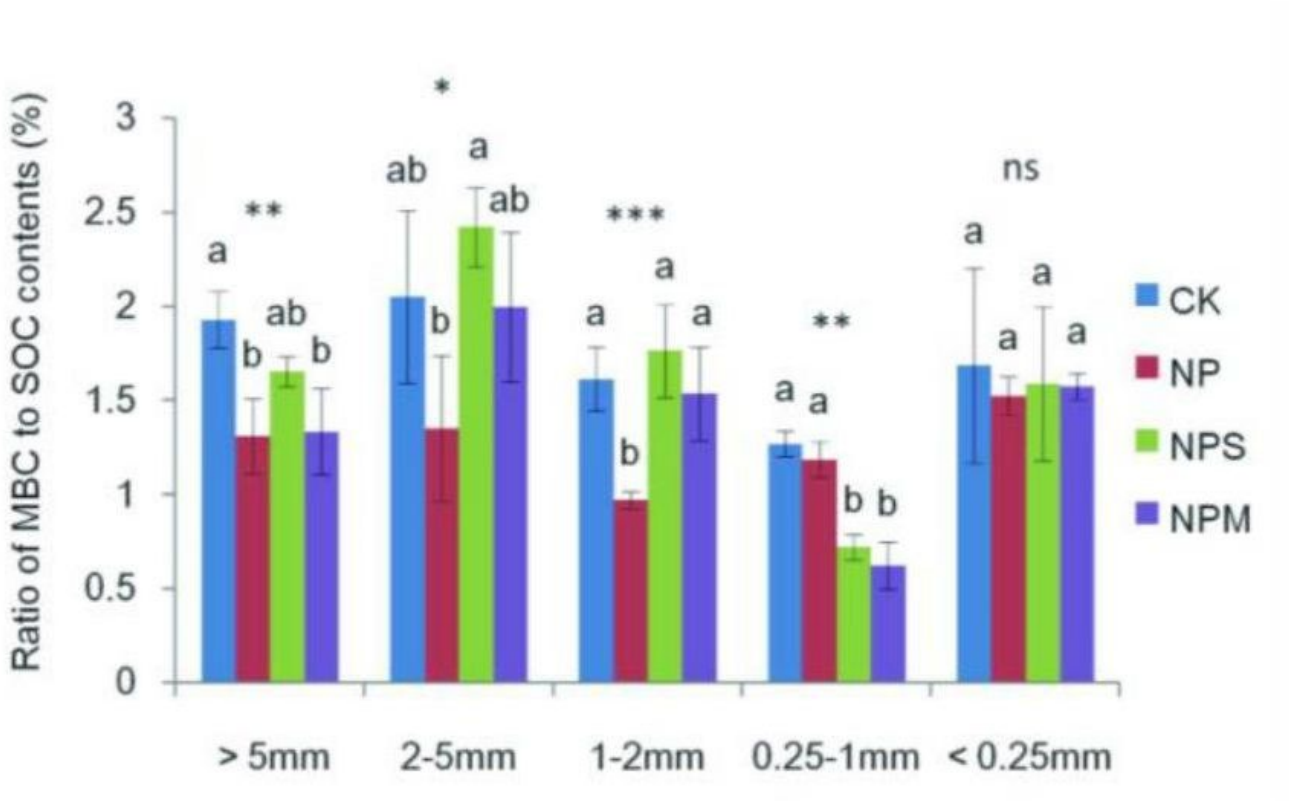


Figure 2

The ratio of MBC to SOC in different aggregates under 4 fertilization managements (CK, NP, NPS and NPM). Values in the same aggregate size followed by the same lowercase letters are not significantly different ($p < 0.05$) according to LSD test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant.

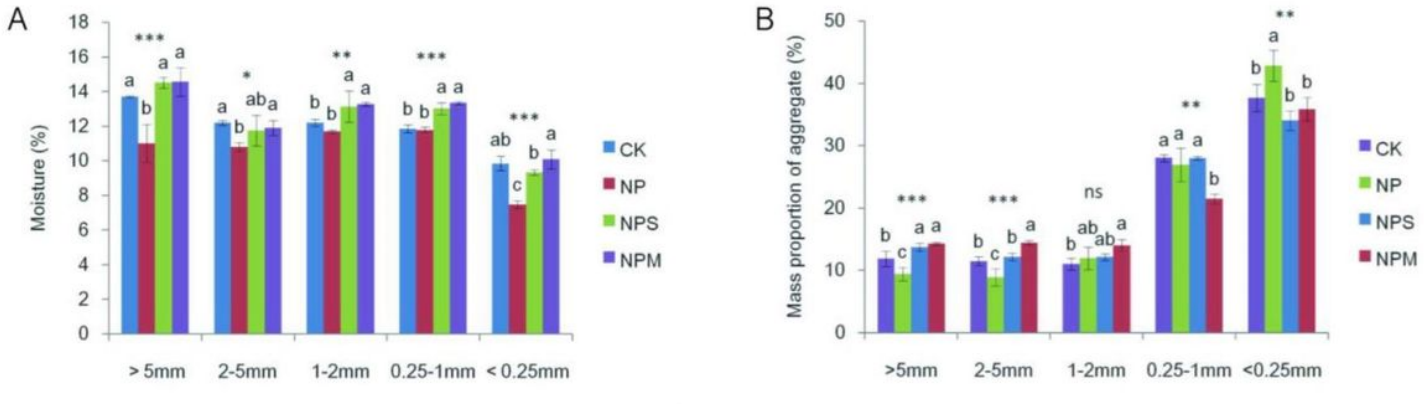


Figure 3

The moisture (A) and mass proportion (B) of different aggregates under 4 fertilization managements (CK, NP, NPS and NPM). Values in the same aggregate size followed by the same lowercase letters are not significantly different ($p < 0.05$). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant.

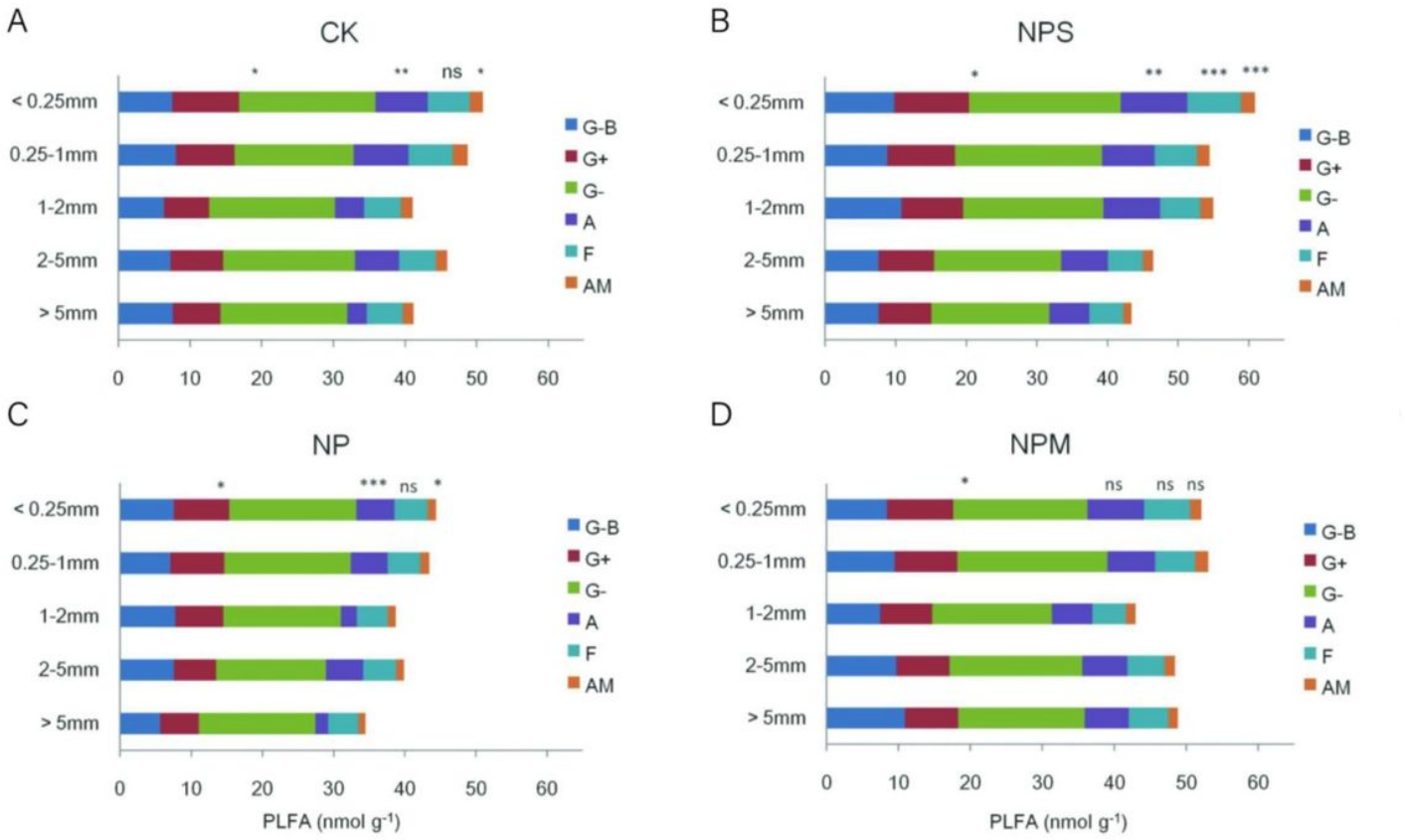


Figure 4

Microbial biomass and the phospholipid acid biomarkers in different sized aggregates under 4 fertilization managements, CK (A), NPS (B), NP (C), and NPM (D). G-B: general bacterial PLFA; G+: Gram-positive bacterial PLFA; G-: Gram-negative bacterial PLFA; A: actinomycetic PLFA; F: general fungal PLFA; AM: arbuscular mycorrhizal fungi. The differences in total bacteria (the sum of G-B, G+ and G- bacteria), actinomycetes, general fungi and AM PLFA among the different sized aggregates were showed at * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$ and ns: not significant.

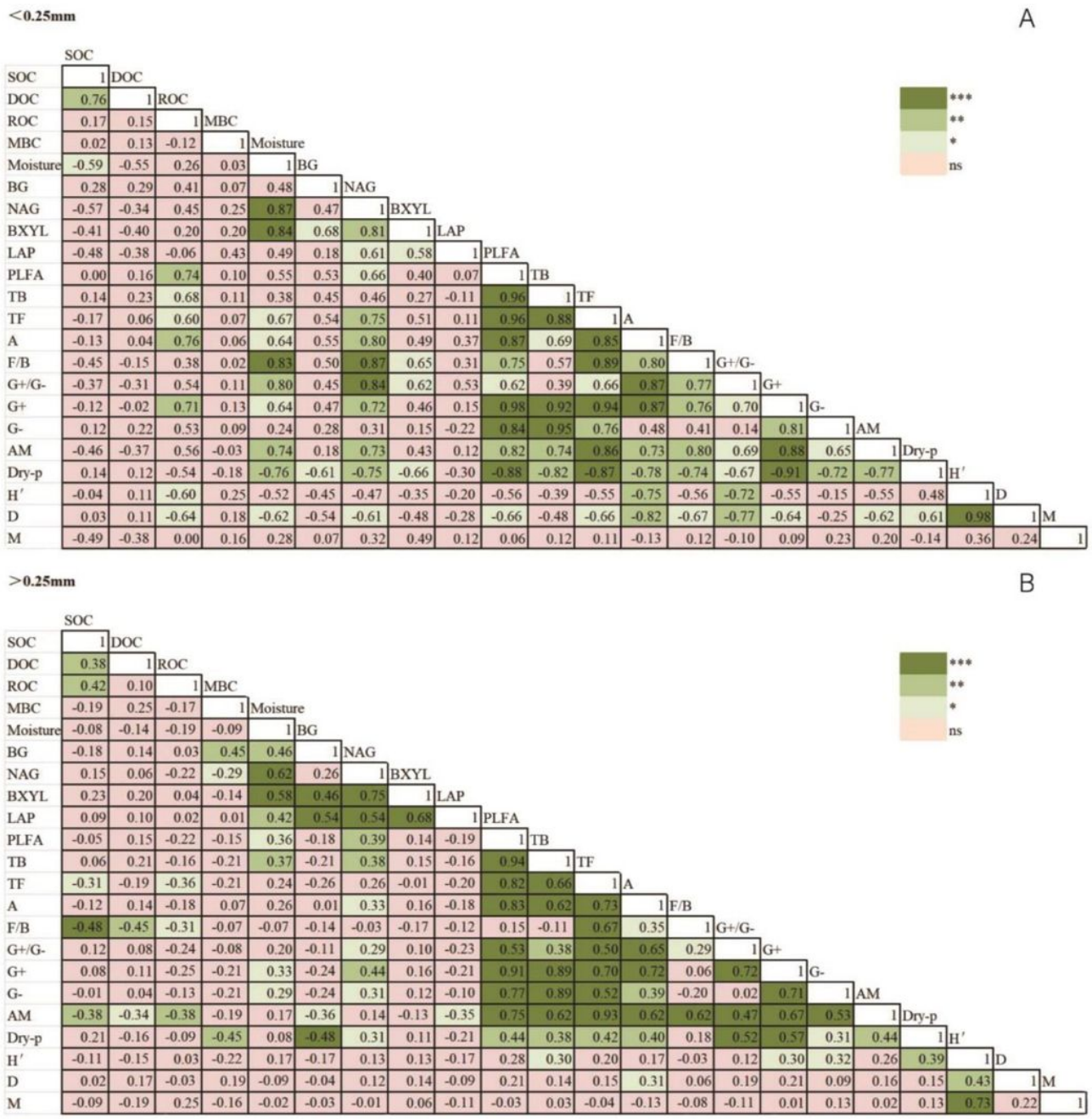


Figure 5

Correlative coefficients among the properties of soil organic C, microbial indices and enzyme activity in < 0.25 mm (A) and > 0.25 mm (B) aggregates, respectively. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

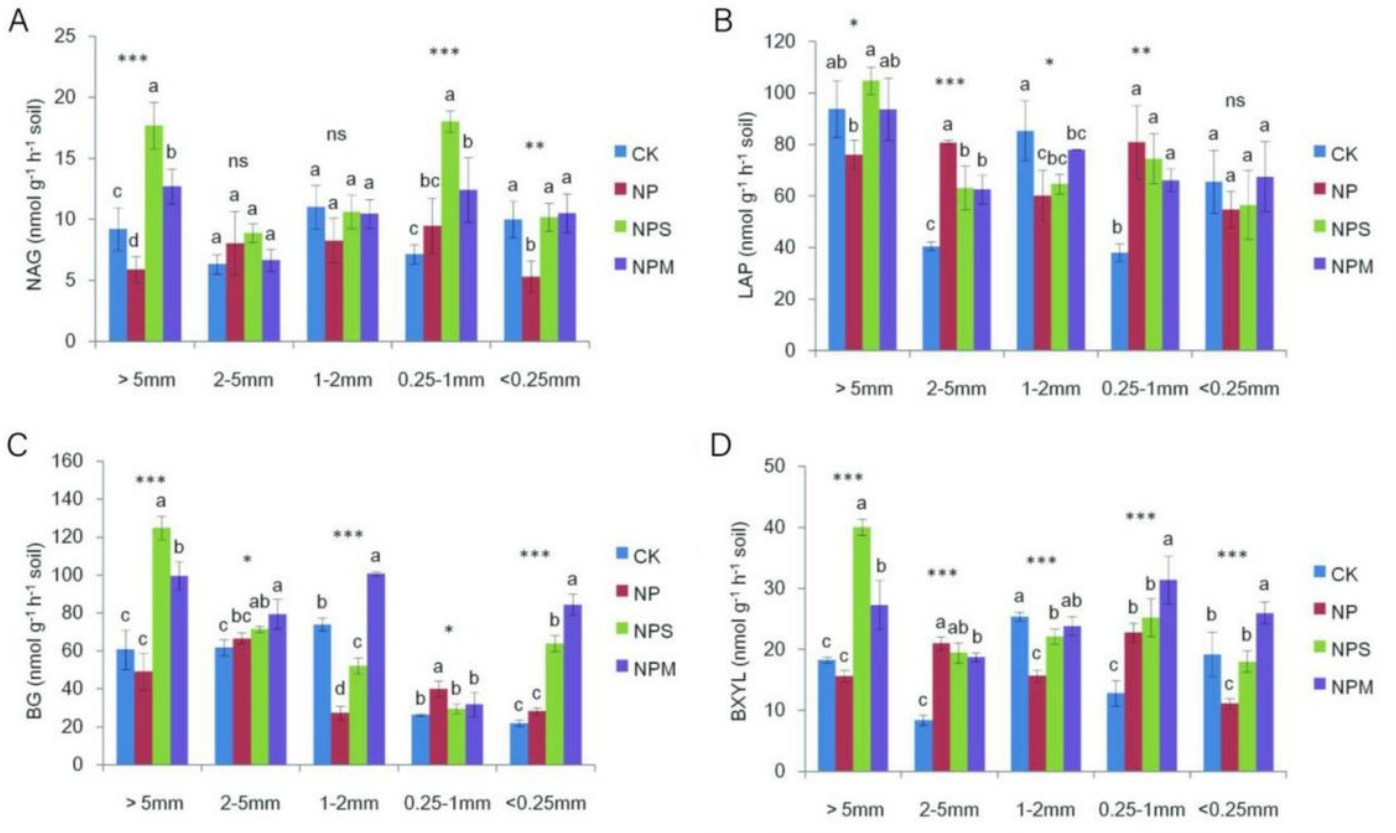


Figure 6

Histogram of N-acetyl-glucosaminidase (A), leucine aminopeptidase (B), β-glucosidase (C) and β-xylosidase (D) activities in aggregates in different treatments. NAG, N-acetyl-glucosaminidase; LAP, leucine aminopeptidase; BG, β-xylosidase; BXYL, β-xylosidase. Different lowercase letters mean signifcant differences among aggregate size fractions ($p < 0.05$). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant.

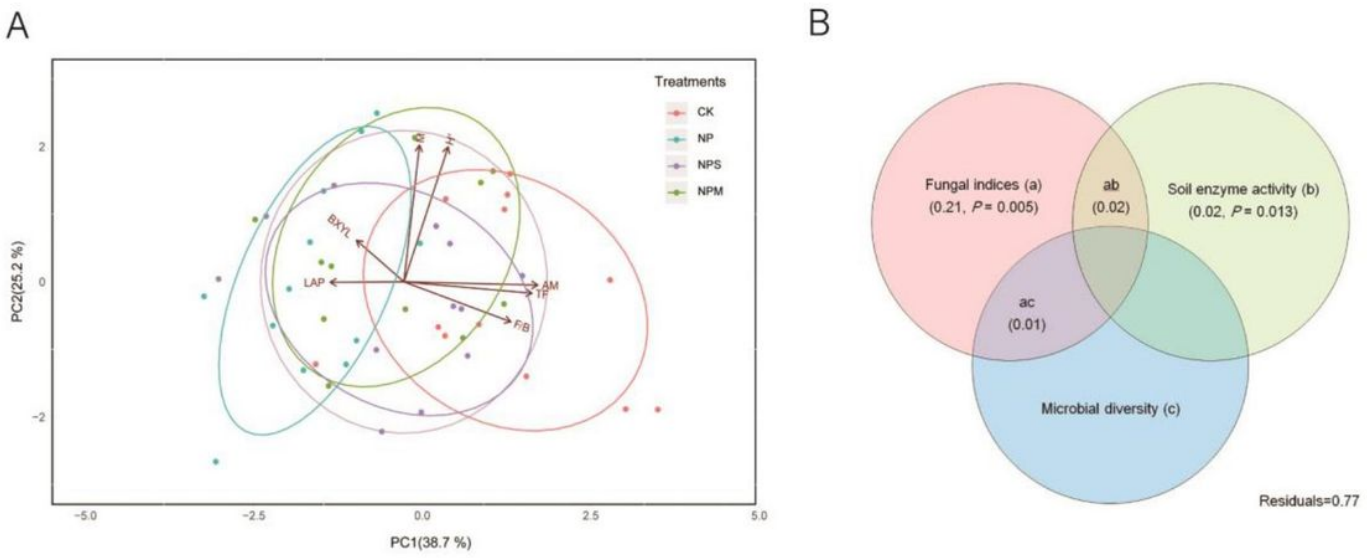


Figure 7

PCA analysis (A) of soil microbial properties and enzyme activity in macroaggregates (> 0.25 mm) under 4 fertilization managements (CK, NP, NPS and NPM). Variation-partitioning Venn diagram (B) of SOC accumulation variance partitioning among fungal indices (a), soil enzyme activity (b), and microbial diversity (c) predictor matrices in macroaggregates (> 0.25 mm). Fungal indices, soil enzyme activity, and microbial diversity contained three, two, and two factors, respectively.