Soil organic carbon dynamics under long-term fertilization in a black soil of China: Evidence from stable C isotopes

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Effects of different fertilizers on organic carbon (C) storage and turnover of soil fractions remains unclear. We combined soil fractionation with isotope analyses to examine soil organic carbon (SOC) dynamics after 25 years of fertilization. Five types of soil samples including the initial level (CK) and four fertilization treatments (inorganic nitrogen fertilizer, N; balanced inorganic fertilizer, NPK; inorganic fertilizer plus farmyard manure, MNPK; inorganic fertilizer plus corn straw residue, SNPK) were separated into four aggregate sizes (>2000 μm, 2000–250 μm, 250–53 μm, and <53 μm), and three density fractions: free light fraction (LF), intra-aggregate particulate organic matter (iPOM), and mineral-associated organic matter (mSOM). Physical fractionation showed the iPOM fraction of aggregates dominated C storage, averaging 76.87% of SOC storage. Overall, application of N and NPK fertilizers cannot significantly increase the SOC storage but enhanced C in mSOM of aggregates, whereas MNPK fertilizer resulted in the greatest amount of SOC storage (about 5221.5 g C m⁻²) because of the enhanced SOC in LF, iPOM and mSOM of each aggregate. The SNPK fertilizer increased SOC storage in >250 μm aggregates but reduced SOC storage in <250 μm aggregates due to SOC changes in LF and iPOM.

Agro-ecosystem represents around 40% of all land on earth1, which is critical for maintaining agricultural sustainability, environmental stability, and long-term terrestrial carbon (C) sequestration2,3. Soil organic carbon (SOC) plays an important role in cycling plant nutrients, increasing grain yield and improving the physical, chemical properties of soils4. Fertilization as an agricultural management strategy, is being used to promote soil C storage5,6, which could directly or indirectly increase the SOC inputs and thereby change the availability of nutrients and soil turnover7. For instance, inorganic nitrogen (N) fertilizer may indirectly enhance the SOC storage by increased crop residue input to soils5,7, whereas application of organic manure could influence soil organic matter (SOM) owing to the directly inputs of processed organic materials to soils8–11. In contrast, application of organic fertilizers would result in a higher level of soil C and N mineralization than inorganic fertilizers8,10,12.

Some studies have shown that inorganic fertilizers had important effects on crop yield but no significant effect on SOM or SOC8,12,13. However, the combined application of inorganic and organic fertilizers has been shown to improve SOM better than the simple addition of inorganic fertilizer8,12,14. To date, positive5,15,16, negative4, and no obvious effects8,12,13 have been reported under fertilization on soil C sequestration for agro-ecosystems. The above controversy may be explained by the fact that increased SOM input resulting from fertilizers may be offset by the soil C loss from various soil fractions, resulting in zero accumulation of SOC, even a negative deficit6,17. Therefore, insight is urgently needed into soil C dynamics under long-term fertilization.

Generally, soil C turnover mainly depends on the interplay between organic inputs (e.g. plant debris, organic fertilizer) and decomposition of SOM with positive, neutral, or even negative response to fertilizer additions18. Thus, understanding the changes in new soil C inputs and the decay rate of old C will be essential to revealing soil C dynamics under long-term fertilization. Originally, the source and turnover rates of SOC can be assessed since δ13C values of C3 (δ13C ca. −28‰) and C4 (δ13C ca. −12‰) vegetation are distinctly different because of their differences in utilizing C isotopes2,19. The relative contribution of new SOC vs. old SOC can be estimated
Table 1. Soil physicochemical properties (0–20 cm) and plant biological traits under long-term fertilization in Gongzhuling, Jilin Province, China. Data are expressed as mean ± SE, n = 3. Different letters indicate statistical significance at P < 0.05 among fertilization treatments. Abbreviations: TC, total carbon; TN, total nitrogen; SOC, soil organic carbon; BD, bulk density; N, inorganic N fertilizer; NPK, balanced inorganic fertilizers of N, P and K; SNPK, balanced inorganic fertilizers plus corn straw residue; MNPK, balanced inorganic fertilizers plus farmyard manure; CK, initial soil.

| Treatments | TC (g kg⁻¹) | TN (g kg⁻¹) | SOC (g kg⁻¹) | BD (g cm⁻³) | pH | δ¹³C (%e) - Corn | C:N ratio – Corn |
|------------|-------------|-------------|--------------|-------------|----|----------------|-----------------|
|            | Leaf        | Roots       | Leaf         | Roots       |
| N          | 15.87 ± 0.72b | 1.85 ± 0.06e | 14.49 ± 1.25c | 6.3⁰ | −14.84 ± 0.23a | −13.35 ± 0.17a | 15.39 ± 2.13e | 24.44 ± 3.67m |
| NPK        | 14.59 ± 1.53e | 1.84 ± 0.04e | 14.16 ± 1.69b | 6.4⁰ | −13.36 ± 0.25b | −14.21 ± 0.21a | 16.17 ± 0.86m | 28.33 ± 2.19m |
| MNPK       | 23.47 ± 2.64e | 2.87 ± 0.34e | 22.40 ± 1.17c | 7.4⁰ | −16.23 ± 0.34a | −14.35 ± 1.16c | 19.65 ± 1.43e | 28.45 ± 4.09m |
| SNPK       | 17.04 ± 0.28e | 2.15 ± 0.09b | 16.79 ± 1.06c | 7.7⁰ | −14.76 ± 0.56a | −12.67 ± 0.33e | 19.09 ± 3.08e | 29.56 ± 2.01m |
| CK         | 16.50 ± 1.23c | 2.11 ± 0.27c | 16.14 ± 0.60a | 7.6⁰ | −12.67 ± 0.33e | −14.35 ± 1.16c | 16.23 ± 0.86m | 24.44 ± 3.67m |

Results

The soil physicochemical properties and plant biological traits. The soil total C and N, SOC content was greater in MNPK- and SNPK-treated soils and lower in N- and NPK-treated soils than in the initial level (CK) (Table 1). The soil bulk density was significantly higher in N- and NPK-treated soils than in MNPK- and SNPK-treated soils and CK, whereas soil pH had an opposite tendency with the lowest pH values (pH = 6.3 and 6.4) in N- and NPK-treated soils (Table 1). The δ¹³C values of the leaf and roots varied from −13.36‰ to −16.23‰ and from −12.67‰ to −14.35‰, respectively, in the corn-planted field, which was typical of C₄ plants (Table 1). The C:N ratios in the leaf and roots of the corn decreased in the following order: SNPK > MNPK > NPK > N-treated soils (Table 1).

Size distribution, SOC storage, and δ¹³C of soil aggregates. Long-term fertilization cannot significantly affect the portioning of aggregate distribution across the fertilizer treatments except for <53 μm aggregates (Fig. 1). Overall, application of organic and inorganic fertilizers increased the weight distribution of <53 μm size fraction compared with CK (Fig. 1). In general, the aggregate distribution was dominated by macroaggregates (2000–250 μm; 48.31–64.10%) across all the fertilizer treatments. Overall, long-term application of N and NPK fertilizers resulted in no remarkable increases in SOC storage across all aggregates compared with CK, except for 2000–250 μm aggregates in N-treated soils (Table 2). Long-term fertilization increased the SOC storage by an average of 466.0 g C m⁻² in all aggregates. The SNPK fertilizer increased SOC by an average of 191.1 g C m⁻² in macroaggregates (>250 μm) but decreased it by an average of 131.4 g C m⁻² in microaggregates (250–53 μm) compared with CK (Table 2). Besides, the SOC storage showed a decrease in 250–53 μm aggregates compared with other aggregate sizes in the fertilized soils except for MNPK treatment. Generally, the SOC storage in macroaggregates (>250 μm) was greater than in microaggregates (<250 μm) across the fertilizer treatments (Table 2). Long-term fertilization resulted in no significant changes in the C:N ratios across all the aggregate sizes (Fig. 2). The δ¹³C values of all of the aggregates were less negative in the fertilized soils than in CK due to the C₄ residue inputs, whereas the least negative δ¹³C values appeared in SNPK-treated soils (Table 3). Overall, the least negative δ¹³C values were found in 250–53 μm aggregates across all the aggregate sizes (Table 3).
Density fractionation: SOC storage and δ¹³C in LF, iPOM and mSOM. The iPOM stored the largest C fraction of the total SOC pool across all the aggregate sizes in the fertilized soils, which accounted for 80.79–90.32% in >2000 μm and 250–53 μm aggregates, and as well as accounted for 49.59–63.89% in 2000–250 μm aggregates (Table 2). The mSOM accounted for the smallest fraction (1.54–4.92%) of total organic C in >2000 μm aggregates, whereas the LF accounted for the smallest fraction (4.10–6.74%) of total organic C in 250–53 μm aggregates (Table 2). The greatest SOC storage in the LF, iPOM, and mSOM of all the aggregates was found in NPK-treated soils (Table 2). The SNPK fertilizer obviously enhanced SOC storage mainly in LF and iPOM of macroaggregates (>250 μm) but decreased SOC in LF and iPOM of microaggregates (<250 μm) (Table 2). Additionally, inorganic N and NPK fertilizers significantly increased the SOC storage in mSOM of all the aggregates. Moreover, inorganic fertilizers increased the LF in >2000 μm aggregates but decreased it in 250–53 μm aggregates (Table 2). No significant changes of SOC storage in iPOM were found in N- and NPK-treated soils compared with CK across all aggregate sizes (Table 2). The higher C:N ratios occurred in LF while the lower C:N ratios occurred in mSOM among soil density fractions across all fertilization treatments (Fig. 3). Long-term fertilization led to less negative δ¹³C values in LF, iPOM, and mSOM compared with CK, and the least negative δ¹³C values were found.
Soil C turnover. Long-term fertilization stimulated both new C inputs and decay rate of old C in soil fractions (Table 4). The new C inputs into all of the aggregates were greatest in SNPK-treated soils followed by MNPK-treated soils (Table 4). Accordingly, the fastest decay rates of old C were found in SNPK-treated soils across all aggregate sizes (Table 4). Overall, the greatest new C inputs into the soil aggregates and the fastest C decay rates were found in 250–53 μm aggregates (Table 4). The new C inputs were greater in mSOM than LF and iPOM in macroaggregates (>250 μm), whereas the fastest soil C turnover occurred in mSOM of 2000–250 μm aggregates (Table 4).

Discussion
Our stable isotope analysis confirmed that the abundance of δ13C in SOM fractions in the fertilized soils was more enriched than in CK (Table 3), resulting from a higher contribution of C3 residues. Overall, we found that the greatest SOC storage was found in MNPK-treated soils, followed by SNPK and then by inorganic fertilizers across all the aggregates (Table 2), which fully supported our previous study and others. Furthermore, the
SOC storage in macroaggregates (>250 μm) was greater than in microaggregates (<250 μm) overall across the fertilization treatments (Table 2), suggesting that the presence of macroaggregates (>250 μm) is usually associated positively with SOC content as an important component for C sequestration in soils30. In our study, the aggregate distribution was dominated by macroaggregates (2000–250 μm; 48.31–64.10%); meanwhile, the iPOM accounted for the largest C fraction (76.87% on average) of the total SOC pool across all the treatments (Table 2). Thus, we may draw the conclusion that 25 years of fertilization significantly increased the SOC storage, mainly by enhancing the soil C of the macroaggregates (2000–250 μm) with most of the C and N stored in the iPOM in the black soils of northeast China.

The SOC storage showed a decrease in 250–53 μm aggregates in the fertilized soils except for the MNPK treatment, maybe due to the fastest decay rates of old C in 250–53 μm fractions among aggregate sizes17 (Tables 2 and 4). Moreover, the SOC storage in LF with a less stable fraction was susceptible to be decomposed by microorganisms, and indeed maintained the same trend as that in 250–53 μm aggregates (Table 2). The findings suggested that despite the better physically protection against soil C decomposition in microaggregates, SOC within microaggregates may be susceptible to microbial breakdown6. Additionally, higher C:N ratios of LF reflected more recent litter inputs, while mSOM with much lower C:N ratios suggested decreasing C:N ratios in soil C fractions have been associated with increasing SOM decomposition and mineral association23,24 (Fig. 3). Indeed, the decay rates of old C were relatively fast in the mSOM across all the fractions in our study (Table 4). Overall, the C decay rates were relatively slow in N- and NPK-treated soils than in MNPK- and SNPK-treated soils (Table 4), indicating that application of organic fertilizers combined with inorganic fertilizers would accelerate the soil C turnover rate when compared with the addition of inorganic fertilizers alone4,29.

Overall, we found that there were no significant changes in SOC storage across all the aggregates relative to CK except for in the 2000–250 μm aggregates in N-treated soils after long-term application of N and NPK fertilizers (Table 2), which indicated that long-term N and NPK fertilizers decreased the SOC content, but significantly increased soil density in the 0–20 cm layer31 (Table 1). This result confirmed previous studies that 25 years of continuous inorganic fertilization was not capable of increasing the total SOC relative to the control8,10. Previous study showed this occurred for the two reasons as stated below29. First, inorganic N and NPK fertilizers were insufficient for preserving SOC levels under conventional management due to no above-ground crop residues returning to soil, although inorganic fertilizers may indirectly enhance SOM by increasing plant biomass production and C return to soils34. Second, the simple addition of inorganic N and NPK fertilizers lead to the soil acidification13, which correspondingly affected soil microbial activity, microbial biomass C and thus affected the SOC pool32 (Table 1). Generally, mSOM was shown to be a major sink for C storage with a more stable fraction because of the presence of a more recalcitrant component26. Soil density fractionation revealed that soil C storage was greater in mSOM fractions of each aggregate in N- and NPK-treated soils than in CK (Table 2), suggesting that soil inorganic N input may stabilize soil C in heavier fraction to a certain extent33,34. Additionally, we found that inorganic N and NPK fertilizers cannot increase the SOC storage in microaggregates (<250 μm), probably because of the offsetting effects of enhanced the mSOM and decreased the LF in microaggregates. The above findings further supported the previous analysis, which showed that no apparent changes in SOC storage of total organic pools occurred in N- and NPK-treated soils, mainly owing to the offsetting effects between enhanced SOC in the recalcitrant pool and decreased SOC in the labile pool33.
In contrast, a long-term application of MNPK fertilizer resulted in the largest soil C storage (about 5221.5 g C m⁻²) among fertilization treatments, which strongly increased the SOC storage on average by 1863.9 g C m⁻² at the black soil region of northeast China (Table 2), which further supported the evidence that long-term addition of manure significantly increased SOC content, regardless of combining inorganic fertilizers or not⁸. The SOC storage in MNPK-treated soils was fully coincided with our previous study that corn straw combined with inorganic fertilizers could accelerate the soil C turnover, largely through various sizes of soil aggregates including macroaggregates and microaggregates (Table 4). Furthermore, the positive effect of organic C in response to the MNPK fertilizer addition was beneficial for the accumulation of SOC and labile organic C content compared with the MNPK-treated soils was related to the increased SOC in both recalcitrant and labile pools ²⁹. In present study, soil density fractionation further confirmed that corn straw combined with inorganic fertilizers (SNPK fertilizer) could accelerate the SOC storage in both recalcitrant old C and labile SOM pool at the same site¹⁰. Whereas our previous analysis by chemical fractionation showed the enhanced organic C pool in MNPK-treated soils was related to the increased SOC in all density fractions (LF, iPOM, and mSOM) of each aggregate (Table 2).

Table 4. New C input (fnew) and decay rate (k, yr⁻¹) of soil C in aggregate size classes and density fractions (0–20 cm) under long-term fertilization. Data are expressed as mean ± SE, n = 3. Different letters indicate statistical significance at P < 0.05 among fertilization treatments. Abbreviations: LF, Light fraction; iPOM, intraaggregate POM; mSOM, mineral associated SOM; The abbreviations for fertilization treatments are the same as presented in Table 1.

| Fractions | N | NPK | MNPK | SNPK | N | NPK | MNPK | SNPK |
|-----------|---|-----|------|------|---|-----|------|------|
| >200 μm   | 19.84 ± 3.66  | 21.55 ± 2.81  | 21.16 ± 1.43  | 30.49 ± 4.09  | 0.009 ± 0.002  | 0.006 ± 0.001  | 0.010 ± 0.001  | 0.015 ± 0.002  |
| LF        | 9.66 ± 1.58   | 21.90 ± 1.98  | 12.85 ± 2.03  | 36.33 ± 5.09  | 0.004 ± 0.001  | 0.011 ± 0.002  | 0.006 ± 0.002  | 0.018 ± 0.004  |
| iPOM      | 15.89 ± 1.75  | 21.61 ± 3.42  | 21.45 ± 2.44  | 26.45 ± 6.52  | 0.007 ± 0.001  | 0.005 ± 0.001  | 0.010 ± 0.001  | 0.012 ± 0.003  |
| mSOM      | 16.09 ± 2.60  | 49.33 ± 9.79  | 61.99 ± 14.07 | 71.76 ± 18.87 | 0.007 ± 0.002  | 0.029 ± 0.005  | 0.058 ± 0.012  | 0.063 ± 0.011  |
| 2000–250 μm| 11.94 ± 2.53  | 4.88 ± 0.20   | 13.58 ± 1.65  | 23.99 ± 3.97  | 0.005 ± 0.001  | 0.002 ± 0.001  | 0.006 ± 0.001  | 0.011 ± 0.002  |
| LF        | 13.81 ± 2.69  | 10.73 ± 2.84  | 16.27 ± 3.06  | 17.68 ± 3.13  | 0.002 ± 0.002  | 0.001 ± 0.001  | 0.007 ± 0.002  | 0.008 ± 0.002  |
| iPOM      | 24.99 ± 2.97  | 15.08 ± 1.56  | 26.78 ± 4.74  | 28.38 ± 6.94  | 0.012 ± 0.003  | 0.007 ± 0.001  | 0.013 ± 0.002  | 0.013 ± 0.002  |
| mSOM      | 58.50 ± 5.70  | 68.39 ± 6.22  | 80.05 ± 14.91 | 76.64 ± 5.23  | 0.035 ± 0.006  | 0.046 ± 0.001  | 0.074 ± 0.016  | 0.059 ± 0.009  |
| 250–53 μm | 23.51 ± 0.99  | 30.61 ± 0.47  | 54.74 ± 3.42  | 83.35 ± 16.23 | 0.011 ± 0.000  | 0.015 ± 0.000  | 0.032 ± 0.003  | 0.087 ± 0.025  |
| LF        | 10.81 ± 1.81  | 3.77 ± 0.90   | 14.88 ± 0.69  | 5.82 ± 1.50   | 0.005 ± 0.001  | 0.002 ± 0.000  | 0.006 ± 0.000  | 0.002 ± 0.000  |
| iPOM      | 11.81 ± 2.62  | 12.49 ± 2.38  | 19.67 ± 2.43  | 22.66 ± 1.25  | 0.005 ± 0.001  | 0.005 ± 0.001  | 0.009 ± 0.001  | 0.010 ± 0.001  |
| mSOM      | 23.12 ± 4.65  | 8.79 ± 1.79   | 18.14 ± 3.95  | 18.23 ± 2.55  | 0.011 ± 0.002  | 0.004 ± 0.001  | 0.008 ± 0.002  | 0.008 ± 0.002  |
| <53 μm    | 11.90 ± 2.44  | 4.39 ± 0.04   | 16.89 ± 3.63  | 18.02 ± 2.45  | 0.005 ± 0.001  | 0.002 ± 0.001  | 0.007 ± 0.002  | 0.008 ± 0.001  |

The previous studies showed that short-term (e.g. 2–4 years) straw return treatment combined with inorganic fertilizer addition was beneficial for the accumulation of SOC and labile organic C content compared with the no straw addition treatment at the top soil⁷,⁸,⁰. Our present results further revealed long-term SNPK fertilization eventually increased SOC storage by an average of 191.1 g C m⁻² in macroaggregates (>250 μm) compared with CK, mainly because of the enhanced organic C in LF and iPOM, while it reduced SOC storage in microaggregates (<250 μm), mainly due to the decreased C in LF and iPOM (Table 2). Straw was a low-quality organic resource with a high C:N ratio, and thus has a slow decomposition rate⁶,⁹. In fact, the fastest decay rates of old C were found in SNPK-treated soils across all the aggregates (Table 4), which was inconsistent with previous studies that slow aggregate turnover had been observed with low-quality organic resources⁴⁰. However, the above findings fully coincided with our previous study that corn straw combined with inorganic fertilizers could accelerate the soil C turnover, and thus result in a larger new C input and faster decay rate of old C compared with the simple addition of inorganic fertilizers or straw alone⁴⁰. This is because that straw decomposed slowly, but the addition of N fertilizers could negate some effects of this type of low-quality organic resource⁹. Our present physically fractionation further confirmed that corn straw combined with inorganic fertilizers (SNPK fertilizer) could accelerate the soil C turnover, largely through various sizes of soil aggregates including macroaggregates and microaggregates (Table 4).

To conclude, we build on our previous findings and utilize the natural abundance of δ¹³C together with soil physical fractionation technique to evaluate dynamics in the SOC fractions after 25 years of fertilization. The present results further confirmed the previous study conducted by soil chemical fractionation technique²⁹ and suggested that long-term application of fertilization could alter the SOC storage, consequently affecting the dynamics of soil C pools in agro-ecosystem. These findings will be helpful for monitoring long-term C accumulation through ecosystem processes under agricultural management practices in a black soil of Northeast China.
Materials and Methods

Study area. A long-term fertilization experiment presented for monitoring black soil fertility and fertilizer efficiency with monoculture maize (Zea mays L.) has been conducted since 1989 at Goungshuling, Jilin Province, China (124°48′33″E, 43°30′23″N)10,31. This region has a north temperate and semi-humid climate with an annual average temperature of 5.6 °C. The annual precipitation is approximately 562 mm, 80% of which falls between June and September31. The soil is a clay loam [Typic Hapludoll (Mollisol) in USDA Soil Taxonomy] developed from Quaternary loess-like sediments with 39% sand, 30% silt and 31% clay at the beginning of the experiment10. A randomized complete block design was used with three replicates in this long-term experiment with each replicate plot covered 130 m². The experiment included five treatments: (1) Initial soils (CK); (2) inorganic nitrogen fertilizer at the rate of 165 kg N ha⁻¹ (N); (3) balanced inorganic fertilizers at 165 kg N ha⁻¹, 82.5 kg P₂O₅ ha⁻¹, and 82.5 kg K₂O ha⁻¹ (NPK); (4) balanced inorganic fertilizers at 50 kg N ha⁻¹, 82.5 kg P₂O₅ ha⁻¹, and 82.5 kg K₂O ha⁻¹ plus farmyard manure at the rate of 2.3 × 10⁵ kg ha⁻¹ (MNPK), and (5) balanced inorganic fertilizers at 112 kg N ha⁻¹, 82.5 kg P₂O₅ ha⁻¹, and 82.5 K₂O kg ha⁻¹ plus corn straw residue at the rate of 7.5 × 10⁵ kg ha⁻¹ (SNPK)31,41. The N contents in corn straw and farmyard manure were 7.0 and 5.0 g kg⁻¹, respectively, and thus the total N application rates for N, NPK, SNPK, and MNPK treatments were kept at 165 kg ha⁻¹ (dry weight basis)31. The organic C content of farmyard manure (mostly, pig manure) was about 112 g kg⁻¹31, the δ¹³C of farmyard manure was measured with an average value of −21.59‰. The sources of inorganic N, P, and K fertilizers were urea, triple superphosphate (TSP) and muritate of potash (MoP)31. One third of the urea and total amounts of TSP and MoP were applied as a basal dose. The application of fertilizers was approximately 10 cm of soil depth. The remaining two thirds of the urea was used for side dressing at the corn jointing stage, whereas the chopped corn straw was also applied in the SNPK plots with the top 25 cm of soil at that time every year. The farmyard manure was applied in the MNPK plots after corn harvesting in autumn each year. Corn was sown in late April and harvested in late September. Aboveground plant residues were removed at harvest31. Prior to the long-term experiment, the field had been continuously cultivated corn for some years, and then was homogenized by growing corn for 3 years without fertilizer application31. The soil physiochemical properties (pH, bulk density, C and N content) were shown in Table 1. The pH and bulk density of soil were measured as previously described by Song et al. (2015)31.

Field sample collection and soil fractionations. In August 2014, we randomly placed three sub-plots (2 m × 2 m) around the corn rhizosphere within each treatment plot; the distances between the sub-plots were approximately 5 m. Soil samples (0–20 cm) from each treatment plot were collected using a 5-cm diameter stainless steel soil corer. Newly produced corn leaves were collected in each treatment plot. Root sampling blocks were the samples were centrifuged (1250 g) at 20 °C for 60 min. The floating material (light fraction-LF) was aspirated sides of the centrifuge tube were washed into the suspension with 10 ml of SPT. After 20 min of vacuum (138 kPa), the material remaining on the cap and sieve and the remaining material on the sieve, i.e., the intra-aggregate particulate organic matter (iPOM) while phosphate by shaking for 18 h on a reciprocal shaker. The dispersed heavy fraction was then passed through a 53-μm sieve and the remaining material (heavy fraction-HF) was rinsed twice with 50 ml of deionized water and dispersed in 0.5% sodium hexametaphosphate by shaking for 24 h at room temperature to remove any soil carbonates23. The C and N content of plant materials (leaves and roots) was measured by using a solution of 1.85 g cm⁻³ sodium polytungstate (SPT), following the methods described in Six et al. (1998)42. The δ¹³C values were measured for all soil fractions, plant materials and farmyard manure. The above oven-dried plant materials and collected soil samples were ground to pass through 20-mesh (0.84 mm) sieves. Subsamples from all soil fractions were treated with 1N HCl for 24 h at room temperature to remove any soil carbonates23. The C and N content of plant materials (leaves and roots), the whole soil and soil fractions were measured. The δ¹³C values were measured for all soil fractions, plant materials and farmyard manure. Subsamples of leaf, root, and soil fractions were weighed and analyzed using an isotope ratio mass spectrometer (Thermo Finnigen, Delta-Plus, Flash, EA, 1112 Series, USA). The carbon isotope ratio of the soil fractions and plant materials was expressed as follows:

\[
\delta^{13}C = \frac{X_{sample}}{X_{standard}} \times \left( \frac{X^h}{X^l} \right) - 1 \times 1000
\]

(1)

C content and C isotope analyses. The above oven-dried plant materials and collected soil samples were ground to pass through 20-mesh (0.84 mm) sieves. Subsamples from all soil fractions were treated with 1N HCl for 24 h at room temperature to remove any soil carbonates23. The C and N content of plant materials (leaves and roots), the whole soil and soil fractions were measured. The δ¹³C values were measured for all soil fractions, plant materials and farmyard manure. Subsamples of leaf, root, and soil fractions were weighed and analyzed using an isotope ratio mass spectrometer (Thermo Finnigen, Delta-Plus, Flash, EA, 1112 Series, USA). The carbon isotope ratio of the soil fractions and plant materials was expressed as follows:
where \( X \) is carbon, \( h \) is the heavier C isotope, and \( l \) is the lighter C isotope. The CO\(_2\) samples were analyzed relative to the internal working gas standards. The carbon isotope ratios (\( \delta^{13}C \)) are expressed as relative values to the Pee Dee Belemnite (\( \delta^{13}C = 0.0112372\% \)). The standards (acetanilide and spinach) were analyzed after every ten samples; the analytical precision of the instrument was \( \pm 0.13\% \) for \( \delta^{13}C \).

With respect to the plots of different fertilization treatments, the \( \delta^{13}C \) values of the SOM were used to calculate the proportion of new C (\( f_{\text{new}} \)), i.e. the C derived from current corn residuals or fertilizers) and of old C (\( f_{\text{old}} = 1 - f_{\text{new}} \), soil C previous to fertilization, i.e., C in the initial soil) with a mass balance equation \(^{22}\):

\[
f_{\text{new}} = \frac{\delta^{13}C_{\text{new}} - \delta^{13}C_{\text{old}}}{\delta^{13}C_{\text{veg}} - \delta^{13}C_{\text{old}}} \times 100\%
\]  

(2)

where \( \delta^{13}C_{\text{new}} \) is the \( \delta^{13}C \) values of organic C in soil fractions under fertilization, \( \delta^{13}C_{\text{old}} \) is the \( \delta^{13}C \) values of organic C from initial soils, i.e. the soil samples previous to fertilization, and \( \delta^{13}C_{\text{veg}} \) is the \( \delta^{13}C \) values of the mixed plant materials of corn; Specially, \( \delta^{13}C_{\text{veg}} \) is the \( \delta^{13}C \) values of the mixed sample including plant materials and manure in MNPK fertilizer treatment \(^{19,22}\).

Because the \( \delta^{13}C_{\text{veg}}, \delta^{13}C_{\text{new}} \) and \( \delta^{13}C_{\text{old}} \) are independently measured, the standard errors (SE) of \( f \) associated with the use of the mass-balance approach can be calculated using partial derivatives \(^{22}\) as follows:

\[
\sigma_f^2 = \left( \frac{\partial f}{\partial \delta^{13}C_{\text{veg}}} \sigma_{\delta^{13}C_{\text{veg}}}^2 + \left( \frac{\partial f}{\partial \delta^{13}C_{\text{new}}} \sigma_{\delta^{13}C_{\text{new}}}^2 + \left( \frac{\partial f}{\partial \delta^{13}C_{\text{old}}} \sigma_{\delta^{13}C_{\text{old}}}^2 \right) \right) \right)
\]  

(3)

This equation can be reduced to:

\[
\sigma_f^2 = \frac{1}{(\delta^{13}C_{\text{new}} - \delta^{13}C_{\text{old}})^2} \left[ \sigma_{\delta^{13}C_{\text{veg}}}^2 + f^2 \sigma_{\delta^{13}C_{\text{new}}}^2 + (1-f)^2 \sigma_{\delta^{13}C_{\text{old}}}^2 \right]
\]  

(4)

where \( \sigma_{\delta^{13}C_{\text{veg}}}^2, \sigma_{\delta^{13}C_{\text{new}}}^2 \) and \( \sigma_{\delta^{13}C_{\text{old}}}^2 \) represent the variances of the mean \( \delta^{13}C_{\text{veg}}, \delta^{13}C_{\text{new}} \) and \( \delta^{13}C_{\text{old}} \) respectively. The \( \sigma_f \) is the SE of the proportion \( f \) estimate \(^{22}\).

The decay rate constant (\( k \)) for the old C (i.e. the C of the organic matter before fertilization) of the soil fractions was calculated based on Cheng et al. (2011) \(^{23}\):

\[
\ln(f_{\text{old}}) = -kt
\]  

(5)

where \( f_{\text{old}} = (1 - f_{\text{new}}) \) is the proportion of old C, \( k \) is the net relative decay rate constant for old C, and \( t \) is the age of fertilization (i.e. for 25 years).

**Statistics.** The SOC content, C:N ratios, \( \delta^{13}C \) values, the new C input (\( f_{\text{new}} \)), and the decay rate (\( k \)) of the old C of the soil fractions for each treatment were calculated by averaging the three replicates for each sample plot. All of the data were examined for the normality by Kolmogorov–Smirnov test and for the homogeneity of variance by one-way analysis of variance (ANOVA). An analysis of variance (ANOVA) of multiple comparisons was conducted to examine the effects of various fertilization treatments on the on total C and N, bulk density and pH of the whole soil, and the SOC content, the \( \delta^{13}C \) values, the C:N ratios in all soil fractions, and the weight distribution (LSD; \( P = 0.05 \)). One-way ANOVA tests were performed to examine the differences in SOC content, \( \delta^{13}C \) value, weight distribution of the soil fractions, new C input (\( f_{\text{new}} \)), and the decay rate of the old C among fertilization treatments (LSD; \( P = 0.05 \)). All of the statistical analyses were performed using SPSS (version 16.0) and OriginPro (version 8.0) for Windows.

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**Author Contributions**

X.L.D., W.Z. and P.Z. designed the research. X.L.D. conducted the experiments, analysed the data, and drafted the manuscript. P.H. and W.Z. helped interpret the results of the study.

**Additional Information**

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