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Soil organic carbon fractions comparison after 40-year long-term fertilisation in a wheat-corn rotation field

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Abstract: Several experimental methods have been developed to fractionate soil organic carbon (SOC) into functional sub-pools. However, which fractions had the potential to better reflect the SOC dynamics responding to fertilisation are still under discussion. Thus, we compared different SOC fractions (microbial biomass carbon, MBC; dissolved organic carbon, DOC; permanganate-oxidisable carbon, POXC; particle organic carbon, POC, and aggregation organic carbon fractions) and the soil respiration rate in a wheat-corn rotation field after 40 years of manure and N fertilisation in North China to search for the most sensitive SOC fractions to fertilisation. Manure increased the organic carbon (OC) contents of all the soil fractions (26.5 to 362.8%) and the POC (18.0 to 43.7%) and macro-aggregation percentages (3.0 to 4.4%), which indicated an increasing physical-protected aggregated OC fraction. N fertilisation alone slightly increased the OC contents of all the soil fractions and DOC percentage, but decreased the macro-aggregation OC percentage, which suggests the increasing possibility that the SOC is exposed to microbial communities causing a decreasing aggregation formation. However, when a high level of both the manure and N fertiliser were applied, the excessive N in the soil stimulates the soil microbial activity and decreases the SOC content comparing it to the same level of the manure fertiliser addition.

Keywords: aggregation organic carbon; dissolved organic carbon; microbial biomass carbon; particle organic carbon; permanganate-oxidisable carbon

About three-quarters of the organic carbon (OC) in terrestrial ecosystems are stored in the soil (Smith et al. 2008), and agricultural ecosystems have been an important sink and source of atmospheric CO_2 (West & Post 2002). Therefore, understanding the soil organic carbon (SOC) dynamics responding to different agricultural management practices and seeking the optimal way to enhance the agricultural soil carbon (C) stock have always been a focus of research. Fertilisation (mineral or organic), as a common agricultural management practice to enhance the SOC sequestration, has been extensively investigated. Previous studies have reported both positive (Purakayastha et al. 2008; Gong et al. 2009; Zhu et al. 2015; Blanchet et al. 2016; Li et al. 2018) and no effects (Huang et al. 2010; Lou et al. 2011) of long-term fertilisation on the SOC content. These conflicts might be caused by both the fertilisation conditions (type, quantity, and application time) and the SOC state (Giacometti et al. 2021).

Thus, to help better predict the dynamics of SOC under different fertilisation schemes, several phys-

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ical and chemical methods have been developed to fractionate SOC into functional sub-pools (von Lützow et al. 2007). For example, using chemical fractionation procedures based on SOC hydrophilicity could help in separating the dissolved organic carbon (DOC) fraction (Marschner & Kalbitz 2003); using the chloroform-fumigation method could help in separating the microbial biomass carbon (MBC) fraction (Brookes & Jenkinson 1987); using chemical fractionation procedures based on soil organic matter (SOM) resistance to potassium permanganate ($KMnO_4$) could help in separating the permanganate-oxidisable carbon (POXC) fraction (Culman et al. 2012); using physical fractionation procedures based on wet sieving with or without dispersion would help in separating the particle organic carbon (POC) (Six et al. 2002); and using the physical SOC fractionation method based on the aggregation size would help in separating several different aggregation factions (e.g., macro-aggregation with a diameter > 250 μm and micro-aggregation with a diameter 53–250 μm) (Gupta & Germida 2015).

However, which fractionation methods or which SOC fractions are "the best" are still under discussion; and more importantly, how to define "the best"? Thus, although several research studies have been undertaken on how fertilisation affects the different SOC physical and chemical fractions (Hai et al. 2010; Huang et al. 2010; Chaudhary et al. 2017; Li et al. 2018; Yang et al. 2018; Balík et al. 2020), few research studies have focused on the sensitivity of different SOC fractions to the fertilisation and its relationship with the SOC pool dynamics after long-term fertilisation use. To fill this gap, we compared different soil organic carbon fractions (MBC, DOC, POXC, POC, and aggregation OC fractions) in a 40-year long-term manure and nitrogen (N) fertilisation experiment in North China and attempted to answer the following questions:

(1) How does long-term organic and inorganic fertilisation influence the SOC content.

(2) Which were "the best" SOC fractions that are most sensitive to the fertilisation and could better reflect the SOC pool dynamics under the long-term use of manure and N fertilisation.

MATERIAL AND METHODS

Site and samples. The study site is in the Laiyang long-term field experiment station of Qingdao Agricultural University in Laiyang, Shandong Province, China (36.9°N, 120.7°E). The climate in this location is a warm temperate sub-humid monsoon climate with a mean annual temperature of 11.2 °C and a mean annual precipitation of 779.1 mm. The soil type is fluvo-aquic with a texture of light loam. This experiment started in 1978 with a total nine fertilisation treatments, interactive fertilisation with three levels of manure addition (M_0 for no manure fertiliser addition, M_1 for 30 t/ha of manure fertiliser addition, M_2 for 60 t/ha of manure fertiliser addition) and three levels of nitrogen fertiliser addition (N_0 for no N fertiliser addition, N_1 for 138 kg/ha of N fertiliser addition, N_2 for 276 kg/ha of N fertiliser addition). Moreover, at that time, the 0 to 20 cm soil layer had 4.10 g/kg of organic matter, 0.50 g/kg of total N, 0.46 g/kg of total P and a pH of 6.8. Each treatment was repeated three times in a randomised block design with 33.3 m^2 of each experimental plot; and a 1.0 m glass plate was used to separate each plot. A winter wheat-summer maize rotation was carried out in the experimental flied, and the winter wheat season was usually from October to June the following year, while the summer maize season was usually from June to October after the wheat harvest. Conventional tillage was carried out in the experimental field, and both the wheat and maize straw were removed after harvest.

The manure fertiliser was pig manure with a water content of 70–75%, total N content of 2–3 g/kg, total P content of 0.5–2 g/kg and an organic matter content of 20–50 g/kg. The manure was used as a base fertiliser and applied into the soil during the sowing period of the winter wheat, and fresh pig slurry was broadcasted and ploughed into soil after fermenting for one week. The N fertiliser used urea, and the urea was applied into the soil at the sowing period and rising stage of the winter wheat and jointing stage and bell stage of the summer maize.

Soil samples (the 0–20 cm soil layer) were collected with a stainless-steel soil sampler (3 cm inner diameter) on April 20th, May 8th, May 26th and June 18th during the wheat season and on August 15th, August 30th, September 15th and October 1st during the maize season in 2018. The soil temperature was also measured as the same time. In each plot, twenty-four individual soil cores were mixed to form a single composite sample, and all the soil samples were kept in sealed bags before being taken back to the laboratory within 2 h, and the gravel, roots, and large organic residue were manually removed before sieving by a 2 mm sieve. Each sample was separated into two parts: one part was stored at –20 °C for the microbial biomass carbon analysis, and the other part was air-dried for the soil physicochemical analyses. At harvest, plant samples were collected, and the panicles were hand-threshed and oven-dried at 80 °C until a constant weight was obtained to determine the grain yield.

SOC fractionation methods. The DOC, POC, POXC, MBC and total soil organic carbon content were determined for all the samples from of each sampling date, except the MBC concentration on May 8th and May 26th. The OC content of the aggregation fractions were determined using samples from October 1st.

For the DOC fractionation, 10 g of the air-dried soil sample was added into 50 mL of deionised water and was shaken for 30 min at 200 rpm, the suspension was then centrifuged at 4 000 rpm for 30 min and filtered through a 0.46 μm pore polycarbonate filter. The solutions were analysed to get the DOC concentration. For the POC fractionation, 20 g of the air-dried soil sample was added into 100 mL of 0.5 mol/L sodium hexametaphosphate and was shaken for 18 h at 90 rpm, the suspension was then filtered through a 53 μm sieve. After repeatedly being washed by deionised water, the particles left on the sieve were collected, dried at 60 °C and weighed. The soil MBC concentration was measured by the fumigation-extraction method, using 0.5 mol/L K_2SO_4 as the extracting agent (Brookes & Jenkinson 1987). The soil aggregation fractions was determined by a wet sieve. One hundred grams of the air-dried soil was placed in a series of automatic vibrating sifters (from top to bottom: 2 000, 250 and 53 μm), deionised water was added to about 5 cm above the soil. After shaking for 5 min at 30 rpm, the fraction with a diameter of 250–2 000 μm (macro-aggregate), 53–250 μm (micro-aggregate) and < 53 μm were collected, dried at 50 °C and weighed. The OC concentration of the original soil samples, DOC, POC, MBC and each aggregation fraction were all analysed by the $K_2Cr_2O_7$-H_2SO_4 calefaction method (Nelson & Sommers 1982), which calculates the OC content according to the remaining specific concentration of potassium dichromate titrated by ferrous sulfate after it reacted with the SOC under an external heating condition (180 °C, boiling for 5 min). The POXC concentration determination was tested with an ultraviolet spectrophotometer (MAPADA V-1200, SH, Meipuda, Shanghai) at 565 nm using 2.5 g of the air-dried soil sample and 25 mL of a 333 mmol/L $KMnO_4$ stock solution (Culman et al. 2012).

The soil respiration rates of each sample date were tested by the AA method (Kirita 1971); 5 mL of a 0.5 M NaOH solution was placed in a small beaker in a chamber (18 cm in diameter, 30 cm in height) of each plot. The measuring periods were normally 24 h to avoid any daily influence on the soil respiration estimate. An NaOH solution was titrated with 0.1 mol/L HCl, and the soil respiration rate was calculated by the volume of the HCl used.

Statistical analyses. The contents of all the OC fractions were calculated by dividing the OC amount of each fraction (OC concentration multiplied by the dry weight) by the weight of the original soil sample. The percentages of all the OC fractions were calculated by dividing the OC contents by the total SOC content. To better understand the SOC output rate, the soil respiratory quotient was calculated by dividing the soil respiration rate by the MBC, and the ratio of the soil respiration rate and the SOC was also calculated. All the variables were evaluated by an analysis of variance (ANOVA) and a repeated measures analysis of variance (RMANOVA) with the manure and N fertiliser treatments as the main factors for each sampling time. Tukey's range test was used to separate the differences among the means. Pearson's correlation analysis was used to analyse the relationship of the soil temperature, crop yields and the other soil variables. All the data were analysed using R software (Ver. 3.6.1) (www.r-project.com) with a significance defined as $\alpha < 0.05$, and the figures were created in R 3.6.1 with the "ggplot2" and "fmsb" packages.

RESULTS AND DISCUSSION

Gain yield. The manure fertilisation significantly increased the wheat and maize yield (Table 1). The N fertiliser had no significant influence on the wheat and maize yield, but the manure and N fertiliser had a significant interactive influence on the wheat and maize yield. With no manure fertilisation treatments, the low N addition had the highest wheat yield, and the high N addition had the highest maize yield; with the low manure fertilisation treatments, the low and high N additions had the higher wheat yield than the no N addition treatment, and the low N addition had the highest maize yield; and with the high manure fertilisation treatments, the high N addition had the highest wheat and maize yield.

Soil organic carbon content of different fractions. Considering all the fertilisation treatments, the total SOC content of our experiment field was

Table 1. Wheat and maize yield of the different manure and nitrogen fertiliser treatments in 2018

Treatments	Wheat	Maize
	(kg/ha)	
M$_0$N$_0$	1 632 ± 254^{Cc}	3 087 ± 42^{Cc}
M$_0$N$_1$	3 765 ± 263^{Ca}	4 046 ± 251^{Cb}
M$_0$N$_2$	2 675 ± 271^{Cb}	5 276 ± 88^{Ca}
M$_1$N$_0$	5 484 ± 18^{Bb}	6 844 ± 423^{Bb}
M$_1$N$_1$	8 430 ± 401^{Ba}	7 986 ± 135^{Ba}
M$_1$N$_2$	8 714 ± 341^{Ba}	6 840 ± 435^{Bb}
M$_2$N$_0$	7 956 ± 34^{Ac}	8 188 ± 146^{Ab}
M$_2$N$_1$	9 342 ± 327^{Ab}	8 459 ± 608^{Aab}
M$_2$N$_2$	10 235 ± 210^{Aa}	9 159 ± 48^{Aa}

N$_0$ – no N fertiliser addition; N$_1$ – low amount of N fertiliser addition; N$_2$ – high amount of N fertiliser addition; M$_0$ – no manure fertiliser addition; M$_1$ – low amount of manure fertiliser addition; M$_2$ – high amount of manure fertiliser addition; values are means (n = 3), with standard error; different capital letters represent significant differences among the different manure fertilisation treatments with the same nitrogen fertilisation, different lowercase letters represent significant differences among the different nitrogen fertilisation treatments with the same manure fertilisation ($P < 0.05$)

9.59 ± 0.30 g/kg for the whole year average. From high to low, the OC contents for the whole year average of the different fractions were 6.71 ± 0.19 g/kg soil for the macro-aggregation, 2.52 ± 0.09 g/kg soil for the POXC, 1.93 ± 0.19 g/kg soil for the micro-aggregation, 1.89 ± 0.08 g/kg soil for the POC, 0.941 ± 0.022 g/kg soil for the fraction < 53 μm, 0.373 ± 0.000 g/kg soil for the MBC, 0.242 ± 0.001 g/kg soil for the DOC (Figure 1A).

The POC was more closely related to the larger (250–2 000 μm) and lighter (< 1.7 g/cm^2) soil fractions predominated by carbohydrates (Christensen 2001; Stewart et al. 2007), but the POXC mainly consisted of aromatic and humic substances (Tirol-Padre & Ladha 2004; Culman et al. 2012), and the DOC mainly contained active OC fractions that could both be used by the soil microbial community or absorbed by the mineral particles (Marschner & Kalbitz 2003; Angst et al. 2021). Thus, POXC and DOC might more represent the chemical-protected or mineral-associated or unprotected microbial sourced soil carbon fraction, while POC might more represent the free or physically-protected OC fraction derived from the partly decomposed plant residue (Oades et al. 1987; Christensen 2001).

The sample time had significant influences on the OC contents of the total SOC and all the OC fractions. The POC content had similar variation trend with regards to the total SOC content, but the POXC and DOC contents were opposite to the SOC variation (Figure 1B). The higher total SOC and POC contents on Aug 30th and Apr 20th (Figure 1B) might be because the application of the nitrogen fertiliser on Apr15th and Aug 20th. These applications increased the OC input and aggregation formation by stimulating crop growth and root exudation (Christensen 2001; Gupta & Germida 2015). However, the extra OC input caused by the nitrogen fertiliser application gradually decomposed, thus, the chemical recalcitrant POXC and decomposed DOC gradually accumulated over

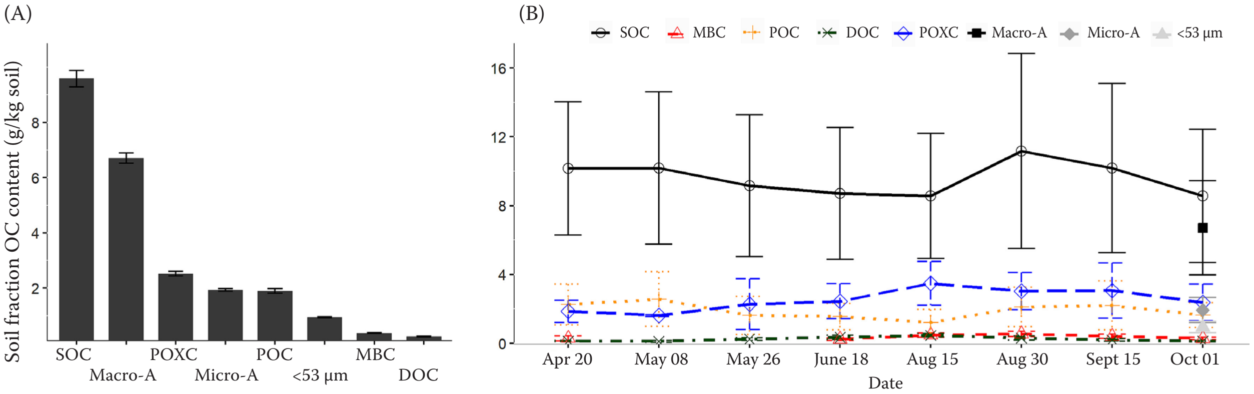

Figure 1. The mean organic carbon (OC) content of the soil organic carbon (SOC), microbial biomass (MBC), particle organic carbon (POC), dissolvable organic carbon (DOC), potassium permanganate oxidisable organic carbon (POXC), macro-aggregation (Macro-A), micro-aggregation (Micro-A) and soil fraction < 53 μm (<53 μm) for the whole year average (A); and each sample time (B)

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time, as a result, POXC and DOC had an opposite variation trend compared to SOC. Also, the MBC content was higher in August and September and lower in June, April and October (Figure 1B), which was likely affected by the soil temperature as the Pearson correlation analysis showed (Figure S3 in the Electronic Supplementary Material (ESM)).

Fertilisation influences on different SOC fractions and soil respiration rate. Manure could increase the soil C input directly by itself and indirectly by the increasing the crop biomass (Matsuda & Schnitzer 1972). In our experimental field, after 40 years of fertilisation, the manure fertilisation significantly increased crop growth (Figure S4 in ESM), the OC contents of the total soil and all the soil fractions and the soil respiration rate (Figure 2A and for more detial of soil repiration variation see Figure S1A in ESM). Compared to the no manure fertilisation, the low manure fertilisation increased the SOC, POC, POXC, DOC, MBC, macro-aggregation, micro-aggregation, fraction < 53 µm OC contents and the soil respiration rate by 126.2, 172.4, 68.5, 26.5, 51.3, 133.9, 103.5, 127.3 and 15.9%, respectively, while the high manure fertilisation increased them by 219.5, 362.8, 134.6, 35.6, 85.4, 205.3, 170.0, 151.7 and 30.3%, respectively. These results correspond

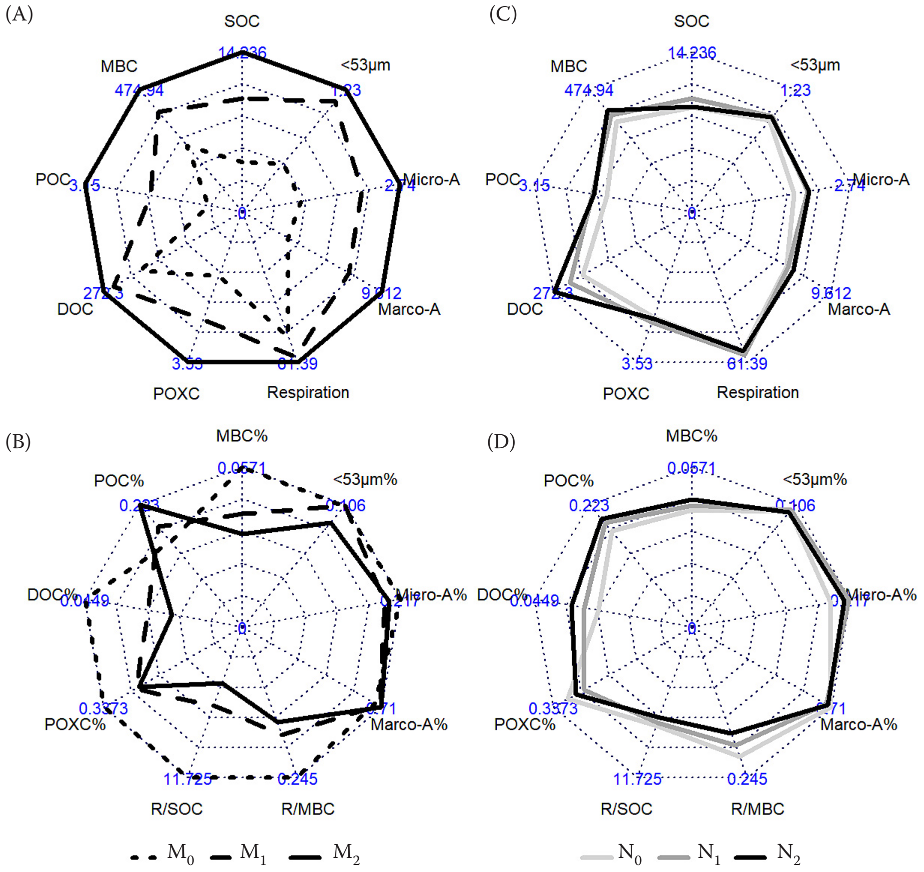

Figure 2. The mean content of the soil organic carbon (SOC), microbial biomass carbon (MBC), particle organic carbon (POC), dissolvable organic carbon (DOC), potassium permanganate oxidisable organic carbon (POXC), macro-aggregation (Macro-A), micro-aggregation (Micro-A), soil fraction < 53 µm (<53 µm) and soil respiration rate (Respiration) with the different levels of manure (A) and nitrogen fertiliser treatments (C); and the mean percentages of the MBC, POC, DOC, POXC, Macro-A, Micro-A, <53 µm, the ratio of the soil respiration rate and the MBC (soil respiratory quotient, R/MBC), and the ratio of the soil respiration rate and the SOC (R/SOC) with the different levels of the manure (B) and nitrogen fertiliser treatment (D)

Each axis ranges from 0 to the maximum value of the corresponding variable, the remaining values are plotted according to their proportion to the maximum value, the maximum value was drawn at the end of each axis in a blue colour

M_0 – no manure fertiliser addition; M_1 – low amount of manure fertiliser addition; M_2 – high amount of manure fertiliser addition; N_0 – no N fertiliser addition; N_1 – low amount of N fertiliser addition; N_2 – high amount of N fertiliser addition to other studies (Purakayastha et al. 2008; Gong et al. 2009; Chaudhary et al. 2017; Li et al. 2018). Moreover, the manure fertilisation significantly influenced the OC percentages of all the soil fractions, the soil respiratory quotient and the ratio of the soil respiration rate and the SOC (Figure 2A and for more detial of soil repiration variation see Figure S1A in ESM). However, compared to the no manure fertilisation, the low manure fertilisation increased the POC and macro-aggregation OC percentages by 18.0 and 3.0%, and the high manure fertilisation increased them by 43.7 and 4.4% (Figure 2B). Other studies also indicated that the manure application could increase the aggregation of the OC or POC content (Hai et al. 2010; Huang et al. 2010; Wang et al. 2015; Yang et al. 2018). This indicated that, in our field, the chemical-protected resistant OC sub-pool might asymptotically reach saturation after forty years of manure fertilisation (Stewart et al. 2007), and the manure fertilisation mainly increased the physical-protected aggregated OC content (dominated by the POC).

Although the manure fertiliser application could enhance the soil microbial biomass and soil respiration rate (Figure 2A), which indicated the increasing soil carbon output (Matsuda & Schnitzer 1972), the SOC output rate (the ratio of the soil respiration rate and the SOC) of the manure addition treatment was relatively lower than the no manure treatment (Figure 2B). Moreover, the relatively lower soil respiration quotient of the manure treatment (Figure 2B) also indicated that the microbial communities invested more carbon sources into the biomass growth than the metabolic activities under the manure fertilisation (Kaiser & Kalbitz 2012). As the microbial-derived smaller organic matter helps format the soil organo-mineral complexes, which further physically protects SOC from decomposing (Munoz et al. 2008); thus, the soil microbial organisms with the manure fertilisation help immobilise more carbon into the soil instead of mineralising it into the atmosphere.

The N fertiliser could only increase the soil C input indirectly by crop biomass improvement (Matsuda & Schnitzer 1972), thus, the influence of the N fertilisation on the SOC and its fractions in our study were not as great as the manure, similar to previous research studies (Hai et al. 2010; Huang et al. 2010; Yang et al. 2018). The N fertilisation significantly affected the OC contents of the SOC, DOC, POC, macro-aggregation, micro-aggregation and fraction < 53 μm. The SOC content was significantly higher in the low N addition treatment (10.11 g/kg compared to 9.37 for N_2 and 9.29 for N_0 treatments, Figure 2C), but the OC contents of the DOC, POC, macro-aggregation, micro-aggregation and fraction < 53 μm were all highest in the high N addition treatment (271.42 mg/kg soil, 2.00, 6.99, 2.03 and 0.92 g/kg soil) and lowest in the no N addition treatment (215.03 mg/kg soil, 1.72, 6.54, 1.77 and 0.95 g/kg soil, Figure 2C). Furthermore, the N fertilisation significantly affected the OC percentages of the POC, macro-aggregation and micro-aggregation, soil respiratory quotient and the ratio of the soil respiration rate and the SOC. The POC percentage was highest in the high N addition treatment (19.7%) and lowest in the no N addition treatment (17.5%); the macro-aggregation OC percentage and soil respiratory quotient were highest in the no N addition treatment (71.0% and 58.23 mg per m^2 h) and lowest in the low N addition treatment (68.4% and 56.45 mg per $m^2 \cdot h$); the micro-aggregation OC percentage was lowest in the no N addition treatment (19.0%) and highest in the low N addition treatment (21.5); the ratio of the soil respiration rate and the SOC was highest in the no N addition treatment (10.11 g/kg) and lowest in the high N addition treatment (9.29 g/kg); and although not significantly, the N fertilisation increased the DOC percentage (Figure 2D). As a readily bioavailable SOC fraction, DOC often represents a labile substrate for microbial activity (Burford & Bremner 1975; Marschner & Kalbitz 2003); Thus, our results indicated that although the N fertilisation could also increase the SOC content, it would further increase the possibility of the soil C being exposed to the microbial communities and decrease the formation of the aggregation.

Also, the OC contents of the SOC, macro-aggregation, micro-aggregation and fraction < 53 μm, the OC percentages of the POC, POXC, macro-aggregation, micro-aggregation and fraction < 53 μm, soil respiratory quotient and the ratio of the soil respiration rate and the SOC were significantly interactively affected by the manure and N fertilisation (Figure 3), while others were not (Figure S2 in the ESM). For the total soil and most fractions except for the POC, the N addition usually increased their OC contents with the no manure treatment, but decreased their OC contents with the low or high manure treatment (Figure 3). These results imply that when only N or the manure fertiliser was applied, the crop productivity increased and, thus, increased the soil C content; however, when a high amount of both manure and N fertiliser were applied, the excessive N in the soil would stimulate

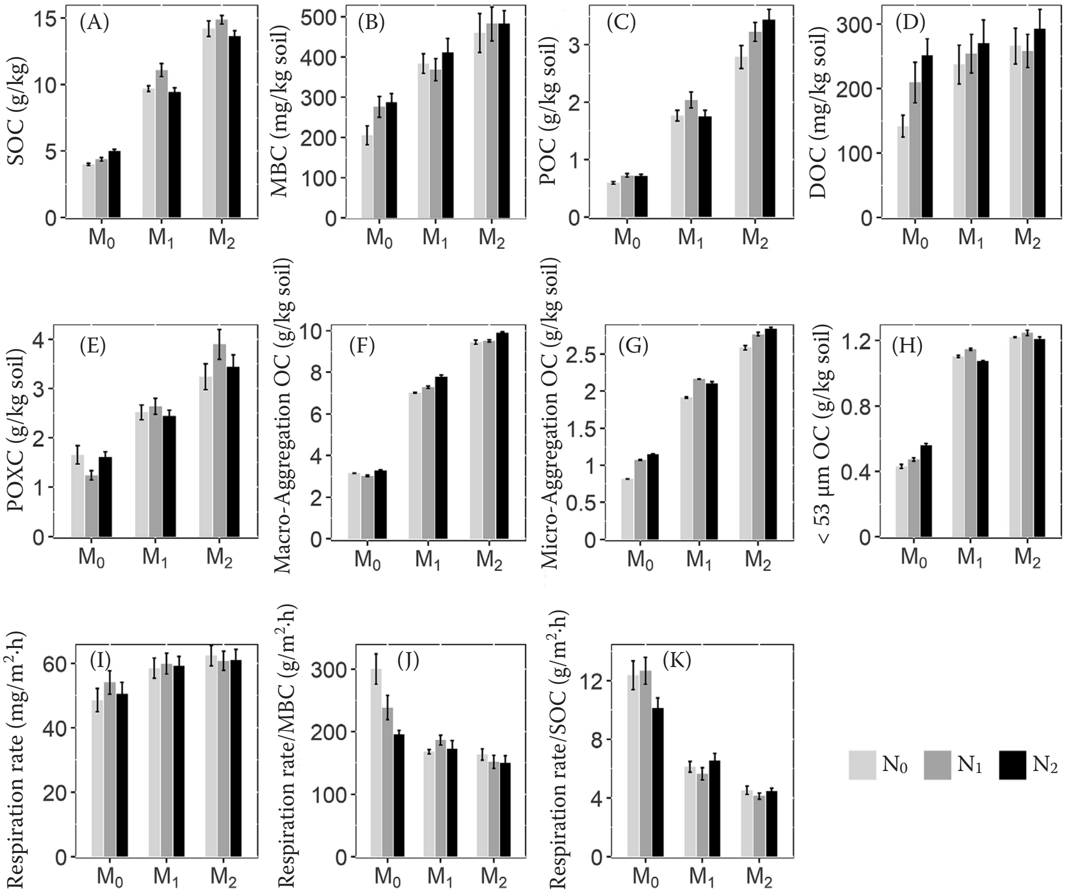

Figure 3. The mean organic carbon (OC) content of the soil total organic carbon (SOC) (A), microbial biomass carbon (MBC) (B), dissolved organic carbon (DOC) (C), particle organic carbon (POC) (D), potassium permanganate oxidisable organic carbon (POXC) (E), macro-aggregation OC (F), micro-aggregation OC (G), the OC content of fraction < 53 μm (H), and the mean value of the soil respiration rate (I), the ratio of the soil respiration rate and the MBC (soil respiratory quotient) (J), the ratio of the soil respiration rate and the SOC with the different levels of the manure and nitrogen fertiliser treatments (K)

M_0 – no manure fertiliser addition; M_1 – low amount of manure fertiliser addition; M_2 – high amount of manure fertiliser addition; N_0 – no N fertiliser addition; N_1 – low amount of N fertiliser addition; N_2 – high amount of N fertiliser addition

the soil microbial activity by a priming effect and then decreased the SOC content by the microbial mineralisation compared to the same level of manure fertilisation.

CONCLUSION

Manure fertilisation increased the POC and macro-aggregation OC percentages and helped format the soil organo-mineral complexes, while the N fertilisation mainly increased the unprotected readily bioavailable C fractions (DOC) and decreased the aggregation formation (macro-aggregation OC percentage). However, when a high level of both manure and N fertiliser were applied, the excessive N in the soil would stimulate the soil microbial activity and decrease the SOC content compared to the same level of the manure fertiliser addition.

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