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Effects of the Replacement of Chemical Fertilizers with Organic Fertilizers in Different Proportions on Microbial Biomass and Enzyme Activities of Soil Aggregates in Gravel-Mulched Field

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Abstract: Gravel-mulched fields are a unique form of drought-resistant agriculture in the northwest region of China. In recent years, continuous cropping obstacles caused by the perennial cultivation of a single crop have seriously constrained the sustainable development of sand fields. This study aimed to explore the distribution patterns of different particle sizes of aggregates (>2, 1–2, 0.25–1, and <0.25 mm) and the relationships between their microbial biomass and enzyme activities under different organic fertilization and to explore the effective measures for improving soil fertility in a gravel-mulched field with an 8-year positioning test. The results indicate that the mass percentage of soil aggregates of ≥ 1 mm and their mean weight diameter (MWD), microbial biomass (carbon and nitrogen, bacteria, fungi, actinomycetes, and total phospholipid fatty acids), and their related enzyme activities (leucine aminopeptidase, LAP; N-acetyl-β-d-glucosidase, NAG; β-glucosidase, BG; and polyphenol oxidase, PPO) in aggregates of different particle sizes increased with the increase in the proportion of organic fertilizers replacing the N fertilizer. Among them, the organic fertilizer replacing more than 50% of chemical nitrogen fertilizers exerted the most significant effect. With the decrease in agglomerate particle size, the contents of microbial carbon and nitrogen showed a decreasing trend, whereas LAP, NAG, and BG activities followed an increasing trend, and the change in microbial biomass was not obvious. The correlation analysis showed highly significant positive correlations between the MWD of soil aggregates, microbial biomass, and the activities of LAP, NAG, BG, and PPO. Therefore, the replacement of more than 50% of chemical fertilizer with organic fertilizer was observed to be conducive to promoting the formation of large aggregates in sandy soils and increasing the microbial biomass and enzyme activities in different sizes of aggregates.

Keywords: gravel-mulched fields; soil aggregates; microbial biomass carbon and nitrogen; microbial biomass; enzyme activity

1. Introduction

Soil aggregates provide soil microorganisms with physical habitats, on which they are dependent for their survival, as well as an abundance of food and water [1,2]. Furthermore, they directly contribute to the spatial segregation of microbes that share the same habitats in the soil, which influences the degree of organic substrate availability to microorganisms, as well as the predatory interactions among microbial communities, subsequently affecting the biochemical processes occurring in the soil (e.g., nutrient cycling, mineralization of carbon and nitrogen, nitrification, denitrification, etc.) [3]. Microorganisms influence the

formation and stability of soil aggregates. For exampere, the AMF (arbuscular mycorrhizal fungi) cement clay particles by secreting glomalin (a polysaccharide) and immobilize them into aggregates by hyphal twining, which is an important mechanism for the formation and stabilization of soil aggregates [4]. Li Jing [5] studied the effects of high carbon and nitrogen inputs on the organic carbon and microbial characteristics of soil aggregates in agroecosystems where large aggregates (0.25–2 mm) are the most active sites for microbial mineralization and decomposition of the closed-state organic particles as the main source of carbon, which improve the turnover rate of organic carbon. Soil enzymes, as the main biological catalysts for organic matter decomposition, turnover, and mineralization, participate in all biochemical processes taking place in the soil. The level of enzyme activity not only reflects the microbial community functions but also indicates the utilization rates of soil nutrients and the degree of biodegradation [6]. On the other hand, soil aggregates directly affect the activity of soil enzymes by controlling the accessibility of organic matter to microbes that inhabit the soil aggregates of different particle sizes, as well as the composition of microbial communities, moisture, and other factors [7,8]. Lei et al. [9] investigated the distribution characteristics of enzyme activities in soil aggregates in mixed pine forests of different Sargassum species and indicated that the five hydrolytic enzymes had the highest activities in microaggregates, while two oxidoreductases had the highest activities in macroaggregates. Therefore, the changes in the microbial biomass and enzyme activities in different soil aggregates are important indicators of changes in soil nutrients and the ability of aggregates to maintain soil fertility.

The gravel-mulched field is a mixture or a single layer of pebbles, gravel, coarse sand, and fine sand, with a thickness of 5–16 cm, laid on the soil surface to reduce water evaporation and surface runoff, with great potential for temperature rise, water storage capacity, and moisture conservation efficiency, and it has undergone unique and traditional drought-resistant plowing in arid and semi-arid regions of northwestern China [10]. In recent years, planting watermelons in gravel-mulched fields has contributed to the development of a new green industry that lifts local farmers out of poverty, increases their income, and reduces the risk of disasters. However, watermelon cropping and a long-term single application of chemical fertilizers have led to a decline in soil quality and increased crop failure, seriously threatening the sustainable development of the watermelon industry. Our previous study demonstrated that with equal total nitrogen inputs, the replacement of 50% to 100% chemical fertilizers with organic fertilizers increased survival by 26.47% to 34.16%, yield by 63.2% to 156.6%, and nitrogen utilization rate by 16.5% to 18.5% in watermelon plants, compared to chemical fertilizers alone [11]. Numerous studies have found that organic fertilizers and the long-term application of combined organic and chemical fertilizers can increase soil organic matter content, promote the formation of macroaggregates [12,13], improve microbial biomass and accelerate microbial metabolism and reproduction [14,15], and enhance the activity of various enzymes in the soil and its agglomerates [16,17]. However, variations in soil microbial communities are closely linked to crop species and the soil environment, and the effects of soil physicochemical properties (pH, salinity, carbon content, availability of nutrients, soil type, etc.) on microbial communities vary across different ecological environments and crop planting patterns [18,19]. To this end, this study selected traditional gravel-mulched fields in arid and semi-arid regions of northwest China with obvious regional characteristics as the research object. The purpose is to clarify the distribution patterns of soil aggregates, microorganisms, and enzyme activities under the mode of long-term replacement of chemical fertilizers with different proportions of organic fertilizers and reveal their interrelationships with a positioning experiment for 8 consecutive years, thereby obtaining the effective fertilization measures to improve soil quality in continuous cropping fields. Our hypothesis is that improving soil fertility with the long-term application of organic fertilizer can effectively promote the formation of macroaggregates and improve the microbial biomass and enzyme activity in aggregates, thereby increasing the soil quality in gravel-mulched fields. The innovation of

this study lies in the research of the effects of different fertilization measures on microbial and extracellular enzyme activities in the micro-domain space of soil aggregates.

2. Materials and Methods

2.1. Overview of the Experimental Site

The experimental site is located in Sanping Village (36°13′ N, 103°42′ E), Jiuhe Town, Anning District, Lanzhou City, China, at an altitude of 1830 m. It has a temperate semi-arid climate, with an average annual precipitation of approximately 260 mm, an average air temperature of 7.0 °C, a $\geq$10 °C active accumulated temperature of 2798 °C, and a frost-free period of 142 days. A gravel-mulched field with a 20-year planting history was used for the experiment. The soil feature is sandy-textured soils. Before setting up the experiment in 2014, the basic nutrient contents of soil at a 0–20 cm depth were as follows: organic matter of 2.64 g·kg^{-1}, total nitrogen of 0.31 g·kg^{-1}, alkaline dissolved nitrogen of 36.36 mg·kg^{-1}, quick-acting phosphorus of 8.48 mg·kg^{-1}, quick-acting potassium of 60.42 mg·kg^{-1}, and pH of 8.78.

2.2. Experimental Design

The positioning experiment was started in 2014 in a gravel-mulched field, under the same amount of watermelon fertilizer (N 200 kg·hm^{-2}, P_2O_5 170 kg·hm^{-2}, and K_2O 260 kg·hm^{-2}), and six treatments were set up:

No nitrogen (N) fertilizer, control (CK, N 0 kg·hm^{-2});
Only chemical N fertilizer (CF);
Organic fertilizer replacing 25% of chemical N fertilizer (OF-25%);
Organic fertilizer replacing 50% of chemical N fertilizer (OF-50%);
Organic fertilizer replacing 75% of chemical N fertilizer (OF-75%);
Organic fertilizer replacing 100% of chemical N fertilizer (OF-100%).

Each treatment was repeated three times, with 64 m^2 plots arranged in a randomized complete block design (RCBD). Organic fertilizer was derived from rotten cow dung containing organic matter of 311 g·kg^{-1}, total nitrogen of 15 g·kg^{-1}, total phosphorus of 12 g·kg^{-1}, and total potassium of 19 g·kg^{-1} and chemical fertilizers comprising urea (N: 46%), superphosphate, which is a universal phosphorus-based fertilizer (P_2O_5: 12%), and potassium sulfate (K_2O: 52%). Moreover, the nutrient dosages for the treatments were adjusted according to equal inputs of inorganic nitrogen (N), phosphorus (P), and potassium (K) fertilizers. Among them, 100% organic fertilizer, 30% N fertilizer, 100% P fertilizer, and 50% K fertilizer were used as base fertilizers, while 30% N fertilizer and 20% K fertilizer were spread at the vine elongation stage of watermelon, and 40% N fertilizer and 30% K fertilizer were applied at the early stage of fruit development.

2.3. Sample Collection

Before watermelon sowing in 2022 (April 10th), an undisturbed soil sample (10 cm × 10 cm × 20 cm) was collected from the 0–20 cm depth in the watermelon rows of each experimental plot according to the "S"-type 5-point sampling method. After removing the part of the soil that had been disturbed by using a soil shovel, the samples were immediately transferred to the laboratory. Meanwhile, the larger soil samples were broken into small pieces with a diameter of approximately 1 cm along the natural fracture surface, and debris such as gravel and plant roots were removed. Five samples from each plot were mixed into one homogeneous sample and then passed through a standard sieve of 8 mm.

2.4. Classification of Soil Aggregates

Soil aggregates of different particle sizes were graded using wet soil—dry sieve method [20]. The 500 g pre-treated soil samples were placed on the top sieve of a set of sieves (2 mm, 1 mm, and 0.25 mm) of Soil Dry Sieve instrument (DM185, Shanghai Dema Information Technology Co., Ltd., Shanghai, China) and sieved at an amplitude of 1.5 cm and a frequency of 30 times/min. After 1 min of sieving, soil samples retained on the sieve

surface for each particle size were collected, which resulted in the formation of aggregates of >2 mm, 1–2 mm, 0.25–1 mm, and <0.25 mm. The above steps were repeated several times to obtain sufficient aggregate samples of different sizes, which were then weighed and used to determine their distribution.

2.5. Determination of Microbial Biomass

Soil microbial biomass carbon and nitrogen were determined using the chloroform fumigation and potassium sulfate (K_2SO_4) extraction method [21,22].

A modified version of the method of Li et al. [23] was used to extract the microbial lipids from soil aggregates and analyze the phospholipid fatty acids (PLFAs). Around 3.0 g of soil sample was transferred to a glass test tube, and the citric acid buffer (pH 4.0) and chloroform–methanol were added sequentially. Thereafter, the tube was shaken to suspend the mixture, and total lipids were extracted by centrifugation. After blow drying by nitrogen, chloroform was added and separated by SPE silica gel column to obtain phospholipid fatty acids. Then, a mixture of methanol and toluene (1:1) was added for esterification to obtain phospholipid fatty acid methyl ester. After blow drying by nitrogen, the internal standard (methyl n-n-nineteenthanedioate) was added. An Agilent 6890 N gas chromatograph was used to analyze the composition of extracted PLFAs, and each component of fatty acids was identified by the MIDI Sherlock Microbial Identification System (MIS). Fatty acids were named using the Francisco et al. [24] method and quantified with the internal standard calibration curve based on the received peak area. Different fatty acids were assigned to different microbial taxonomic groups according to their molecular structure. The biological characteristics of PLFAs are shown in Table 1.

Table 1. The PLFAs used to evaluate the microbial biomass.

Microbial Group	Phospholipid Fatty Acid Signatures	Reference
Bacteria	14:0, i15:0, a15:0, 15:0, 16:0, i16:0, i17:0, 16:1ω7c, cy17:0, 17:0, 18:0, cy19:0, 20:0	[25]
G^+ bacteria	i15:0, a15:0, i16:0, i17:0	[26]
G^- bacteria	16:1ω7c, cy17:0, cy19:0	[25]
Fungi	18:1ω9c, 18:1ω9t, 18:2ω6	[27]
Actinomycete	10Me16:0, 10Me18:0	[25]

2.6. Measurement of Enzyme Activities

The microtiter plate fluorescent assay was used to measure the activities of β-glucosidase (BG), β-xylosidase (β-XYS), N-acetyl-β-d-glucosidase (NAG), and leucine aminopeptidase (LAP) [26]. The catalase (CAT) and polyphenol oxidase (PPO) activities were determined by the addition of the substrate L-3,4-dihydroxyphenylalanine (DOPA) using the colorimetric method with an enzyme marker [27]. Details of the substrates used and the measured wavelengths are shown in Table 2.

Table 2. Soil enzyme names and substrates for assays.

Soil Enzyme	Substrate	Concentration of Substrate/ ($\mu mol \cdot L^{-1}$)	Measure Wavelength/nm
β-glucosidase (BG)	4-MUB-β-D-glucoside		
β-Xylosidase (β-XYS)	4-MUB-β-D-xylosidc		
N-acetyl-β-D- glucosidase (NAG)	4-MUB-N-acetyl-β-D-glucosaminide	200	365, 450
Leucine aminopeptidase (LAP)	L-Leucine-7-amido-4-methylcoumarin		
Catalase (CAT) Polyphenoloxidase (PPO)	L-3,4-dihydroxyphenylalanine	25,000	465

Determination of mean weight diameter:

$$\text{Mass percentage of agglomerates of different particle sizes} = \frac{\text{agglomerate mass of each particle size}}{\text{quality of total soil samples}} \times 100\% \tag{1}$$

$$\text{Mean weight diameter of agglomerates(MWD)} = \frac{\sum_{i=1}^{n}(x_i w_i)}{\sum_{i=1}^{n}(w_i)} \tag{2}$$

where x_i is the average diameter of each particle size of each grain-level agglomerate and w_i is the mass percentage of each particle size of each grain-level agglomerate [28,29].

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) and the Duncan multiple range test were used for multiple comparisons at $\alpha = 0.05$ [30]. Furthermore, the Pearson correlation method was used for correlation analysis [31], and data given in the table are presented as mean $\pm$ standard deviation. Data processing was performed using the SPSS 18.0 software, and Origin 9.0 was used to create graphs.

3. Results

3.1. Effects of Different Fertilizer Treatments on the Distribution of Soil Aggregates

In terms of the mass percentage of soil aggregates of different particle sizes, microaggregates (<0.25 mm) were dominant with the non-organic fertilizer treatment, whereas macroaggregates (>2 mm) were dominant with organic fertilizers providing 50% and more nitrogen fertilizer (Table 3). Within the same range of particle size of aggregates (≥ 1 mm), soil aggregate content increased with the increasing proportion of organic fertilizer replacing chemical fertilizer, whereas the opposite trend was observed for aggregates with particle sizes of <1 mm. The mean weight diameter (MWD) of agglomerates increased with the increase in the proportion of organic fertilizers replacing chemical fertilizers, significantly higher by 10.13%, 13.92%, and 21.52% in the OF-50%, OF-75%, and OF-100% treatments, respectively, compared to the CF treatment.

Table 3. Effect of different treatments on the distribution of aggregates (%).

Treatments	>2 mm	1–2 mm	0.25–1 mm	<0.25 mm	MWD (mm)
CK	28.62 ± 0.76 dB	11.01 ± 0.73 cD	24.43 ± 1.71 aC	35.95 ± 1.47 aA	0.78 ± 0.02 d
CF	29.71 ± 0.92 cdB	11.41 ± 1.33 bcD	23.57 ± 0.94 abC	35.30 ± 1.22 aA	0.79 ± 0.03 d
OF-25%	31.48 ± 1.29 cdA	12.21 ± 0.80 bcC	22.99 ± 1.28 abB	33.33 ± 1.21 aA	0.81 ± 0.02 cd
OF-50%	34.41 ± 2.96 bcA	13.66 ± 1.29 abcD	22.05 ± 1.88 abC	29.87 ± 1.74 bB	0.87 ± 0.04 bc
OF-75%	37.20 ± 3.54 abA	14.10 ± 1.01 abD	21.47 ± 1.72 abC	27.24 ± 2.72 bB	0.90 ± 0.03 ab
OF-100%	40.63 ± 1.95 aA	14.96 ± 1.33 aC	21.08 ± 1.10 bB	23.34 ± 0.39 cB	0.96 ± 0.03 a

MWD: Mean weight diameter of agglomerates. Different small letters indicate significant differences among the same size aggregates of different treatments ($p < 0.05$). Different capital letters indicate significant differences among the different size aggregates of the same treatment ($p < 0.05$). The same below.

3.2. Effects of Different Fertilizer Treatments on Microbial Biomass Carbon and Nitrogen in Soil Aggregates

The microbial biomass carbon and nitrogen contents in soil aggregates of the gravel-mulched field gradually decreased with decreasing particle size (Figure 1), and the differences between macroaggregates (≥ 2 mm) and microaggregates (<0.25 mm) were significant ($p < 0.05$). Within the same range of aggregate size, the contents of microbial biomass carbon and nitrogen increased with the increase in the proportion of organic fertilizer application, and the values for the nitrogen fertilizer application treatments were significantly higher than those for the non-nitrogen fertilizer (CK). However, except for no significant difference in the microbial biomass nitrogen content among different nitrogen application treatments in aggregates with particle sizes within the range of 0.25–1 mm, the microbial biomass

carbon and nitrogen contents for the OF-75% and OF-100% treatments were significantly higher than those for the chemical fertilizer alone (CF).
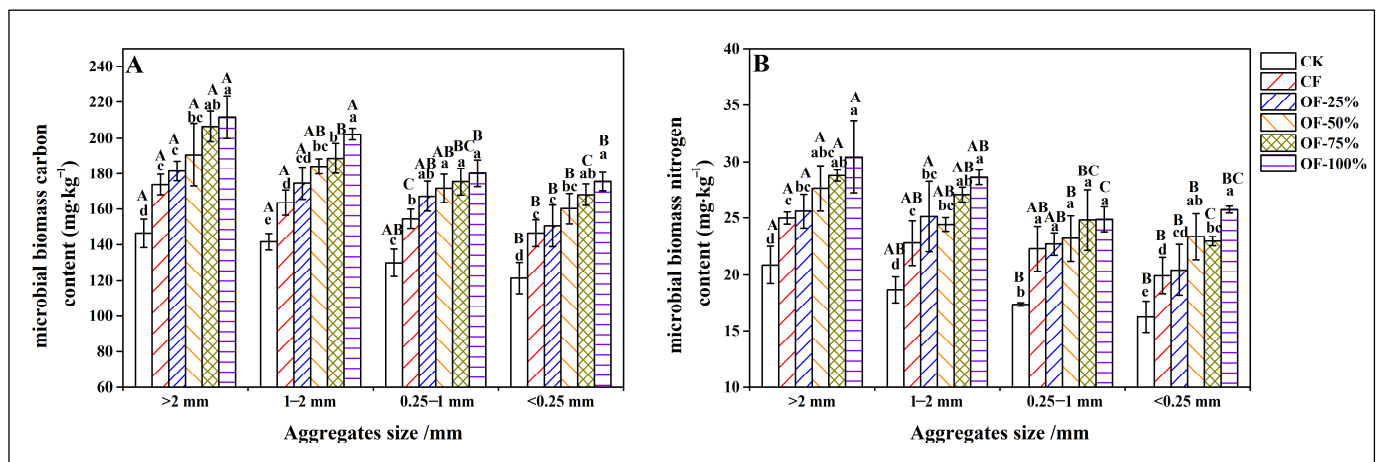

Figure 1. The effects of different treatments on microbial biomass carbon and nitrogen of aggregates. (**A**) Microbial biomass carbon; (**B**) microbial biomass nitrogen. Different small letters indicate significant differences among the same size aggregates of different treatments ($p < 0.05$). Different capital letters indicate significant differences among the different size aggregates of the same treatment ($p < 0.05$).

3.3. Effects of Different Fertilization Treatments on Microbial Biomass in Soil Aggregates

There was no significant difference in the microbial biomass (bacteria, fungi, actino-mycetes, and total phospholipid fatty acids) among soil aggregates of different particle sizes (Figure 2). In aggregates with the same particle size, however, the microbial biomass increased with the increase in the proportion of organic fertilizer used instead of chemical fertilizer, and the values for the nitrogen application treatment were significantly higher than those for the non-nitrogen fertilizer (CK) treatment. The values for the organic fertil-izer treatments providing 50% or more nitrogen fertilizer were significantly higher than those for chemical fertilizer (CF). Except for the OF-100% treatment, the ratio of Gram-positive bacteria to Gram-negative bacteria (G^+/G^-) in aggregates of the same particle size decreased with the increase in the proportion of organic fertilizers substituting chemical fertilizers. In addition, the treatment without nitrogen fertilizer (CK) exhibited significantly higher values than the nitrogen application, and the treatments of organic fertilizer pro-viding 50% or more nitrogen fertilizer had significantly lower microbial biomass than the single application of chemical fertilizer (CF) (Figure 2E). The soil fungal-to-bacterial ratio (F/B) increased at first and then decreased with the increase in the proportion of organic fertilizer applied instead of chemical fertilizer, among which the OF-75% treatment showed the highest value (Figure 2F).
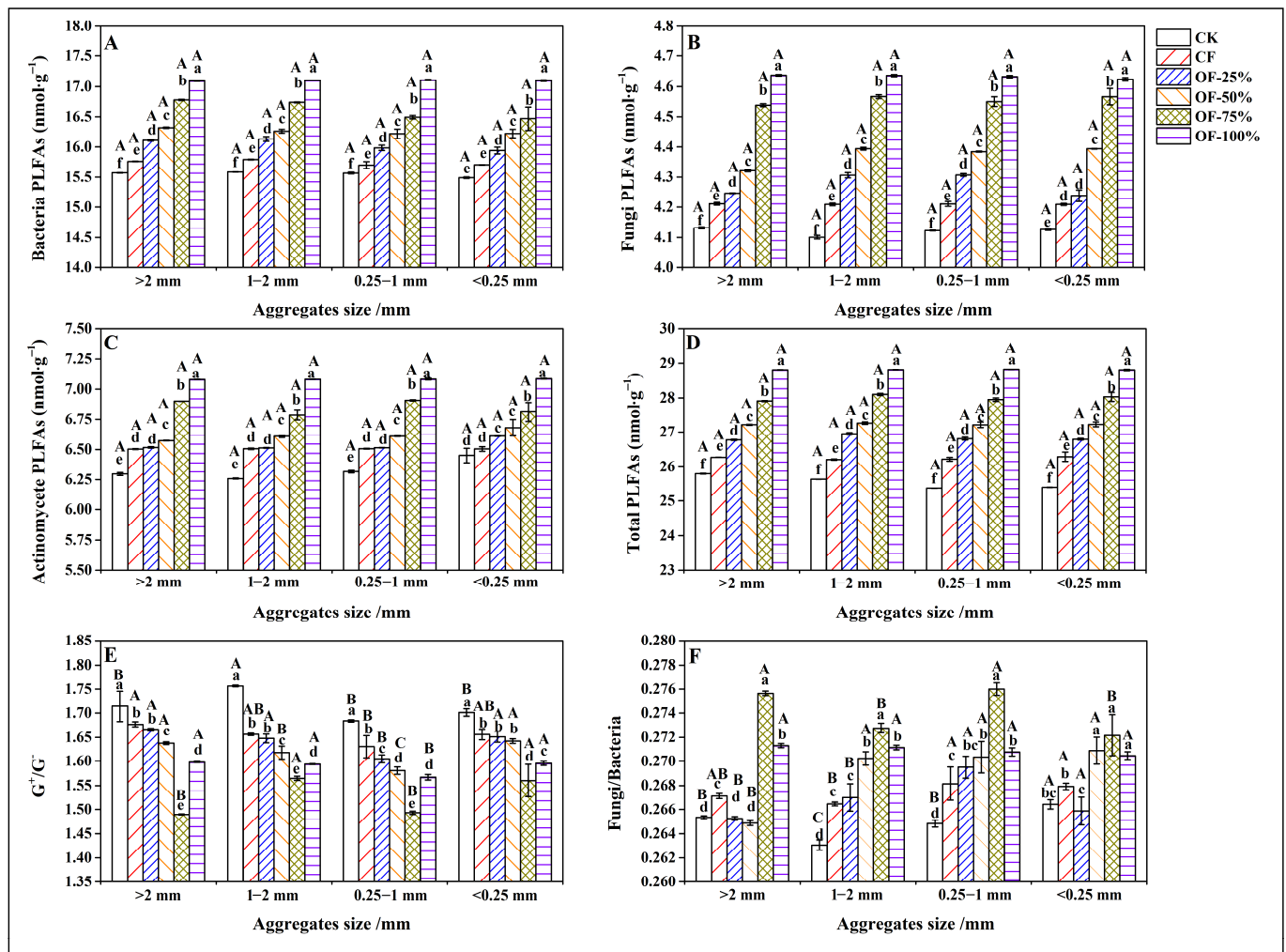
Figure 2. Effects of different fertilization on microbial biomass of soil aggregates. (**A**) Bacteria PLFAs; (**B**) fungus PLFAs; (**C**) actinomyces PLFAs; (**D**) total PLFAs; (**E**) Gram-positive/Gram-negative bacteria (G+/G−); (**F**) fungus/bacteria (F/B). Different small letters indicate significant differences among the same size aggregates of different treatments ($p < 0.05$). Different capital letters indicate significant differences among the different size aggregates of the same treatment ($p < 0.05$).

3.4. Effects of Different Fertilization Treatments on Microbial Enzyme Activities of Soil Aggregates

As shown in Figure 3, the activities of leucine aminopeptidase (LAP), N-acetyl-β-d-glucosidase (NAG), and β-glucosidase (BG) among the four hydrolases increased with the decrease in aggregate size or the increase in nitrogen proportion of organic fertilizer replacing chemical fertilizer. Meanwhile, with the decrease in the particle size of aggregates, the activity of these three hydrolytic enzymes increased with the inputs of organic fertilizers (Figure 3A–C). The distribution of the activity of β-xylosidase (β-XYS) in different aggregates was affected by fertilization treatments, i.e., microaggregates (<0.25 mm) displayed the highest activities in CF, OF-25%, and OF-50% treatments; in 0.25–1 mm aggregates, the highest activities were achieved in OF-75% and OF-100% treatments, whereas in macroaggregates (1–2 mm), the highest activities were achieved in organic N fertilizer treatments (OF-25%–100%); in >2 mm aggregates, the highest activities were achieved in the OF-25% treatment, and the activity of this enzyme increased first and then decreased with the increase in the proportion of nitrogen replaced by organic fertilizer (Figure 3D). The activity of catalase (CAT) decreased with the decrease in aggregate size, and the application of all organic fertilizers resulted in lower values than the treatment without organic fertilizer (Figure 3E). The activity of polyphenol oxidase (PPO) was the highest in aggregates with

1–2 mm particle sizes, and it generally increased with the increase in the proportion of organic fertilizer used in different aggregate particle sizes (Figure 3F).
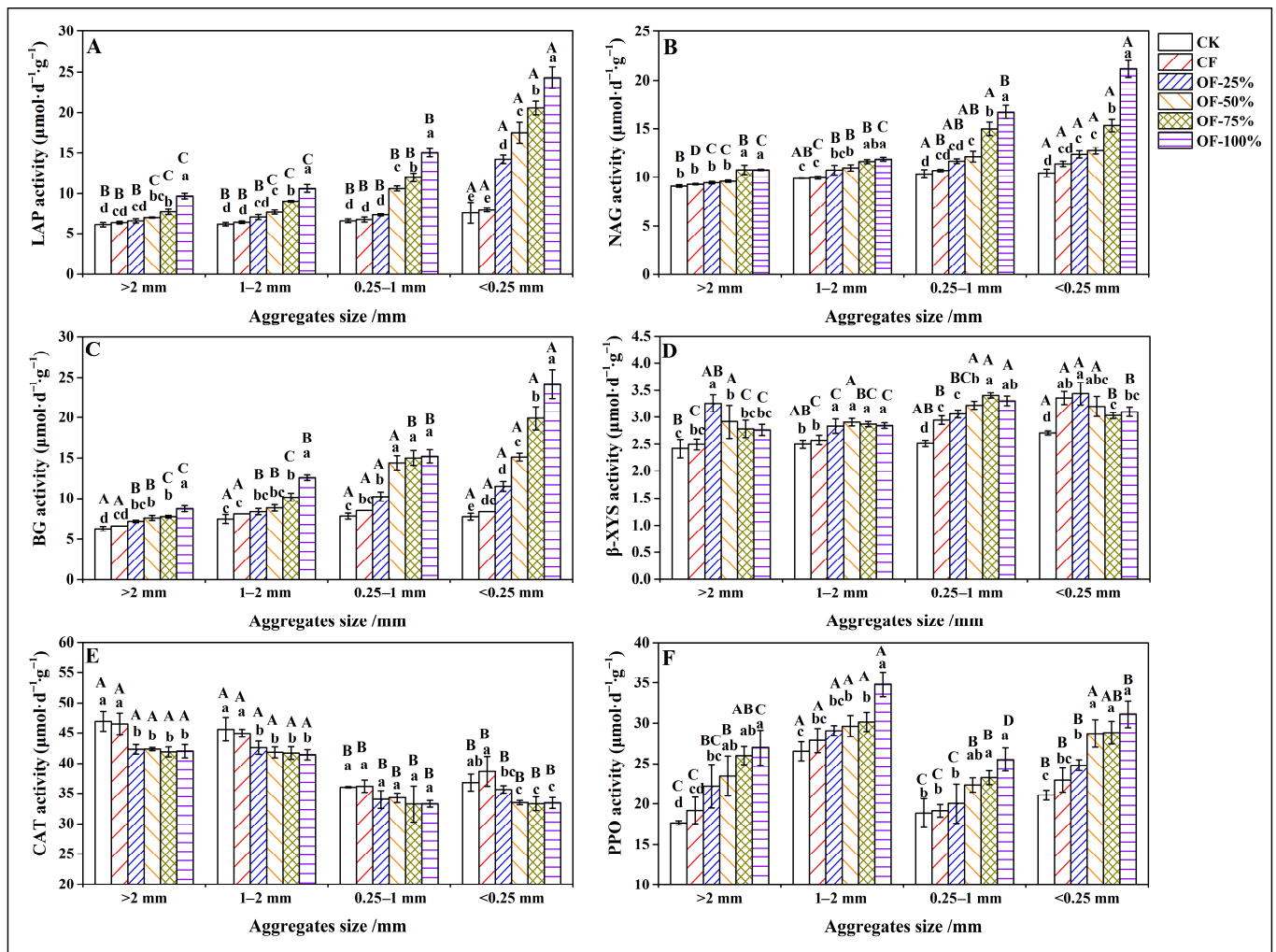

Figure 3. Effects of different fertilization on enzyme activities of soil aggregates. (**A**–**F**) represent the activity of LAP, NAG, BG, β-XYS, CAT, and PPO, separately. Different small letters indicate significant differences among the same size aggregates of different treatments ($p < 0.05$). Different capital letters indicate significant differences among the different size aggregates of the same treatment ($p < 0.05$).

3.5. Correlation Analysis

The parameters, including the mean weight diameter (MWD) of soil aggregates and the microbial biomass (carbon and nitrogen, bacteria, fungi, actinomycetes, and total phospholipid fatty acids), had significantly or highly significantly positive correlations with the activities of three hydrolases (LAP, NAG, and BG) and polyphenol oxidase (PPO) but significantly negative correlations with the catalase (CAT) activity (Tables 4 and 5). The correlation analysis between the microbial biomass and enzyme activity of aggregates revealed that the activities of LAP, NAG, BG, and PPO were significantly positively correlated with the microbial biomass (bacteria, fungi, actinomycetes, and total phospholipid fatty acids), and positive correlations were also observed between the PPO activity and the soil microbial biomass carbon and nitrogen (Table 6). In addition, CAT activity showed no significant correlation with other microorganism biomass except for a significant positive correlation with G^+/G^- and a significant negative correlation with fungi/bacteria.

Table 4. The correlation analysis between microbial biomass and mean weight diameter of soil aggregates.

	Microbial Biomass—C	Microbial Biomass—N	Bacteria	Fungi	Actinomycete	Total PLFA	G$^+$/G$^-$	Fungi/Bacteria
MWD	0.892 *	0.888 *	0.986 **	0.977 **	0.968 **	0.972 **	−0.789	0.827 *

MWD: Mean weight diameter of agglomerates. Total PLFA: Total phospholipid fatty acid. G$^+$: Gram-positive microbes. G$^-$: Gram-negative bacteria. * and ** indicate significant correlation between two index parameters at $p < 0.05$ and $p < 0.01$, respectively.

Table 5. The correlation analysis between enzyme activities and mean weight diameter of soil aggregates.

	Catalase (CAT)	Polyphenoloxidase (PPO)	Leucineaminopeptidase (LAP)	N-Acetyl-β-D-Glucosidase (NAG)	β-Glucosidase (BG)	β-Xylosidase (β-XYS)
MWD	−0.843 *	0.985 **	0.994 **	0.967 **	0.995 **	0.500

MWD: Mean weight diameter of agglomerates. * and ** indicate significant correlation between two index parameters at $p < 0.05$ and $p < 0.01$, respectively.

Table 6. The correlation analysis between microbial biomass and enzyme activities of soil aggregates.

	Microbial Biomass—C	Microbial Biomass—N	Bacteria	Fungi	Actinomycete	Total PLFA	G$^+$/G$^-$	Fungi/Bacteria
Catalase (CAT)	0.173	0.184	−0.229	−0.335	−0.354	−0.289	0.442 *	−0.414 *
Polyphenoloxidase (PPO)	0.512 *	0.492 *	0.668 **	0.651 **	0.609 **	0.663 **	−0.338	0.386
Leucineaminopeptidase (LAP)	0.113	0.142	0.544 **	0.602 **	0.620 **	0.600 **	−0.397	0.413 *
N-acetyl-β-D-glucosidase (NAG)	0.133	0.149	0.595 **	0.643 **	0.669 **	0.634 **	−0.502 *	0.481 *
β-glucosidase (BG)	0.142	0.156	0.565 **	0.642 **	0.629 **	0.625 **	−0.492 *	0.482 *
β-Xylosidase (β-XYS)	0.143	0.123	0.326	0.370	0.402	0.420 *	−0.471 *	0.385

Total PLFA: Total phospholipid fatty acid. G$^+$: Gram-positive microbes. G$^-$: Gram-negative bacteria. * and ** indicate significant correlation between two index parameters at $p < 0.05$ and $p < 0.01$, respectively.

4. Discussion

4.1. Effects of Different Fertilization Treatments on the Distribution of Soil Aggregates of Different Size Fractions

Microaggregates are the foundation of soil aggregates, while macroaggregates are weak binding between microaggregates, and their content is linearly related to the stability of soil aggregates [32,33]. Microaggregates can gradually form large aggregates under the action of various cementitious substances [34]. In this study, the content of macroaggregates (≥1 mm) gradually increased, whereas the content of microaggregates (<0.25 mm) gradually decreased, and the MWD of agglomerates gradually increased as the proportion of organic fertilizers increased. This is similar to the results of previous research into the effect of cattle manure on the distribution of soil aggregates in black soil [35] and yellow soil [36]. The reason for this change may be that the addition of exogenous organic materials can effectively promote the formation of large aggregates and improve their stability by increasing the content of organic cementing substances in the soil [37–39]. However, Xie et al. [40] found that the high input of swine manure (27 mg·hm^{-2}·y^{-1}) reduced the content of large aggregates in brown soil compared to the small amount of swine manure (13.5 Mg·hm^{-2}·y^{-1}) in a 30-year locational study. This difference in the distribution of soil aggregates in response to the application of organic fertilizers may be because of factors such as the type of soil and organic fertilizer, quantity of inputs, and the duration of the experiment.

4.2. Effects of Different Fertilization Treatments on Microbial Biomass of Soil Aggregates

The soil microbial biomass carbon and nitrogen are important parameters that characterize the nutrient and quality status of soils. Compared with microaggregates, macroaggregates can maintain a higher nutrient level, which is conducive to microbial colonization [41]. Therefore, in general, large aggregates contain higher microbial biomass carbon and nitrogen than microaggregates. This is consistent with the results of the present study (Figure 2). Additionally, the microbial biomass carbon and nitrogen contents increased with the increasing proportion of organic fertilizers replacing the chemical fertilizer. This is mainly due to the improvement in soil quality by the long-term application of organic fertilizers, as well as creating a favorable environment for the reproduction and metabolism of microorganisms, thus increasing microbial biomass carbon and nitrogen contents [42].

Meanwhile, our experiment showed that long-term organic fertilization dramatically increased the microbial biomass (bacteria, fungi, actinomycetes, and total PLFAs) of soil aggregates in gravel-mulched fields, with the most pronounced effect with high organic fertilizer inputs. Analyzing the reasons, the application of organic fertilizer can increase the accumulation of active organic carbon in different soil aggregate sizes in gravel-mulched fields, so as to facilitate microbial colonization [43,44]. On the other hand, organic fertilizer contains a lot of organic matter, which provides a rich carbon source for the reproduction of microorganisms [45,46]. And, organic fertilizer itself contains a large number of living microorganisms, thus facilitating the "inoculation" and "introduction" of microorganisms [47]. The ratio of G^+/G^- is often used as an indicator of soil nutrient status, with lower values indicating more optimized nutrient status [48]. Changes in the fungal/bacterial ratios can better reflect the buffering capacity of soils [49]. Organic fertilization treatments were found to be effective in improving the nutrient status of soil aggregates in gravel-mulched fields and increasing soil buffering capacity. This is similar to the findings obtained by Guo Yun et al. [47], who carried out a study on the long-term impact of fertilization on small soil aggregates. In contrast, Wei et al. [50] found that long-term substitution of chemical fertilizers with pig manure significantly reduced the fungal/bacterial ratios in black soil farmland. This difference may be related to characteristics of soil types, basal fertilization, and differences in exogenous inputs of organic fertilizers and their respective organic matter contents.

4.3. Effects of Different Fertilization Treatments on Enzyme Activities of Soil Aggregates

As one of the most important indicators of soil fertility, the activity of soil enzymes is affected by soil aggregate size and fertilization regimes [7,8]. In this study, the activities of three hydrolases (LAP, NAG, and BG) increased with the decrease in the particle size of aggregates, which is consistent with the results published by Lei et al. [9], who carried out a study in forest soils. It is possible that the smaller particle size of aggregates makes it easier for substrates and water required for enzymatic reactions to enter the aggregates through diffusion, and the structural stability is not easily disturbed by environmental and mechanical effects, resulting in the accumulation of more reaction substrates [51]. Meanwhile, inputs of organic fertilizers could provide more substrates for soil enzymes, and the activities of three hydrolytic enzymes (LAP, NAG, and BG) also increased with the increase in the proportion of organic fertilizers replacing chemical fertilizers, which is in line with the results of the study carried out by Li et al. [52]. However, the excessive accumulation of extracellular enzymatic matrix can inhibit enzyme activity [53], which may be one of the main reasons for the change in the β-xylosidase (β-XYS) activity in this paper. Unlike hydrolases, oxidoreductases are mainly produced by fungi, which mainly exist in large aggregates because their hyphae cannot penetrate the pores of soil microaggregates [54], and thus, the activities of two oxidoreductases (CAT and PPO) in macroaggregates were the highest in this study. Among them, the activity of aggregate catalase (CAT) in the organic fertilizer treatment was lower than that in the chemical fertilizer treatment, while there was no significant difference between the two chemical fertilizer treatments (CK and CF) and between the four organic fertilizer treatments (OF-

25%, OF-50%, OF-75%, OF-100%). The reason for this may be that peroxidase activity did not have any significant correlation with soil organic carbon, but it was highly significantly and positively correlated with soil pH [9,55]. After being added to the soil, organic fertilizers mainly neutralize alkaline soils through the decomposition and release of organic acids and other substances, thereby significantly reducing the pH of the soil [56]. The PPO activity increased with the increase in the proportion of organic fertilizers, which may be mainly attributed to the positive promoting effect of these organic fertilizers on the soil fungal community. Jastrow et al. [57] believed that fungi were the main contributors to the activity of PPO in the soil polyphenol oxidase.

4.4. Correlation between Soil Aggregate Particle Size and Microbial Biomass and Enzyme Activities

Soil aggregates and their associated microorganisms and enzymatic activities are inseparable, with aggregates providing the habitat for the survival of the microbes, while the microorganisms and their enzyme activities contribute to the formation of aggregates [58,59]. This study showed highly significant positive correlations between the mean weight diameter (MWD) of soil aggregates and the microbial biomass (bacterial, fungal, actinomycetes, and total phospholipid fatty acids) and the enzyme activities (LAP, NAG, BG, and PPO). MWD is one of the main indicators for measuring the stability of soil aggregates; the larger the value of MWD, the more stable the aggregates, which means the stronger the physical isolation and protection effect on the microbial and enzyme activities in the aggregates [60]. On the other hand, LAP, NAG, BG, and PPO activities serve as regulators for soil humus and carbon and nitrogen chemical transformation, soil microbial metabolism, and the decomposition of organic matter and polysaccharides, which positively affect the formation and stability of aggregates [9,61,62].

5. Conclusions

Under equal nitrogen conditions, chemical fertilizer substitution with organic fertilizers significantly promoted the formation of soil aggregates with particle sizes of > 1 mm in the sandy field. In aggregates of different sizes, compared with the single application of chemical nitrogen fertilizer, the application of equal or higher proportions of organic nitrogen fertilizer (OF-50%–100%) significantly promoted the accumulation of microbial carbon and nitrogen, increased the microbial biomass (bacteria, fungi, actinomyces) and fungal/bacterial ratios, and improved the activities of hydrolytic enzymes (LAP, NAG, BG, and β-XYS) and polyphenol oxidase (PPO) to varying degrees but decreased the G^+/G^- ratios and catalase (CAT) activity and then enhanced the stability of the aggregates. This confirms our established research hypothesis. Therefore, the replacement of more than 50% of the chemical nitrogen fertilizer with organic manure is an effective measure to improve the physical structure of continuous cropping soil in gravel-mulched fields, promote the microorganism reproduction and the extracellular enzyme activity of aggregates, and enhance soil fertility.

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