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Long-Term Chemical and Organic Fertilization Differently Affect Soil Aggregates and Associated Carbon and Nitrogen in the Loess Plateau of China

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Abstract: Fertilizer sources may have variable effects on soil aggregation, aggregated-associated C and N, and wheat yield. A 34-year field experiment was performed to evaluate the influences of chemical and organic fertilization on soil aggregates and associated carbon and nitrogen under winter wheat in a Cumulic Haplustoll of the Loess Plateau, China. Treatments included unfertilized control (CK), inorganic N fertilizer (NF), inorganic P fertilizer (PF), inorganic N and P fertilizer (NP), organic manure (M), inorganic N fertilizer plus manure (NM), inorganic P fertilizer plus manure (PM), and inorganic N plus P fertilizers plus manure (NPM). Compared to CK, long-term fertilization significantly increased the proportion of soil macro-aggregates, mean weight diameter (MWD), and geometric mean diameter (GMD), but decreased the proportion of micro-aggregates and fractal dimension, especially fertilizer plus manure. Compared to CK, manure treatments (M, NM, PM, and NPM) had a better improvement on soil organic carbon (SOC), soil total nitrogen (STN), particle organic C, and microbial biomass C in all aggregates than the fertilizer alone. The SOC in different aggregates increased with the increased aggregate size, which was because the larger aggregates formed by the binding of the smaller aggregates and organic matter. PON increased in NM and NPM, and MBN was more sensitive to N fertilizer. The C/N ratio in bulk soil and aggregates decreased with fertilization, especially in fertilizer with manure and in macro-aggregates. The improved soil structure was related to the increased SOC and STN, which was proved by the positive correlations among SOC and STN with macro-aggregates and MWD. A correlation analysis also showed that the contribution rate of SOC and STN in macro-aggregates was positively associated with the macro-aggregate and stability. Therefore, the sequestration of C and N in soil was related to aggregate size and was mainly affected by larger aggregates. The results demonstrated that fertilizer with manure improved the soil structure and fertility better than fertilizer alone, thus increasing crop yield.

Keywords: long-term fertilization; soil aggregates; aggregated-associated C and N; wheat yield

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1. Introduction

Maintaining soil good structure and high organic matter is essential for the continuous supply of soil nutrients, plant growth, and ecosystem balance [1,2]. Soil aggregation promotes root growth and microbial activity by optimizing the soil structure [3]. Materials secreted by roots and microorganisms that act as adhesives can promote soil aggregations [4,5]. Soil organic carbon (C) and nitrogen (N) are the core substances of

nutrient cycling, and the sequestration of C and N in soil is considered to be a potential mechanism for reducing the emissions of greenhouse gases in agricultural systems [6,7]. It has been reported that nearly 90% of soil organic C is in the aggregates [3,8]. Aggregates protect soil C and N from mineralization, and soil organic C and N provides the adhesives for the formation of aggregates [9]. Therefore, the changes in soil aggregates affect the sequestration of C and N in soil, and C and N pools that are protected by soil aggregates are more stable than that in the unprotected pools [10].

The storage and transformation of C and N in aggregates vary with the aggregate size [11]. Soil organic C (SOC) and total N (STN) can be greater in macro-aggregates (>2 mm) than in micro-aggregates (<0.25 mm) because fresh crop residues are incorporated first in macro-aggregates [9,12,13]. However, Udom et al. [14] reported that SOC can be higher in micro-aggregates because of the more stable structure and slower decomposition. Large aggregates provide an ecological niche for storing labile organic C while small aggregates contain more stabilized C [15–17]. Therefore, C and N in large aggregates have greater dispersion and turnover rates under the influence of external conditions than those in small aggregates [18–20]. Particle organic C (POC) and N (PON) are intermediate fractions of C and N that are more labile than SOC and STN, which provide substrates for soil microorganisms and contribute to aggregate stability [21,22]. Soil microbial biomass C (MBC) and N (MBN) are labile C and N fractions that are stored in the microbial body and change rapidly within a growing season [11,16,23]. Therefore, it is necessary to know how aggregate stability and turnover under management practices affect C and N storage, mineralization, and availability for plant uptake.

Fertilization, as an important management to improve crop yield, has a great impact on soil aggregates and associated C and N fractions [17,24]. Previous studies have shown that frequent use of chemical fertilizers deteriorates soil aggregation and reduces the formation and stability of large aggregates, thereby exposing previously protected organic C [25]. In contrast, the application of organic fertilizer is beneficial to the formation of large aggregates that are rich in organic C and increases the retention of organic C [16,24,26]. Wang et al. [27] found that the combined application of chemical and organic fertilizer had a positive effect on improving aggregate stability and nutrient accumulation in dryland greenhouses. In addition, Duan et al. [28] found that the long-term application of manure led to an increase in macro-aggregates (>250 μm) and the contents of plant available N in aggregates, thus contributing to high yield. However, Das et al. [29] and Hati et al. [30] reported the long-term application of chemical fertilizers improved macro-aggregation and stability of aggregates. Therefore, the response of soil aggregates' associated C and N fractions to fertilization practices may vary with the fertilizer types and soil properties [2,31].

In the Loess Plateau of China, different fertilization practices, mainly with chemical fertilizers, have been used since the 1970s to improve soil quality and enhance winter wheat (*Triticum aestivum* L.) yield [32]. This has degraded the soil quality by reducing aggregation, resulting in a decline in the crop yield [32,33]. Improved management strategies are needed to enhance soil aggregation, quality, and crop yields. This study evaluated a 34-year field experiment that received chemical fertilizers, organic manure, and a combination of both on soil aggregation and associated C and N in a Cumulic Haplustoll. The objectives were to determine the long-term effect of chemical fertilizers and organic manure on soil aggregation and stability, aggregated-associated C and N, and winter wheat yields in the Loess Plateau of China. We hypothesized that organic manure, with or without chemical fertilizers, would enhance soil aggregation and stability, the associated C and N, and the winter wheat yield compared to chemical fertilizers alone or no fertilization.

2. Materials and Methods

2.1. Site Description

A field experiment was established in 1984 that evaluated the effects of chemical fertilizers and organic manure on winter wheat yield at the Shaanxi Changwu Agro-Ecological Station of China (107°44.70′ E, 35°12.79′ N) [32,34]. The site (elevation 1200 m)

had a sub-humid temperate climate with an average (30 year) annual precipitation of 584 mm, an average annual air temperature of 9.1 °C, and a frost-free period of 171 days. The soil was classified as an aridic and loamy, Cumulic Haplustoll, according to USDA Soil Taxonomy [35], with sand, silt, and clay concentrations of 35, 656, and 309 g kg^{-1}, respectively, pH 8.4, soil organic C (SOC) 6.5 g kg^{-1}, total N (STN) 0.80 g kg^{-1}, total phosphorus (P) 1.26 g kg^{-1}, and $CaCO_3$ 105 g kg^{-1} at the 0–30 cm depth at the initiation of the experiment.

2.2. Experimental Design

Treatments were (1) unfertilized control (CK), (2) inorganic N fertilizer (NF), (3) inorganic P fertilizer (PF), (4) inorganic N and P fertilizer (NP), (5) organic manure (M), (6) inorganic N fertilizer plus organic manure (NM), (7) inorganic P fertilizer plus organic manure (PM), and (8) inorganic N and P fertilizers plus organic manure (NPM). All treatments were arranged in a randomized block design with three replications. The plot size was 10.3 m × 6.5 m, with a 0.5 m strip between plots. Following the local practices, N fertilizer as urea (46% N), P fertilizer as triple superphosphate (45% P), and organic manure were applied, and the doses of N and P were at a rate of 120 and 90 kg ha^{-1}, respectively, in 75 t ha^{-1} of organic nutrients. The organic manure had an average C, N, and P concentrations of 800, 87, and 44 kg ha^{-1}, respectively. Because the soil was rich in K concentration, no K fertilizer was applied. However, organic manure also supplied K at 30 kg K ha^{-1}.

Before planting, chemical fertilizers and organic manure were broadcast and incorporated at a depth of 20 cm with a rotary tiller in late September each year [33]. Seed beds were prepared by cultivating soil at a depth of 20 cm using a rotary tiller. Immediately after tillage operation, winter wheat (cv. Changwu 131) was sown at 195 kg ha^{-1} with a 20 cm row-spacing. No irrigation was applied. Weeds were controlled manually and pesticides were applied as needed. Winter wheat was harvested in June of the following year and grain yield was determined by cutting all plants at 2 cm above the ground with a sickle within the plot, threshing plants on the ground, and oven drying at 70 °C for 7 days.

2.3. Soil Sampling

Soil samples were collected from 0–20 cm after wheat harvest in late June of 2018. In each plot, five cores were collected randomly with a hand probe (5 cm inside diameter), and composited within a plot. After air-drying, one part was gently broken along the natural destruction surface to obtain aggregates of <10 mm for the aggregate analysis. Another part was sieved to 2 mm to perform C and N analysis.

2.4. Soil Aggregate Analysis

Dry sieving is an appropriate method that separates aggregates in dryland cropping systems with limited precipitation where aggregates are disrupted more by wind than water erosion [7]. Briefly, we prepared a set of upper and lower sets of 2.00 mm and 0.25 mm, respectively. A total of 100 g of air-dried soil samples was dry sieved through a nest of sieves containing 2 mm and 0.25 mm sieves and vibrated mechanically for 5 min in a shaker. Aggregates that remained in each sieve after sieving (10–2 mm, 2–0.25 mm, and <0.25 mm) were collected and weighed. The stability index of soil aggregates included aggregate content >0.25 mm ($R_{0.25}$), mean weight diameter (MWD), geometric mean diameter (GMD), and fractal dimension (D). The MWD (mm), GMD (mm), and D were estimated using the following equations [36]:

$$\mathrm{MWD} = \frac{\sum_{i=1}^{3} m_i d_i}{\sum_{i=1}^{3} m_i} \quad (1)$$

$$\mathrm{GMD} = Exp\frac{\sum_{i=1}^{3} (m_i ln d_i)}{\sum_{i=1}^{3} m_i} \quad (2)$$

$$\frac{m_{(d<d_i)}}{\sum_{i=1}^{3} m_i} = \left(\frac{d_i}{d_{max}}\right)^{3-D} \quad (3)$$

where m_i is the mass of aggregate fraction i (g); d_i is the mean diameter of the aggregate fraction i (mm); $m_{(d<di)}$ is the mass of aggregate fraction with diameter of $<d_i$ (g); and d_{max} = 10 mm.

2.5. Soil C and N Fractions Analyses

Soil organic carbon (SOC) and total nitrogen (STN) in bulk soil and aggregates were analyzed using a C and N analyzer (Euro Vector EA3000, Manzoni, Italy) after further grinding the bulk soil and aggregates to 0.15 mm and pretreating with 1.0 mol L^{-1} HCl to remove inorganic C. The POC and PON in aggregates were determined using the method shown by Cambardella and Elliott [37]. A total of 10 g of aggregates was mixed with 30 mL of 5 g L^{-1} sodium hexametaphosphate solution and shaken in a reciprocating oscillator for 16 h. The dispersed solution was passed through a 0.053 mm sieve, dried at 50 °C, and analyzed for C and N concentrations with a C and N analyzer, as described above. The POC and PON concentrations were calculated as the difference between the SOC or STN concentrations in the bulk soil and the particle concentration that passed through the sieve after correction for sand concentration. Soil microbial biomass C and N (MBC and MBN) were determined using the chloroform fumigation leaching method [38]. The contribution rate (CR) and enrichment coefficient (EC) of SOC and STN in soil aggregates were calculated by the following equations [39]:

$$\mathrm{CR} = \frac{SOC_i(\text{or } STN_i) \times M_i}{\mathrm{SOC(or\ STN)}} \times 100\% \quad (4)$$

$$\mathrm{EC} = \frac{SOC_i(\text{or } STN_i)}{\mathrm{SOC(or\ STN)}} \times 100\% \quad (5)$$

where SOC_i (or STN_i) is the content of SOC and STN in aggregate (g kg^{-1}); M_i is the proportion of the aggregate (%); and SOC (or STN) is the content of SOC (or STN) in the bulk soil (g kg^{-1}).

2.6. Statistical Analysis

The SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis of data. A one-way analysis of variance (ANOVA) was carried out to determine significant differences between treatments using LSD's test with a significance level of $p < 0.05$. Treatment was considered as the main plot and aggregate size was considered as split-plot treatment for data analysis. Correlation heatmap was constructed using the pheatmap-package and R package vegan, based on the Spearman correlation matrix. Co-occurrence networks among soil aggregates, the aggregate stability, and aggregate-associated C and N fractions were constructed according to the Pearson's correlation analysis and p value ($p < 0.01$, Bonferroni-corrected). The software Gephi 0.9.2 was used to visualize the networks of co-occurring relationships [40].

3. Results

3.1. Soil's Basic Properties

Compared to CK, fertilizer with manure decreased the soil bulk density (BD) and PM decreased by 7.6% ($p < 0.05$) (Table 1). Inversely, the soil porosity increased by 3.6–6.6% in fertilizer with manure and PM was significant. Compared to CK, all fertilized treatments increased WHC and fertilizer with manure showed a more obvious effect than the fertilizer alone. M, NM, PM, and NPM increased WHC by 36, 39, 38, and 47% ($p < 0.05$), respectively. All fertilized treatments, except NF, had a greater SOC than CK, and the SOC was 19–54% greater in fertilizer with manure than fertilizer alone. Similarly, compared

to CK, all fertilized treatments had a greater STN, which was 46–67% greater in fertilizer with manure than fertilizer alone. As a result, the soil C/N ratio decreased by 17–37% in fertilized treatments compared to CK and NP, and the fertilizer with manure had more obvious changes.

Table 1. Soil's basic properties under different fertilization treatments. Treatments were CK, unfertilized control; NF, inorganic N fertilizer; PF, inorganic P fertilizer; NP, inorganic N plus P fertilizer; M, organic manure; NM, inorganic N fertilizer plus manure; PM, inorganic P fertilizer plus manure; and NPM, inorganic N plus P fertilizers plus manure. Values are means ± standard errors (n = 3). Different letters indicate significant difference between treatments ($p < 0.05$, LSD test).

Treatments	BD (g cm^{-3})	Porosity (%)	WHC (%)	SOC (g kg^{-1})	STN (g kg^{-1})	C/N Ratio
CK	1.19 ± 0.04 b	55.00 ± 1.57 b	20.25 ± 0.61 f	9.29 ± 0.36 ef	0.98 ± 0.07 d	11.09 ± 0.51 a
NF	1.22 ± 0.11 ab	54.04 ± 4.02 bc	24.52 ± 0.72 e	10.11 ± 0.55 de	1.39 ± 0.06 b	7.27 ± 0.09 cd
PF	1.26 ± 0.11 a	52.62 ± 4.17 c	25.62 ± 0.25 de	10.43 ± 0.37 cd	1.02 ± 0.03 cd	9.10 ± 0.06 b
NP	1.15 ± 0.04 b	56.64 ± 1.64 ab	26.30 ± 0.41 cd	10.85 ± 0.32 cd	1.15 ± 0.07 c	10.90 ± 0.02 a
M	1.13 ± 0.06 bc	57.23 ± 2.35 ab	27.54 ± 0.61 bc	12.55 ± 0.71 b	1.12 ± 0.06 cd	9.33 ± 0.15 b
NM	1.12 ± 0.08 bc	57.81 ± 2.93 ab	28.19 ± 0.98 b	11.45 ± 0.35 c	1.64 ± 0.09 a	6.98 ± 0.17 d
PM	1.10 ± 0.07 c	58.62 ± 2.56 a	27.84 ± 0.69 b	11.07 ± 0.69 cd	1.43 ± 0.07 b	7.74 ± 0.13 c
NPM	1.14 ± 0.05 bc	57.00 ± 1.83 ab	29.85 ± 0.45 a	14.27 ± 0.18 a	1.58 ± 0.06 a	9.05 ± 0.21 b

3.2. Aggregate Distribution and Stability

Compared to CK, the proportion of macro-aggregates (10–2 mm) increased by 28, 13, 35, 45, and 61% in NP, M, NM, PM, and NPM ($p < 0.05$, Figure 1), respectively. The proportion of medium aggregates (2–0.25 mm) decreased in all fertilized treatments, which decreased by 9.2, 11, 19, and 22% ($p < 0.05$) in N, NM, PM, and NPM, respectively. The proportion of micro-aggregates (<0.25 mm) was opposite to the changes in macro-aggregates, which decreased by 37, 22, 34, 29, and 49% ($p < 0.05$) in NP, M, NM, PM, and NPM, respectively. Table 2 showed that the $R_{0.25}$ increased by 7.8, 4.5, 7.1, 6.1, and 10% ($p < 0.05$) in NP, M, NM, PM, and NPM, respectively, compared to CK. Meanwhile, NP, M, NM, PM, and NPM significantly increased MWD and GMD by 12–51% and 7.8–29%, respectively. The fractal dimension (D) decreased by 4.2, 4.9, and 7.2% ($p < 0.05$) in NM, PM, and NPM, respectively. These results indicated that soil aggregate stability improved better in fertilizer with manure than in fertilizer alone, especially in NPM.

3.3. C and N Fractions in Aggregates

Compared to CK, the SOC in M, NM, PM, and NPM increased by 3.1–4.7% and 3.3–4.8% in macro- and medium-aggregates, respectively, and all fertilized treatments increased SOC by 1.1–5.1% in micro-aggregates ($p < 0.05$, Figure 2a). The POC in M, NM, PM, and NPM increased by 85–115%, 30–67%, and 3.3–4.8% in macro-, medium-, and micro-aggregates, respectively, while NF, PF, and NPF only increased POC in macro- and micro-aggregates ($p < 0.05$, Figure 2b). Compared to CK, MBC in NM, PM, and NPM increased by 15–40%, 6.7–33%, and 12–54% in macro-, medium-, and micro-aggregates, respectively, and M only increased MBC in macro-aggregates ($p < 0.05$, Figure 2c). However, NF, PF, and NPF decreased MBC in different aggregates. Compared to CK, NP, M, NM, PM, and NPM had significant improvements on aggregated-associated STN, which increased by 39–66%, 36–60%, and 47–76% in macro-, medium-, and micro-aggregates, respectively ($p < 0.05$, Figure 2d). The PON in different aggregates significantly increased in NM and NPM, while other treatments even decreased in macro- and medium-aggregates (Figure 2e). MBN showed the highest value in NPM and increased by 49, 48, and 59% in macro-, medium-, and micro-aggregates, respectively ($p < 0.05$, Figure 2f).

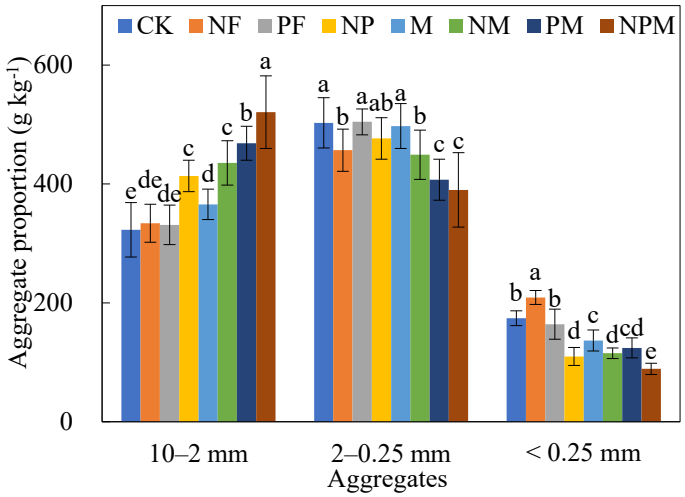


Figure 1. Soil aggregate size distribution under different fertilization treatments. Error bars indicate ±standard deviation. Treatments were CK, unfertilized control; NF, inorganic N fertilizer; PF, inorganic P fertilizer; NP, inorganic N plus P fertilizer; M, organic manure; NM, inorganic N fertilizer plus manure; PM, inorganic P fertilizer plus manure; and NPM, inorganic N plus P fertilizers plus manure. Different letters above error bars indicate significant difference between treatments ($p < 0.05$, LSD test).

Table 2. Soil aggregate stability is measured as the content of aggregates larger than 0.25 mm ($R_{0.25}$), mean weight diameter (MWD), geometric mean diameter (GMD), and the fractal dimension (D) under different fertilization treatments. Treatments were CK, unfertilized control; NF, inorganic N fertilizer; PF, inorganic P fertilizer; NP, inorganic N plus P fertilizer; M, organic manure; NM, inorganic N fertilizer plus manure; PM, inorganic P fertilizer plus manure; and NPM, inorganic N plus P fertilizers plus manure. Values are means ± standard errors ($n = 3$). Different letters indicate significant difference between treatments ($p < 0.05$, LSD test).

Treatments	$R_{0.25}$ (%)	MWD (mm)	GMD (mm)	D
CK	82.58 ± 1.25 d	2.27 ± 0.23 e	1.03 ± 0.04 e	2.65 ± 0.03 a
NF	79.09 ± 1.17 e	2.35 ± 0.14 e	1.01 ± 0.03 e	2.66 ± 0.02 a
PF	83.58 ± 2.53 d	2.32 ± 0.19 e	1.04 ± 0.05 e	2.64 ± 0.03 a
NP	89.01 ± 1.50 b	2.82 ± 0.11 c	1.18 ± 0.03 c	2.55 ± 0.02 c
M	86.32 ± 1.76 c	2.53 ± 0.13 d	1.11 ± 0.03 d	2.60 ± 0.02 a
NM	88.47 ± 0.88 b	2.95 ± 0.17 c	1.21 ± 0.04 bc	2.54 ± 0.02 cd
PM	87.58 ± 1.68 bc	3.17 ± 0.13 b	1.24 ± 0.04 b	2.52 ± 0.03 d
NPM	91.10 ± 0.95 a	3.42 ± 0.25 a	1.33 ± 0.07 a	2.46 ± 0.05 e

3.4. C and N Distributions in Aggregates

Compared to CK, all fertilized treatments decreased the C/N ratio in different aggregates (Table 3). The fertilizer with manure had a more significant effect than fertilizer alone, which reached the lowest value in NPM and decreased by 37, 35, and 41% ($p < 0.05$) in macro-, medium-, and micro-aggregates, respectively. Compared to CK, CR of SOC (C-CR) increased in macro-aggregates and decreased in medium- and micro-aggregates, especially in fertilizer with manure (Table 4). However, the EC of SOC (C-EC) in fertilizer with manure had no significant changes, nor did it decrease. In the macro-aggregates, CR of STN (N-CR) increased by 54, 38, 29, 42, and 66% ($p < 0.05$) in NP, M, NM, PM, and NPM, respectively. However, N-CR in medium-aggregates only increased in NP and M, while it decreased by 18–23% ($p < 0.05$) in NM, PM, and NPM. Fertilizer with manure also decreased N-CR in the micro-aggregates by 25–44% ($p < 0.05$). Consistent with N-CR in the medium-aggregates, the EC of STN (N-EC) significantly increased in NP and M in different aggregates. Meanwhile, N-EC also significantly increased in the micro-aggregates in PM and NPM.

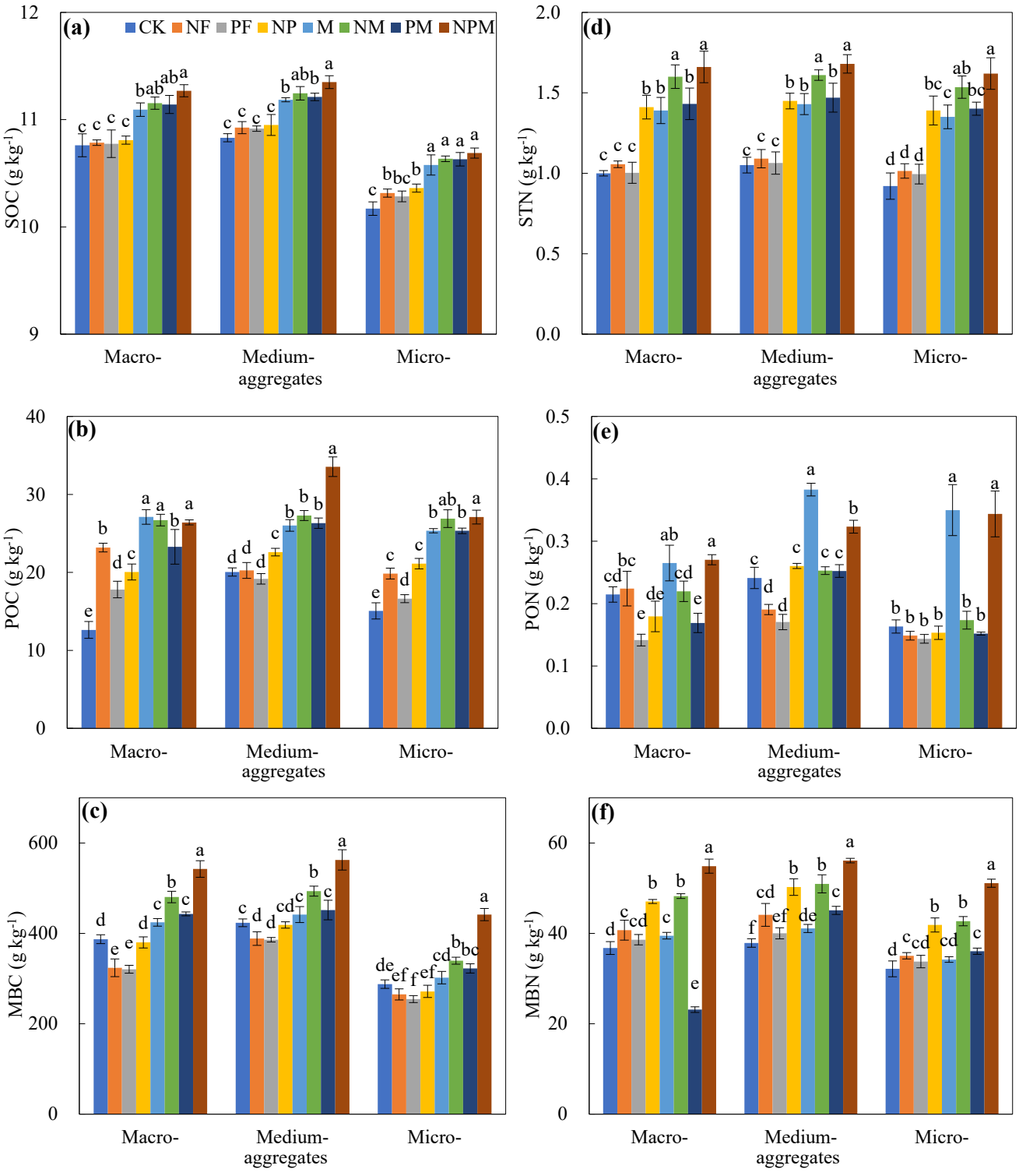


Figure 2. Soil organic C (SOC), particulate organic C (POC), microbial biomass C (MBC), soil total N (STN), particulate organic N (PON), and microbial biomass N (MBN) in different aggregates as affected by chemical fertilization and organic manure. The subfigures (**a**–**f**) represent SOC, POC, MBC, STN, PON, and MBN, respectively. Treatments were CK, unfertilized control; NF, inorganic N fertilizer; PF, inorganic P fertilizer; NP, inorganic N plus P fertilizer; M, organic manure; NM, inorganic N fertilizer plus manure; PM, inorganic P fertilizer plus manure; and NPM, inorganic N plus P fertilizers plus manure. Error bars indicate ±standard deviation. Different letters above error bars indicate significant difference between treatments ($p < 0.05$, LSD test).

Table 3. Soil C/N ratio in aggregates under different fertilization treatments. Treatments were CK, unfertilized control; NF, inorganic N fertilizer; PF, inorganic P fertilizer; NP, inorganic N plus P fertilizer; M, organic manure; NM, inorganic N fertilizer plus manure; PM, inorganic P fertilizer plus manure; and NPM, inorganic N plus P fertilizers plus manure. Values are means ± standard errors (n = 3). Different letters indicate significant difference between treatments ($p < 0.05$, LSD test).

Treatments	Macro-Aggregates (>2 mm)	Medium-Aggregates (2–0.25 mm)	Micro-Aggregates (<0.25 mm)
CK	10.76 ± 0.07 a	10.33 ± 0.45 a	11.13 ± 0.92 a
NF	10.22 ± 0.18 a	10.04 ± 0.48 a	10.18 ± 0.42 a
PF	10.80 ± 0.84 a	10.30 ± 0.65 a	10.37 ± 0.59 a
NP	7.68 ± 0.43 bc	7.56 ± 0.18 bc	7.48 ± 0.46 b
M	8.01 ± 0.42 b	7.84 ± 0.35 b	7.85 ± 0.36 b
NM	6.98 ± 0.28 bc	6.99 ± 0.18 bc	6.94 ± 0.30 b
PM	7.82 ± 0.48 bc	7.66 ± 0.49 bc	7.59 ± 0.18 b
NPM	6.81 ± 0.37 c	6.76 ± 0.19 c	6.62 ± 0.37 b

Table 4. The contribution rate (CR) and enrichment coefficient (EC) of soil aggregate organic C and total N under different fertilization treatments. Treatments were CK, unfertilized control; NF, inorganic N fertilizer; PF, inorganic P fertilizer; NP, inorganic N plus P fertilizer; M, organic manure; NM, inorganic N fertilizer plus manure; PM, inorganic P fertilizer plus manure; and NPM, inorganic N plus P fertilizers plus manure. Values are means ± standard errors (n = 3). Different letters indicate significant difference between treatments ($p < 0.05$, LSD test).

Item	Treatments	Macro-Aggregates (>2 mm)		Medium-Aggregates (2–0.25 mm)		Micro-Aggregates (<0.25 mm)	
		CR (%)	EC	CR (%)	EC	CR (%)	EC
SOC	CK	32.04 ± 3.10 c	0.99 ± 0.02 b	47.17 ± 2.92 bc	0.99 ± 0.02 b	17.42 ± 1.74 b	0.94 ± 0.02 cd
	NF	35.79 ± 3.69 bc	1.07 ± 0.05 b	46.74 ± 2.69 bc	1.07 ± 0.05 b	20.83 ± 1.46 a	1.02 ± 0.05 b
	PF	38.65 ± 5.05 bc	1.16 ± 0.06 a	55.92 ± 1.75 a	1.16 ± 0.06 a	16.92 ± 1.63 bc	1.11 ± 0.04 a
	NP	35.73 ± 1.88 bc	0.86 ± 0.05 c	39.50 ± 2.29 d	0.86 ± 0.05 c	7.98 ± 0.82 fg	0.83 ± 0.05 e
	M	38.92 ± 0.86 bc	1.06 ± 0.03 b	50.52 ± 2.92 ab	1.06 ± 0.03 b	14.22 ± 1.25 cd	1.02 ± 0.02 bc
	NM	42.48 ± 3.30 ab	0.98 ± 0.02 b	41.73 ± 2.31 cd	0.98 ± 0.02 b	10.62 ± 0.19 ef	0.93 ± 0.02 d
	PM	47.28 ± 1.93 a	1.01 ± 0.05 b	39.32 ± 3.36 d	1.01 ± 0.05 b	11.74 ± 1.75 de	0.96 ± 0.05 c
	NPM	41.12 ± 3.70 ab	0.79 ± 0.01 c	29.25 ± 3.81 e	0.79 ± 0.01 c	6.71 ± 1.00 g	0.75 ± 0.01 f
STN	CK	33.03 ± 3.62 d	1.02 ± 0.06 bc	53.96 ± 3.37 b	1.07 ± 0.03 b	16.31 ± 0.31 a	0.94 ± 0.01 d
	NF	25.41 ± 2.17 e	0.76 ± 0.02 d	35.84 ± 1.50 d	0.78 ± 0.01 c	15.26 ± 0.64 ab	0.73 ± 0.00 e
	PF	32.43 ± 1.49 d	0.98 ± 0.03 c	52.54 ± 2.06 b	1.04 ± 0.03 b	16.06 ± 2.75 a	0.97 ± 0.03 c
	NP	50.69 ± 0.17 ab	1.23 ± 0.01 a	60.10 ± 1.77 ab	1.26 ± 0.03 a	13.26 ± 0.54 bc	1.21 ± 0.01 a
	M	45.45 ± 1.26 bc	1.24 ± 0.01 a	63.62 ± 2.32 a	1.28 ± 0.01 a	16.52 ± 1.37 a	1.21 ± 0.00 a
	NM	42.49 ± 2.76 c	0.98 ± 0.01 c	44.16 ± 3.21 c	0.98 ± 0.07 b	10.78 ± 0.65 cd	0.94 ± 0.01 d
	PM	46.90 ± 1.77 bc	1.00 ± 0.02 bc	42.23 ± 5.69 cd	1.03 ± 0.11 b	12.18 ± 0.99 c	0.98 ± 0.02 c
	NPM	54.86 ± 6.50 a	1.05 ± 0.02 b	41.52 ± 5.22 cd	1.06 ± 0.00 b	9.11 ± 0.12 d	1.02 ± 0.03 b

3.5. Correlation between Aggregate Stability and the C and N Fractions

Figure 3 shows the correlations between the soil's basic properties and the aggregate parameters. Soil porosity and WHC were positively correlated with the macro-aggregates, $R_{0.25}$, MWD, and GWD, while they were negatively correlated with the micro-aggregates and fractal dimension (D). However, BD showed opposite relations with the aggregate parameters compared to porosity and WHC. SOC and STN were positively correlated with WHC, macro-aggregates, MWD, and GWD, while they were negatively correlated with the fractal dimension (D). Meanwhile, SOC and STN were negatively correlated with the medium- and micro-aggregates.

The correlations between the aggregate size and stability of bulk soil and the C and N fractions in different aggregates are shown in Figure 4. In macro-aggregates, SOC and MBC were positively correlated with the SOC of bulk soil, with coefficients of 1.00 and 0.77, respectively. Meanwhile, SOC, POC, MBC, and C-CR in macro-aggregates were positively correlated with MWD, with coefficients of 0.71, 0.54, 0.80, and 0.72, respectively. SOC in medium- and micro-aggregates and POC in micro-aggregates showed the highest positive correlations with the STN of bulk soil, with coefficients of 0.77 and 0.79, respectively.

Similarly to macro-aggregates, MBC in medium- and micro-aggregates showed positive correlations with the SOC of bulk soil. However, C-CR in the medium- and micro-aggregates was negatively correlated with MWD, with coefficients of 0.84 and 0.82, respectively. STN in different aggregates was positively correlated with MWD (0.82, 0.83, and 0.83) and GMD (0.85, 0.86, and 0.86). The PON in aggregates was positively correlated with the SOC of bulk soil, with coefficients of 0.51, 0.48, and 0.46 in macro-, medium-, and micro-aggregates, respectively. N-CR was positively correlated with GWD (0.90) in macro-aggregates while negatively correlated with GWD (0.87) in macro-aggregates. The C/N ration in different aggregates was positively correlated with D (0.80, 0.82, and 0.81) and negatively with GMD (0.81, 0.82, and 0.81).

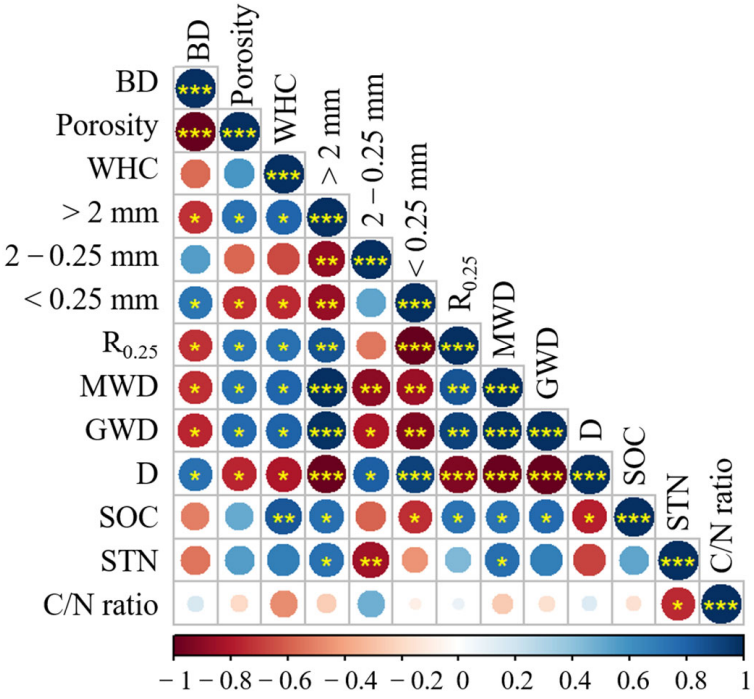


Figure 3. Correlation heatmap between soil's basic properties and aggregate parameters. The intensity of color indicates the correlation coefficient (R) (blue and red indicate a positive and negative correlation, respectively). *, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively.

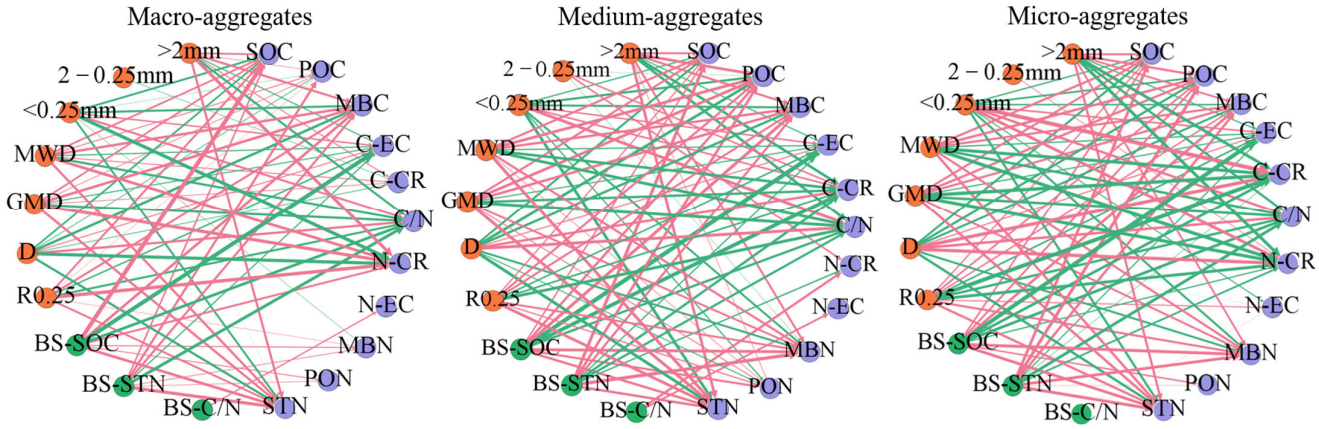


Figure 4. Co-occurrence networks of soil aggregate C/N fractions and the aggregate parameters of bulk soil (BS) under different fertilization based on correlation analysis. A red edge indicates a positive relationship, while a green edge indicates a negative relationship between two individual nodes. An orange node indicates the aggregate size and stability. A green node indicates the C/N fractions of bulk soil. A purple node indicates the soil aggregate C/N fractions.

3.6. Average Wheat Yield

The winter wheat yield, averaged across years, had more obvious improvements in fertilizer with manure than the fertilizer alone (Figure 5). Compared to CK, N and NP increased the yield by 84 and 140% ($p < 0.05$), respectively, while P had no significant effect. M, NM, PM, and NPM increased the yield by 99, 96, 166, and 147%, respectively, compared to CK.

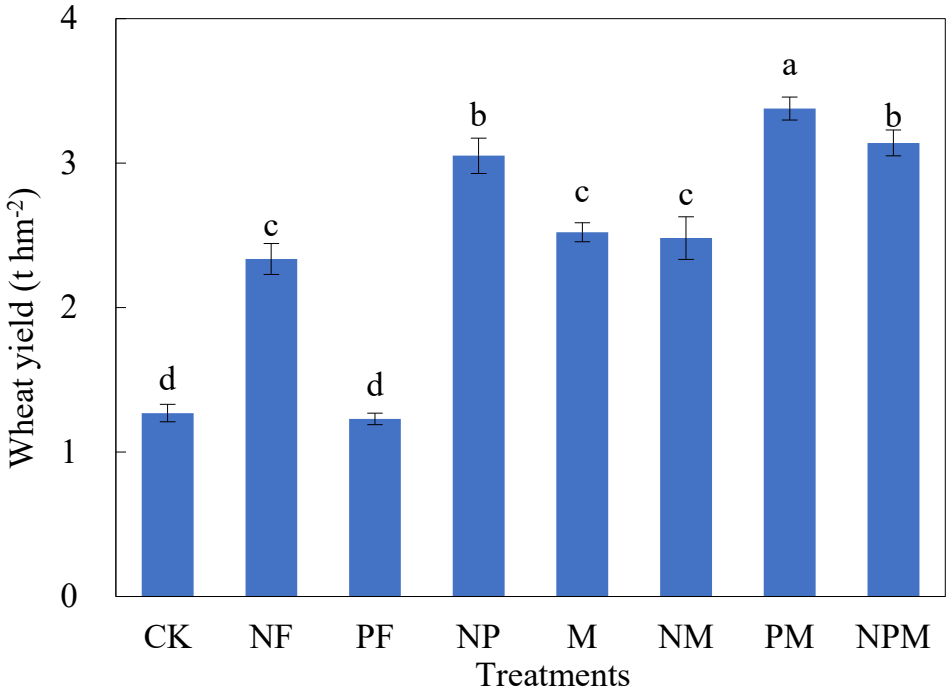


Figure 5. The average wheat yield across 34 years after long-term fertilization. Treatments were CK, unfertilized control; NF, inorganic N fertilizer; PF, inorganic P fertilizer; NP, inorganic N plus P fertilizer; M, organic manure; NM, inorganic N fertilizer plus manure; PM, inorganic P fertilizer plus manure; and NPM, inorganic N plus P fertilizers plus manure. Different letters above error bars indicate significant difference between treatments ($p < 0.05$, LSD test).

4. Discussion

4.1. Effects of Long-Term Fertilization on SOC and STN in Bulk Soil

The manure treatments increased SOC and STN while reducing the C/N ratio more than the chemical fertilizer, especially the NPM (Table 1). The total SOC mainly depended on the balance of the input and degradation of organic C [34]. The chemical fertilizer plus manure could increase the soil microbial activity on the one hand, thus promoting crop growth and biomass, which could increase the return amount of root stubble and secretion [41]. On the other hand, manure directly promoted increases in SOC by the direct input of C [6,42]. However, the study reported that the input of SOM by chemical fertilizer was not enough to make up for the loss of mineralization, and N fertilizer provided a soil microbial N source effectively, which could improve the soil microbial activity and strengthen the decomposition of organic C by physical protection [30]. Thus, the chemical fertilizer alone had no significant changes in SOC, or the promotion was poorer (Table 1). STN increased significantly with the application of N fertilizer. The soil C/N ratio decreased after fertilization, especially with chemical fertilizer plus manure, which was closely related to the improvement of organic matter decomposition and the application of N fertilizer.

4.2. Effects of Long-Term Fertilization on Aggregate Proportion and Stability

The greater proportion of macro-aggregates as well as increased MWD and GWD for NPM than other treatments (Figure 1 and Table 2) were likely due to higher C and N

concentrations (Figure 2). These results were consistent with previous findings that the application of manure increased soil aggregation and stability compared to chemical fertilization [43,44]. The increased soil aggregation and stability could be related to the increased SOM, which acted as a binding agent for soil particles to form aggregates [4,20,45,46], as proved by the positive relationships between SOC and macro-aggregates, MWD, and GWD (Figure 3). Although chemical fertilizer could increase cementing substances indirectly by improving the crop biomass, this effect was far less obvious than with organic fertilizer [47]. Organic fertilizer could not only improve crop biomass by providing various nutrients, but it also contained a lot of organic cementing substances [6,48,49]. The increased microbial biomass and fungal mycelia, as shown by greater POC and MBC, may increase the binding agents and promote soil aggregation for NPM [42]. Manure alone also increased macro-aggregates compared to chemical fertilization; however, the greater macro-aggregate proportion in NPM was also likely due to the increased winter wheat yield (Figure 3) that returned a greater crop residue to the soil, which provided more C inputs along with that provided by organic manure. This subsequently reduced the aggregate proportion for NPM compared to other treatments in medium- and micro-aggregates. Therefore, supplementing exogenous organic matter was an important measure to improve soil structure and maintain soil fertility.

4.3. Effects of Long-Term Fertilization on Aggregate-Associated Carbon and Nitrogen

The manure treatments increased the SOC, POC, and MBC in all aggregates, while chemical fertilizer only increased the POC in macro- and micro-aggregates and decreased the MBC in all aggregates (Figure 3). In addition to N, P, and K, organic manure also supplied 800 kg C ha^{-1} annually. As a result, more C may have been stored in aggregates that were in the treatments receiving manure. The SOC in different aggregates increased with the increased aggregate size. Larger aggregates were formed by the binding of small, free aggregates to temporary cementing agents, and the structure of larger aggregates could better protect the organic C and its components; thus, the larger aggregates contained more organic C [18,50,51]. Larger soil aggregates were also formed by the cementation of organic residues and mycelia, and newly imported organic C that was easily mineralized and decomposed mainly existed in large aggregates and was physically protected, while small aggregates were formed by the cementation of polysaccharides or inorganic colloid [52,53]. However, the chemical fertilizer resulted in a low content of newly input organic C, which reduced cementation material and contents of large aggregates, thus increasing the contribution rate of small aggregates to organic C [25]. At the same time, the effect of chemical fertilizer on POC was inferior to that of manure. On the one hand, POC lacked the physical protection due to poor soil aggregate structure and was easily used by microorganisms. On the other hand, chemical fertilizer could provide direct and available nutrients for microorganisms to enhance microbial activity and accelerate the conversion of POC to humic compounds [21]. In addition, larger aggregates could maintain a higher nutrient level than micro-aggregates and powder clay particles, which was conducive to microbial colonization [54]; thus, the MBC content in large aggregates was higher under the application of manure. A correlation analysis also showed that $R_{0.25}$ was positively correlated with the contribution rate of SOC (C-CR) in macro-aggregates, while it was negatively correlated with C-CR in medium- and micro-aggregates (Figure 4), which confirmed the physical protection theory of aggregates to a certain extent.

The changing trends of STN in bulk soil and different aggregates were basically consistent with those of SOC, which was due to the fact that 95% N in soil existed in organic matter in the form of organic N; thus, the changes in C in organic matter would inevitably lead to changes in organic N [11,55] (Figure 3). STN in different aggregates significantly increased in NP, M, NM, PM, and NPM. The application of manure not only directly increased the input of N, but also indirectly increased STN through the increase in roots and exudates. MBN was more sensitive to the addition of N fertilizer, which was due to the microorganisms that were no longer limited by the N element after N input,

thus increasing the transformation rate and improving the absorption and utilization of N [30]. The fertilizer plus manure was more effective in reducing the C/N ratio than the fertilizer and manure alone, especially in macro-aggregates (Table 3). With the input of exogenous nutrients, although SOC increased, the soil microbial activity also improved, which accelerated the decomposition of organic matter, thus resulting in the accumulation of N in soil faster than that of organic C [23,56]. Li et al. [57] showed that SOC and STN in subtropical paddy soil reserved in large aggregates ($\geq$0.25 mm) accounted for 64–81% and 54–82% of the total soil, respectively, which was because C and N in large aggregates were not easily degraded by physical action, microorganisms, or enzymes; thus, it is more difficult to be mineralized and detained in soil [48]. A correlation analysis showed that N-CR was positively correlated with $R_{0.25}$, MWD, and GMD in macro-aggregates, while negatively correlated with $R_{0.25}$, MWD, and GMD in micro-aggregates (Figure 4).

4.4. Effects of Long-Term Fertilization on Winter Wheat Yield

A balance of nutrient availability throughout the growing season as well as improved soil aggregation and stability and associated C and N may have increased the winter wheat yield for PM and NPM compared to other treatments, except NP (Figure 3). It is likely that chemical fertilizers supplied nutrients at the beginning of wheat growth, while organic manure supplied them at the later stage of growth, resulting in a consistent availability of nutrients throughout the growing season and an enhanced wheat yield for PM and NPM [58,59]. The improved soil aggregation and stability and associated C and N (Figures 1 and 2) may have also increased water and nutrient movements, root growth, and microbial activity due to greater energy and substrate availability, thereby increasing the wheat yield. As crops require both N and P for enhanced growth and production, the increased wheat yield for NP compared to CK, PF, M, and NM was probably due to the increased availability of both N and P. Soil aggregation and stability and associated C and N were also greater with NP than CK, PF, M, and NM (Figures 1 and 2, Table 2), which may have had a positive effect on the wheat yield, as described above. A decline in the winter wheat yield along with the degradation of soil aggregation due to the continuous application of chemical fertilizers in the Loess Plateau of China have been reported by several researchers [33,34]. A lack of fertilization and manure applications probably reduced the wheat yield for CK. Similarly, the absence of N fertilization reduced the wheat yield for PF.

The decreased C/N ratio in the soil and aggregates indicated that the increased rate of C was lower than that of N, which would accelerate the decomposition of microorganisms and the mineralization rate of N, and was not conducive to the sequestration of C [9]. Weil and Kroontje [60] found that when the C/N ratio was close to 20, most of the organic matter was plant residue in the early stages of decomposition. When the C/N ratio was close to 10, most of the organic matter was humus (the refractory component lignin and the aromatic substances in small aggregates that bind closely to the clay). In this study, the C/N ratio was all close to or lower than 10, indicating that most soil organic matter components were in the later stage of decomposition under long-term fertilization, which converted to humus and existed in soil and aggregates. This result was also verified by the conclusion that the POC was greater in manure (Figure 2). Only the contribution rates of SOC and STN in macro-aggregates were positively correlated with $R_{0.25}$ (Figure 4), indicating that the aggregate of >0.25 mm was more sensitive to fertilization management and was a good indicator of changes in organic C under agricultural management. Therefore, manure could improve soil fertility and physical properties with the increase in SOC, thus improving the soil's ability to supply crop nutrients and increasing crop yield. A reasonable combination of chemical fertilizers and organic manure is an effective fertilization model for the sustainable development of agricultural production.

5. Conclusions

The long-term (34 years) application of chemical fertilizers and organic manure had variable effects on soil aggregation and stability, aggregated-associated C and N, and the winter wheat yield. Soil aggregate stability and the proportion of macro-aggregates increased, but that of micro-aggregates decreased. The manure treatments better improved the contents of SOC, STN, POC, and MBC in different aggregates. The content of SOC in aggregates increased with increased aggregate size, which was because the larger aggregates were formed by the binding of the smaller aggregates and organic matter. PON significantly increased in NM and NPM, and MBN was more sensitive to the addition of N fertilizer. However, the C/N ratio in bulk soil and aggregates decreased with fertilization. The improved soil structure was mainly due to the increased SOC and STN, which was proved by the positive correlation among SOC and STN with macro-aggregates and MWD. A correlation analysis also showed that the contribution rate of SOC and STN in macro-aggregates was positively correlated with aggregate size and stability, while it showed a negative relationship with micro-aggregates. Therefore, the sequestration of C and N fractions in soil was closely related to the aggregate size and was mainly affected by the larger aggregates. The chemical fertilizer plus manure could enhance the ability of the soil to supply nutrients and increase crop yield better than the chemical fertilizer by improving soil structure and fertility.

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