Variations in the profile distribution and protection mechanisms of organic carbon under long-term fertilization in a Chinese Mollisol

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HIGHLIGHTS

- Sole application of manure enhanced SOC content in the subsoil.
- cPOC and iPOC were the most robust indicators of SOC sequestration throughout the profile.
- Physical protection was important for C sequestration in top- as well as in the subsoil.
- Aggregate-related chemical and biochemical protection was dominant across the profile.

GRAPHICAL ABSTRACT
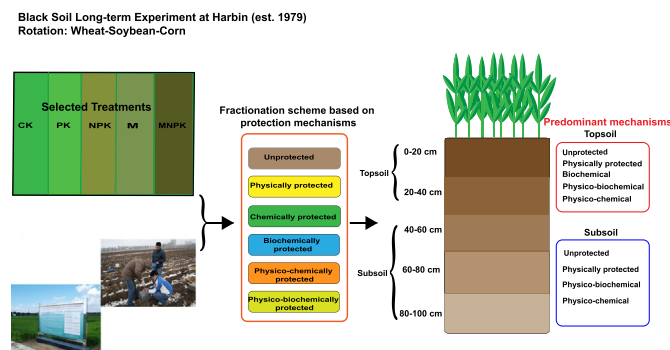
ARTICLE INFO

ABSTRACT

Long term fertilization may have a significant effect on soil organic carbon (SOC) fractions and profile distribution. However, previous research mostly explored the SOC in the topsoil and provided little or no information about its distribution in deeper layers and various protection mechanisms particularly under long-term fertilization. The present study investigated the contents and profile distribution (0–100 cm) of distinct SOC protection mechanisms in the Mollisol (black soil) of Northeast China after 35 years of mineral and manure application. The initial Organic Matter content of the topsoil (0–20 cm) ranged from 26.4 to 27.0 g kg^{-1} soil, and ploughing depth was up to 20 cm. A combination of physical-chemical fractionation methods was employed to study various SOC fractions. There were significant variations throughout the profile among the various fractions and protection mechanisms. In topsoil (to 40 cm), mineral plus manure fertilization (MNPK) increased the total SOC content and accounted for 16.15% in the 0–20 cm and 12.34% in the 20–40 cm layer, while the manure alone (M) increased the total SOC by 56.14%, 48.73% and 27.73% in the subsoil (40–60, 60–80 and 80–100 cm, respectively). Moreover, MNPK and M in the topsoil and subsoil, respectively increased the unprotected coarse particulate organic carbon (cPOC) (48% and 26%, respectively), physically protected micro-aggregate (µagg) (20% and 18%, respectively) and occluded particulate organic carbon (iPOC) contents (279% and 93%, respectively) compared with the control (CK). A positive linear correlation was observed between total SOC and the cPOC, iPOC, physico-biochemically protected NH-µSilt and physico-chemically protected H-µSilt ($p < 0.01$) across the

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whole profile. Overall, physical, physico-biochemical and physico-chemical protection were the predominant mechanisms to sequester carbon in the whole profile, whereas the biochemical protection mechanisms were only relevant in the topsoil, thus demonstrating the differential mechanistic sensitivity of fractions for organic carbon cycling across the profile.

1. Introduction

Soil organic carbon (SOC) plays a key role in sustaining soil productivity and health (Smith et al., 2013), has a positive effect on mitigating climate change (Lal, 2016) and influences many physical, chemical and biological properties of soil (Lal et al., 2011). The mechanisms underlying the maintenance of SOC stocks (in top- as well as in the subsoil) must be determined, and best management practices are required to ensure sustainability without harming other ecosystem functions. Sustainable grain production can be improved by increasing SOC (Pan et al., 2014), and adopting recommended management practices (Shrestha and Lal, 2006).

Black soils (Mollisols) are the most fertile soils in China, and they are primarily distributed in Heilongjiang Province in the northeast region of the country, with a total acreage of 6 million ha and 4.4 million ha under cultivation (Xing et al., 2004). Thus, Heilongjiang Province represents one of the four largest Mollisol areas of the world (Liu et al., 2003; Xing et al., 2004), and it is also one of the major areas famous for corn and soybean production. Removal of above-ground crop residues after harvesting is a common practice in the area. Although, black soils are famous for their inherently high SOC content compared with other soils of China (Ling et al., 2014), a decline in the productivity of these soils has been observed (Xie et al., 2014), due to intensive agriculture; thus, these soils have become unfit for future food production (Kopittke et al., 2019).

Previous studies on the effects of management (e.g., fertilization) on SOC levels were mostly focused on the topsoil (0–20 cm), and few studies have focused on the subsoil (Gregory et al., 2014; Ogle et al., 2005). Subsoil accounts for a large proportion of the total organic carbon and responds to changes in fertilization and other management practices (Rumpel and Kögel-Knabner, 2011). However, the SOC size in the subsoil may surpasses that in the topsoil, and these SOC contents can act as potential contributors towards total SOC reserves (Jandl et al., 2007; Richter and Billings, 2015). Organic carbon inputs in the rhizosphere either in the form of particulate organic matter or root exudates may already have relevance for the topsoil (Angst et al., 2016; Tefs and Gleixner, 2012), but appear to gain importance with increasing depth across the soil profile (Rasse et al., 2005), and may enhance SOC stocks in the subsoil (Lorenz et al., 2007). Nevertheless, the fate of newly added carbon (C) inputs to the existing SOC stabilized in the deep soil layers has not been clarified (e.g., Fontaine et al., 2007; Lorenz and Lal, 2005), and limited studies have focused on stabilization mechanisms based on a detailed organic carbon fractionation. These differences may become more pronounced in the subsoil, where plant inputs are comparatively less important compared with topsoil and clay mineralogy may have a strong effect.

Bulk SOC can have four fractions depending on the mechanisms associated with stabilization: unprotected, physically protected, chemically protected, and biochemically protected (Six et al., 2002). The unprotected fraction is a non-occluded pool (un-aggregated) that is labile in nature and an important energy source (for microbes). The formation of micro-aggregates gives SOC physical protection from decomposition. While SOC is chemically stabilized by adsorption with silt and clay particles, biochemical protection of SOC is related to the formation of recalcitrant organic compounds (Six et al., 2002). The different fractions of SOC are strongly influenced by the stabilization and turnover rates and hence are considered important indices for understanding fertilization management (He et al., 2015).

Hence, detailed studies on changes in SOC contents and stabilizing mechanisms under different fertilization management strategies are needed to realize sustainable food production and mitigate climate change.

This study was conducted to (1) examine the SOC dynamics under different fertilization management, i.e., unbalanced (PK), balanced (NPK), organic manure alone (M) and a combination of chemical fertilizer with manure (MNPK), on the dynamics of variously stabilized soil organic C fractions; and (2) elucidate the association between the C fractions (protected distinctively) and total SOC subjected to long-term (35 years) fertilization to establish a technical rationale for improving soil fertility and quality while promoting sustainability.

2. Materials and methods

2.1. Site description

The experimental site (126°51′ E, 45°50′ N) was established in Harbin City, Heilongjiang Province, Northeast China, in 1979 at an elevation of 151 m. This region has a mid-temperate monsoon climate, with a mean annual temperature of 3.5 °C and mean annual precipitation of 533 mm, and it is characterized by distinct seasons, i.e., long cold winter and warm summer (data from China meteorological sharing service system, http://cdc.cma.gov.cn/). The climate normals for temperature and precipitation (from 1981 to 2010) can be seen in Supplementary Fig. S2. The soil was developed on a diluvial loess clay parent material. The black soil is a sandy clay loam (in the topsoil) and clay loam (in the subsoil) (Xing et al., 2005; see Supplementary Table S1). The soil is classified as a Luvic Phaeozems (FAO Classification, 2010) and characterized by a 2:1 clay mineralogy dominated by montmorillonite. The topsoil (0–20 cm) comprised 16.2 g kg^{-1} of total organic C, 1.48 g kg^{-1} of total nitrogen (TN), 1.07 g kg^{-1} of total phosphorus (TP), 151.1 mg kg^{-1} of alkali-hydrolysable nitrogen, 5.1 mg kg^{-1} available phosphorus, and 200 mg kg^{-1} available potassium, and the pH (H$_2$O) was 7.2.

2.2. Experimental design and set up

A completely random block design was followed with three replicates, and the area of each plot was 36 m^2. The crop rotation consisted of wheat-soybean-corn. The tillage practice was ploughing to a 20 cm depth every year. Five treatments were employed: (1) unfertilized control (CK); (2) unbalanced fertilization: phosphorus (75 kg ha^{-1} year^{-1}) and potassium (75 kg ha^{-1} year^{-1}) (PK); (3) balanced fertilization: nitrogen (150 kg ha^{-1} year^{-1}) phosphorus (75 kg ha^{-1} yr^{-1}) potassium (75 kg ha^{-1} year^{-1}) (NPK); (4) organic manure alone (75 kg N ha^{-1} year^{-1}) (M); and (5) organic manure combined with inorganic N fertilizer (150 kg N ha^{-1} year^{-1}), P fertilizer (75 kg P$_2$O$_5$ ha^{-1} year^{-1}) and K fertilizer (MNPK). The chemical fertilizers (N and P) were applied as urea and diammonium phosphate (DAP) and sulphate of potash (SOP), respectively. The manure was applied on the surface first and then mixed to 20 cm while ploughing in autumn. The organic manure was composed of pure horse manure containing 150 g kg^{-1} total organic C, 10 g kg^{-1} total N, 10 g kg^{-1} P$_2$O$_5$ and 4 g kg^{-1} K$_2$O.

2.3. Soil sampling

Soil samples were taken randomly from each plot at five depths viz. (0–20, 20–40, 40–60, 60–80 and 80–100 cm) using an auger (5 cm

diameter) in September 2014. Crop residues were removed from the soil surface with care prior to sampling. Twelve soil cores were randomly taken from each plot and soil from four random cores was composited to make one sample; consequently, three samples were formed for each treatment. The clumps were gently broken with hand so that the field moist soil could pass through a 2-mm sieve, and the subsamples were dried overnight at room temperature (~27 °C). After drying, these subsamples were stored at room temperature for bulk as well as fractionation analyses.

2.4. Fractionation procedure (physical and chemical fractionation of bulk soil)

The SOC pools were fractionated using a modified method from Six et al. (2002), which is a combination of physical and chemical fractionation schemes, in a three-step process (Fig. S1, Supplementary material). The 1st step was the partial dispersion and physical fractionation of a 20 g subsample that was pre-wetted for 24 h and to acquire three fractions based on size, i.e., coarse non-protected particulate organic carbon, cPOC (>250 μm), and easily dispersible silt and clay, dSilt and dClay (53–250 μm). Soil samples (20 g) were isolated using a micro-aggregate isolator through physical fractionation with 30 glass beads (4 mm diameter) in running water to flush microaggregates and finer fractions. Fraction >250 μm (cPOC) was collected on the 250 μm sieve, while the 53-μm sieve did not allow the micro-aggregates to pass but permitted the d-silt and d-clay particles to pass along with the liquid portion. Centrifugation was performed at 127 ×g (900 rpm) to isolate the d-silt and d-clay fractions for 7 min, and at 1730 ×g (3000 rpm for 15 min) for d-clay fraction. All the fractions were collected in separate aluminium crucibles and oven-dried at 60 °C and weighed.

In the 2nd step, micro-aggregates (obtained after physical fractionation) were mixed with 50 mL 1.85 g cm^{-3} sodium iodide. A density flotation was employed to separate fine POC (fPOC or LF) (Six et al., 1998). The remaining heavy material was mixed with 60 mL 5 g L^{-1} sodium hexameta- phosphate for dispersion and shaken overnight with 12 glass beads and then passed through 53-μm sieve to obtain micro-aggregate occluded POC (iPOC), >53 μm in size (Denef et al., 2004). The sample in the liquid portion (<53 μm) was micro-aggregate derived silt and clay-sized fractions (μ-Silt and μ-Clay). The centrifugation (such as in the case of dSilt and dClay), was used to isolate the μ-Silt and μ-Clay. The final step involved the acid hydrolysis (described in detail by Plante et al., 2006a, 2006b) of each isolated d-silt and d-clay fractions collected from pre-dispersion and physical fractionation of the soil (dSilt and dClay), and density floatation (μSilt and μClay). Acid hydrolysis consisted of refluxing at 95 °C for 16 h in 6 M hydrochloric acid (25 mL) and filtration of the suspension was performed on a glass-fibre filter (0.45 μm) with two to three times continuous washing using distilled water. The residual portions were placed in an oven for drying at 60 °C, weighed, and subsequently subjected to C analysis. The non-hydrolysable C fractions (NH-dSilt, NH-dClay, NH-μSilt, and NH-μClay) were then obtained. However, the fractions (hydrolysed in acid) that consist of (H-dSilt, H-dClay, H-μSilt, and H-μClay) were calculated by the difference method used in the following equation.

$$\text{Hyd.C fractions} = \text{Total org.C content of the fractions} - \text{C contents of the non-hyd.fractions}$$

(1)

2.5. Carbon content analysis

Inorganic C is usually negligible in black soil; thus, the total C concentration was assumed to be total soil carbon as organic carbon (OC). The bulk soil samples, as well as all the fractions (unprotected, physically, chemically and biochemically protected fractions) for all treatments, were analysed for total C using a Eurovector Elemental CHNOS-analyzer,

EA3000 Series, (Milan, Italy). All measurements were performed in triplicates.

2.6. Statistical analysis

Data regarding the fertilizer effects were subjected to an ANOVA (One-way) using IBM SPSS v. 21.0 (Chicago, IL, USA) for Windows and Duncan's test was employed to identify the least significant differences. The p values for both of these analyses were set to 0.05. Correlation and regression analyses were performed with the Sigmastat function in Sigmaplot v.14.0 (Systat Software GmbH, Germany) for Windows. The logarithmic function (in the regression analysis) was used to determine the total SOC (average SOC content of each layer) distributed along with the profile, whereas simple linear regressions were applied to evaluate the relationship between total SOC and its fractions. A polynomial linear model was applied according to first-order equation i.e., (y = a + bx), where a is the intercept term while b is the slope (both of these are constants), proposed by Stewart et al. (2007). Carbon (x) is directly related to the total SOC (y). Results regarding soil dry-mass distribution, and total and fractions SOC content are considered as significant which are different at probability level ($p < 0.05$), while correlation results were regarded as significant at $p < 0.01$.

3. Results

3.1. Relative proportions of isolated fractions

3.1.1. Topsoil

The distribution of the soil dry mass (in grams) into various SOC protected fractions is presented in Table 1. The cPOC and fine POC (fPOC) fractions accounted for approximately 38.8% and 0.89% of the total soil under MNPK compared to CK for the 0–20 cm layer, respectively. The cPOC was significantly higher ($p < 0.05$) in the manure (M) treatment relative to the CK from 20 to 40 cm. However, fPOC accounted for 0.83% and 0.77% in M and MNPK treatments in the 20–40 cm layer, respectively.

From 0 to 20 cm, MNPK significantly increased both the microaggregates (μagg) and intra-aggregate derived particulate organic carbon iPOC proportions (36.9% and 10.5%). Significant differences were not observed among μagg proportions under CK, M, and MNPK in the 20–40 cm layer. However, the application of MNPK increased the iPOC fraction (7.01%) in the same layer. The proportion of H-dSilt was highest (5.12%) under NPK in the 0–20 cm layer, while the H-dClay proportion in both NPK and MNPK treatments was higher (0.28%) than that of the other treatments. No significant differences were found for proportion of the H-dSilt fraction among all treatments in the 20–40 cm layer except in MNPK where it increased significantly and NH-dSilt proportion was increased only in NPK.

There were no significant differences among NH-dSilt proportions in PK, NPK and MNPK treatments in 20–40 cm layer. However, PK increased the NH-dClay proportion (by 0.46%) in the same layer (20–40 cm). A similar trend was found in the top layer for the proportions of H-μSilte, although PK increased the proportion (by 4.59%). Physico-chemically protected (H-μSilt and H-μClay) proportions were almost similar in all the treatments in the 20–40 cm layer. Both NH-μSilt and NH-μClay were notably increased ($p < 0.05$) in the 0–20 cm topsoil layer under CK and M alone. In the lower layer of topsoil, non-significant differences were found among the proportions of NH-μSilt under NPK, M, and MNPK and between proportions of NH- μClay in the 20–40 cm layer.

3.1.2. Subsoil

The proportion of the cPOC fraction increased (38.8% and 38.2%) under MNPK and NPK, respectively in 40–60 cm layer, respectively, whereas the fPOC proportion was increased under MNPK (0.75%). In both the 60–80 cm and 80–100 cm layers, M alone increased the cPOC

Table 1
Distribution of soil dry matter in isolated fractions (%) under long-term fertilization in black soil (Harbin).

	Depth	Treatments	Unprotected		Physically protected		Chemically protected		Biochemically protected		Physico-chemically protected		Physico-biochemically protected	
			cPOC	fPOC	μagg	iPOC	H-dSilt	H-dClay	NH-dSilt	NH-dClay	H-μSilt	H-μClay	NH-μSilt	NH-μClay
Topsoil	0–20	CK	36.6e	0.49c	36.7b	7.90d	4.75ab	0.17c	17.9c	0.30a	3.31b	3.17b	16.7a	3.39a
		PK	37.3d	0.53c	36.1c	8.49c	4.05c	0.19bc	18.6b	0.34a	5.38a	4.59a	14.7bc	2.02d
		NPK	37.5c	0.57c	35.6d	9.99b	5.12a	0.28a	18.4b	0.31a	6.11a	2.20c	14.2c	2.61c
		M	38.1b	0.70b	36.6b	10.1b	4.24bc	0.27ab	18.6b	0.30a	3.48b	3.33b	16.5a	3.38a
		MNPK	38.8a	0.89a	36.9a	10.5a	4.82b	0.28a	20.1a	0.32a	5.42a	4.18b	15.2b	3.13b
	20–40	CK	35.5c	0.61ab	36.2a	6.10c	4.96a	0.27c	19.6b	0.28 cd	6.53a	1.99a	18.6b	1.93b
		PK	33.7e	0.45bc	35.2b	6.19c	5.00a	0.34b	21.1a	0.46a	6.27a	1.88a	18.1b	1.99b
		NPK	34.3d	0.29c	34.6c	6.48b	6.04a	0.27c	19.2b	0.25d	5.58a	2.21a	19.9a	1.65c
		M	37.0a	0.83a	36.2a	5.87d	5.46a	0.23c	21.5a	0.32bc	6.56a	2.62a	20.3a	3.66a
		MNPK	36.2b	0.77a	36.2a	7.01a	5.10a	0.42a	22.2a	0.36b	5.45a	2.50a	19.9a	3.71a
Subsoil	40–60	CK	36.1b	0.45b	36.0b	5.50c	4.94b	0.65a	20.8b	0.22b	5.88a	1.30d	19.7a	1.49c
		PK	36.9b	0.50b	33.6d	6.72b	4.91b	0.38b	22.2a	0.40b	5.12a	2.16c	16.8b	2.20b
		NPK	38.2a	0.47b	31.8e	7.16a	5.83a	0.34b	22.7a	0.41b	6.03a	2.11c	15.3c	1.65c
		M	35.2d	0.62ab	38.1a	7.03ab	4.63b	0.31b	18.7c	0.64a	5.83a	2.93a	19.0a	3.05a
		MNPK	38.8a	0.75a	34.5c	7.26a	5.35ab	0.37b	19.3c	0.43b	3.72b	3.35a	16.4bc	3.20a
	60–80	CK	38.1bc	0.77a	34.4ab	11.0a	4.75b	0.53a	20.2b	0.40a	5.34b	1.07c	16.1b	0.64e
		PK	39.1ab	0.71a	32.3c	8.40d	5.02b	0.31b	22.0a	0.39a	6.41a	1.26c	16.2b	1.25c
		NPK	37.4c	0.39b	32.1c	10.1b	5.83a	0.33b	22.5a	0.31a	3.54c	0.97c	16.7b	0.91d
		M	39.9a	0.99a	35.1a	9.30c	4.75b	0.23b	19.1c	0.35a	3.90c	1.97b	18.8a	1.78b
		MNPK	40.3a	0.80a	33.7b	9.45c	4.93b	0.25b	19.8bc	0.38a	4.09c	2.49a	16.1b	2.34a
	80–100	CK	36.8b	0.72ab	34.4b	4.51c	5.14a	0.41a	21.4a	0.57a	5.93ab	1.41b	19.5a	1.27b
		PK	34.8e	0.71ab	31.0c	4.54c	4.71a	0.34a	21.4a	0.39a	7.52a	1.34b	20.5a	0.96b
		NPK	36.3d	0.58c	34.1b	6.62b	5.54a	0.35a	22.5a	0.44a	5.97ab	1.26b	21.0a	0.94b
		M	37.6a	0.88a	36.9a	9.18a	4.53a	0.29a	19.7a	0.39a	5.69c	2.20a	21.1a	2.37a
		MNPK	36.5c	0.78a	35.8ab	9.01a	5.27a	0.36a	22.7a	0.37a	4.85c	2.36a	21.9a	2.27a

Different lowercase letters indicate significant differences ($p < 0.05$) among treatments.

proportion (39.9% and 37.6%, respectively) relative to the CK, while the other treatments had no effect on cPOC in this layer. In the 80–100 cm layer, the fPOC proportions under M and MNPK were higher (0.78% and 0.88%, respectively) than that of the CK although differences between both treatments were non-significant (p < 0.05; Table 1).

The physically protected fraction (μagg) proportion increased (38.1%, 35.1% and 36.9% in 40–60, 60–80 cm and 80–100 cm, respectively) under manure (M) fertilization whereas the highest proportions of iPOC (7.16% and 11.0% in 40–60 cm and 60–80 cm, respectively) were observed under the NPK and CK treatments. NPK increased the proportions of H-dSilt in sub soil layers (5.83% and 5.83% in 40–60 cm and 60–80 cm, respectively) except the last year (80–100 cm).

The H-dSilt proportion in the last layer presented a similar trend as the NH-dClay proportions in the 60–80 cm and 80–100 cm layers but not the top layer (0–20 cm) in which manure alone (M) increased NH-dClay proportion (by 0.64%). In the first subsoil layer (40–60 cm), the highest proportions of NH-μSilt (19.7% and 19.0%) were found in CK and M, respectively. M and MNPK clearly increased the NH-μClay proportions (by 3.05% and 3.20%, respectively) in the first subsoil layer and presented significant differences for the other treatments. A notable increase (2.34%) in the NH-μClay proportion was observed under MNPK. A trend similar to the top layer of the subsoil was found for the NH-μClay proportion in the last layer (80–100 cm).

In the 40–60 cm layer, no significant difference (p < 0.05) was noted for proportions of H-μSilt among all treatments except MNPK, which is significantly different from the rest of the treatments. MNPK increased the H-μClay proportion (by 3.35%) in the same layer. The highest H-μSilt proportions (6.41% and 7.52%) were found under PK for both the 60–80 cm and 80–100 cm layers, respectively, whereas M increased proportion of H-μClay fraction (by 2.49%) in 60–80 cm and M and MNPK (by 2.27% and 2.37%, respectively) increased this proportion from 80 to 100 cm.

3.2. Distribution of SOC as a function of depth under long-term fertilization

As shown in Fig. 1, both NPK and MNPK fertilization significantly (p < 0.05; Table 1) increased the SOC content of the bulk soil samples in the 0–40 cm topsoil layer compared with the unfertilized control (CK). MNPK contributed 21–22% to the total SOC stabilized in the topsoil layer (0–20 cm), while no increase was observed in the subsoil under NPK and MNPK. Conversely, manure alone (M) significantly (p < 0.05) increased the overall SOC content of the subsoil (40–100 cm) (by 128–156%) as compared to control in each subsoil layer. Stabilized SOC accounted for 55% of the total SOC content in both layers (combined) of topsoil while subsoil SOC content (138.88 g C kg^{-1}) accounted for 45% on average of the total SOC stabilized. A logarithmic decrease in SOC content was observed with increasing soil depth under all treatments (Fig. 1).
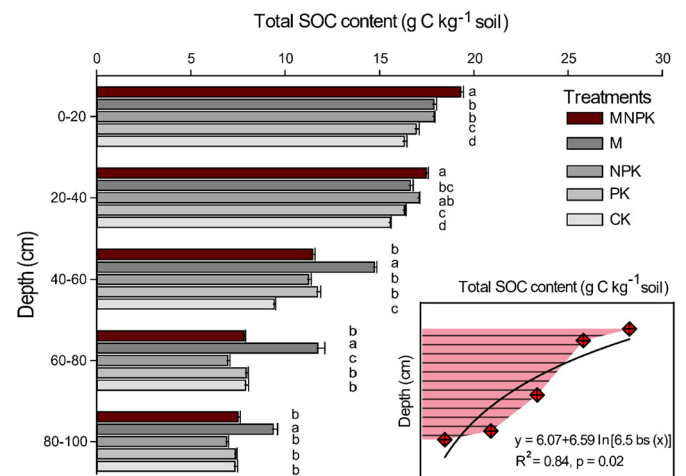

Fig. 1. Profile distribution of the total soil organic carbon contents (g C kg^{-1} soil) in bulk soil under long-term fertilization. Different lowercase letters represent significant differences among different treatments (p < 0.05). Bars represent the standard error of the mean (as SE) (n = 3). Inset graphic shows the logarithmic distribution of soil organic carbon (averaged values) along with the different depth layers.

3.3. SOC content of isolated fractions across the whole profile

3.3.1. Unprotected

The OC content of the cPOC was significantly higher (>49%) in the 0–20 and 20–40 cm layers of topsoil in the MNPK treatment as compared to CK (Fig. 2a). Nonetheless, no significant difference for SOC content was noted between M and MNPK in the 2nd layer (20–40 cm) of topsoil. The OC content in the free particulate organic carbon (fPOC) was increased (124% and 49%) in the M treatment in the topsoil layers

(0–20 cm and 20–40 cm) (Fig. 2b). In all layers of subsoil (40–60 cm, 60–80 cm, and 80–100 cm, respectively), manure alone (M) maintained the highest SOC content for the cPOC and fPOC (Fig. 2a, b).

3.4. Physically protected

The MNPK treatment showed the highest SOC contents in 1st and 2nd layers of topsoil and occurred as physically protected fractions (as μagg and iPOC) in the MNPK treatment, while in the subsoil, the manure
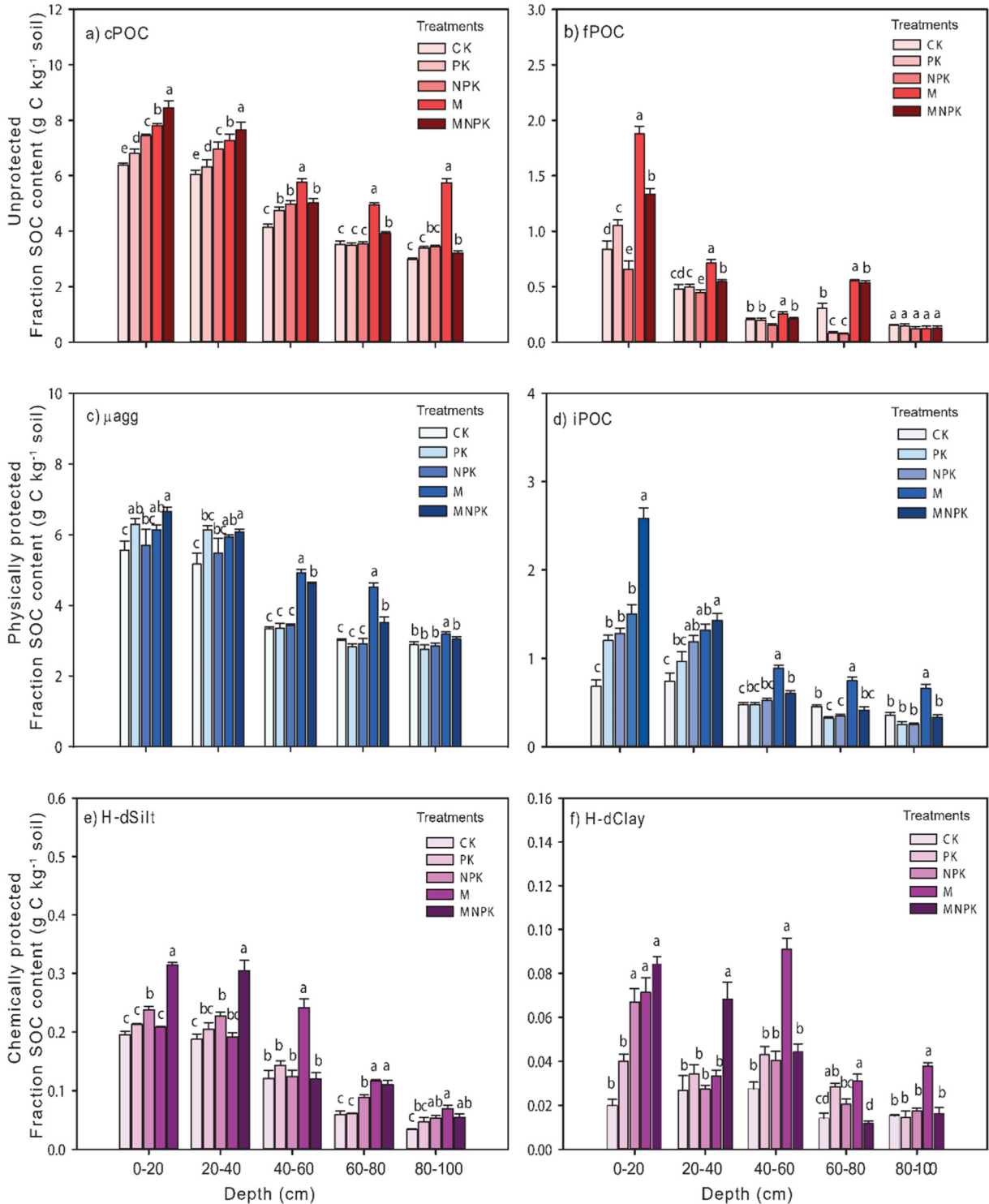

Fig. 2. Profile distribution of the soil organic carbon content in isolated constituent fractions under long-term fertilization (g C kg^{-1} soil). Different lowercase letters represent significant differences within each fraction among different treatments ($p < 0.05$). Bars represent the standard error of the mean ($n = 3$).

(M) treatment increased ($p < 0.05$; Fig. 2c, d) the SOC contents of the physically protected fractions (μagg and iPOC). No significant differences were observed between the PK and M treatments for both physically protected fractions (iPOC and micro-aggregate, μagg). The organic C content was 0.18–0.77 g kg^{-1} in iPOC and 0.05–1.37 g kg^{-1} in μagg in treatments with manure.

3.4.1. Chemically protected

The SOC contents of H-dSilt were highest (61% and 63%) in the topsoil (0–20 cm and 20–40 cm, respectively) in the MNPK treatment compared with the CK, while the rest of the treatments did not differ ($p < 0.05$; Fig. 2e, f). In the subsoil, the SOC contents in the H-dSilt fraction, which is a chemically protected fraction, were highest under M in the 40–60 and 80–100 cm layers. However, no significant difference was observed between M and MNPK in 60–80 cm. OC contents ranged from 0.08 to 0.10 g kg^{-1} for the H-dClay fractions after the application of MNPK up to 1st layer of subsoil. Nevertheless, the lower two depths of subsoil had the highest SOC content as chemically protected H-dClay fraction in the M treatment ($p < 0.05$).

3.4.2. Biochemically protected

The OC content stabilized in the SOC fraction under biochemical protection (NH-dSilt) fraction was considerably enhanced after the MNPK application compared to the CK (Fig. 3a). The SOC content of the biochemically protected clay fraction was highest (0.24 g kg^{-1}) in MNPK and lowest (0.11 g kg^{-1}) in the CK treatment. However, no significant differences were observed for the OC content of the NH-dClay fraction among all treatments in the last two layers (60–80 cm and 80–100 cm) of subsoil ($p < 0.05$; Fig. 3b).

3.4.3. Physico–chemically protected

The OC contents of the H-μSilt fraction were significantly different between M and MNPK, and these were significantly ($p < 0.05$) higher than CK. In the subsoil, the M treatment showed the highest SOC content while the CK showed the lowest SOC content as the H-μSilt fraction. The SOC content as H-μSilt fraction was highest under PK treatment (0.09–0.12 g kg^{-1}) while CK showed lowest SOC content in the 2nd layer of subsoil. In the 80–100 cm layer, the organic C content in H-μSilt fraction was significantly higher (by 68%) under M compared with the CK. The SOC content of H-μClay in surface layers (0–20 cm and 20–40 cm) was higher in the MNPK treatment were higher (105%) than the control (CK), whereas this value was higher in all subsoil layers in the M treatment compared with the CK (Fig. 3d).

3.4.4. Physico–biochemically protected

No differences in the NH-μSilt fraction were observed among all the treatments ($p < 0.05$) (Fig. 3e). The OC content of a physico–biochemically protected fraction (NH-μClay) was considerably greater in the MNPK treatment (1.45–1.55 g kg^{-1} and 0.65–0.69 g kg^{-1} in the 0–20 and 20–40 cm layers, respectively) than the CK, while this value was considerably increased in the manure treatment alone (M) (60%, 101% and 97% in 40–60, 60–80 and 80–100 cm respectively) as the physico-biochemically protected fraction as NH-μClay (Fig. 3f).

3.4.5. Relationship between fractions of SOC and total SOC

The regression analysis revealed a clearly positive linear relationship between the total SOC and unprotected cPOC (Fig. 4a). The cPOC had the highest rate of C accumulation in the topsoil, and the lowest (30%) positive correlation ($p < 0.01$) with the total SOC was found in 60–80 cm layer. Overall, our findings showed significant positive correlations for cPOC and SOC across the whole profile. However, no relationship ($p > 0.01$) was observed between fPOC and SOC in both topsoil layers and the last layer (80–100 cm), although a negative correlation was observed at 40–60 cm and 60–80 cm (Fig. 4b). Similar to cPOC, iPOC also had a highly positive linear correlation with the increase in total SOC (Fig. 4d). Nonetheless, non-hydrolysable dSilt and dClay, physico-

biochemically protected NH-μClay and chemically protected H-dSilt showed a positive linear correlation with SOC but only in the topsoil layers except for NH-μClay and HdSilt, which showed increasing C accumulation tendency in the 40–60 cm layer.

4. Discussion

4.1. Distribution of SOC as a function of depth under long-term fertilization

Significant changes in SOC by land management practices can only be observed after a long time (Gerzabek et al., 2001). Increased SOC mineralization rates caused by continuous tillage and harvesting decrease the total SOC content (Post and Kwon, 2000). The application of organic manure and its combination with mineral fertilizers increases the SOC content because it contains a considerable amount of OC that adds C directly to the soil itself (Kundu et al., 2007), and promotes higher straw, grain, and root biomass yields (Kukal et al., 2009). We observed that the total SOC content in the topsoil layers (0–20 and 20–40 cm) increased in the MNPK treatment. Nitrogen fertilization imposes an indirect positive effect on SOC by enhancing plant biomass production (Gregorich et al., 1996). In the subsoil layers (40–60 cm, 60–80 cm, and 80–100 cm), the SOC content was lower than that of the top layers under both NPK and MNPK treatments. This decrease could be explained by the possible presence of roots in the Ap horizon (near to the soil surface) (Shahbaz et al., 2017). Subsoil receives OM inputs in the form of root litter, root exudates, and dissolved organic carbon (DOC) (Michalzik et al., 2001), and root C is more stable than shoot C due to greater recalcitrance of the tissues (Rumpel and Kögel-Knabner, 2011). Moreover, subsoils are rich in microbially derived C compounds compared to the counterpart (i.e., topsoil), which is enriched in plant-derived material. In the current study, we observed a notable decrease ($p < 0.05$) in the SOC content under NPK and a MNPK but not under manure alone (M) throughout the subsoil. The addition of organic substrates or nutrients (e.g., fructose) that are easily taken up by microbes can accelerate the decomposition of SOC in the form of positive priming (Hamer and Marschner, 2005). Moreover, as the depth increased, C associated with clay-sized particles also increased because of the clay content in the subsoil (Kögel-Knabner et al., 2008). Other studies have shown that subsoil priming due to destabilization is caused by the fresh C supply and N fertilization (Fontaine et al., 2007; Oliveira et al., 2017).

Nitrogen fertilization also strongly influences the microbial community composition (Fanin et al., 2015), and new C integration into the soil is governed by the microbial activity (Lange et al., 2015). Furthermore, Steinmann et al. (2016) reported a decline in the subsoil SOC contents as a result of increased N fertilization over a period of 8 years, although the topsoil OC contents increased. This trend was dissimilar to the general consensus that subsoil OM is relatively more stable than topsoil OM and is not sensitive to the management.

Our study reported an increase in the SOC content of the topsoil due to MNPK and in the subsoil due to manure (M), which could be explained by the quantity of OC input. Initially, the mineralization rate is usually stable; therefore, a direct relationship was observed between SOC changes and C inputs after the application of organic amendments (Jastrow et al., 2007; Li et al., 2010). Second, MNPK significantly enhanced the average crop yields compared with the CK (unfertilized), thus promoting higher root residues and exudates in the soil (Fan et al., 2005) which ultimately increased the SOC content under the MNPK and M treatments (Gregorich et al., 1995).

4.2. Fractional proportions and SOC content under long-term fertilization

4.2.1. Unprotected fraction

The combined application of chemical fertilizers and manure (MNPK) considerably enhanced the proportions as well as the C contents of the cPOC and fPOC fractions throughout the profile irrespective
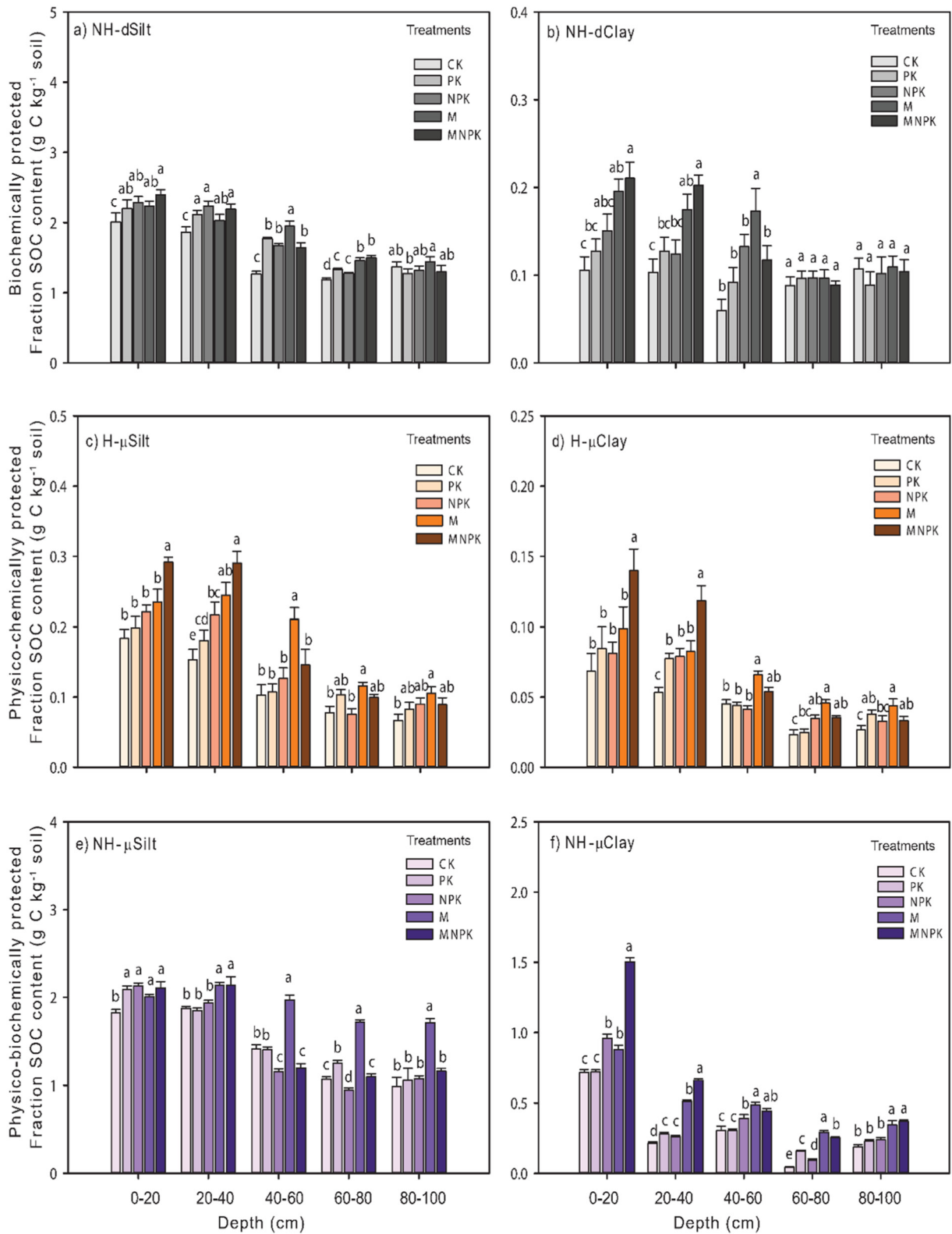
Fig. 3. Profile distribution of the soil organic carbon content in isolated constituent fractions under long-term fertilization (g C kg^{-1} soil). Different lowercase letters represent significant differences within each fraction among different treatments (p < 0.05). Bars represent standard error of the mean (n = 3).

of depth (Table 1; Fig. 2a, b). A similar increase in unprotected fractions with the addition of mineral and organic fertilizers was also reported by Tian et al. (2017) and Yang et al. (2018). However, the effects were prominent only in the topsoil on most of the fractions (similar to Dou et al., 2016; Table 1). Long-term manure application benefits both plants and soil, such as by increasing the OC content in soil and by

increasing crop yields (Whalen and Chang, 2002). Additionally, long-term manure application provides more accretion of SOC in the unprotected fraction which improves aggregation and consequently may widen the scope to enhance SOC linked with minerals (Hai et al., 2010; Ludwig et al., 2003). The increment in cPOC as a result of long-term manure alone or combined with NPK fertilizer addition (Fig. 2a)
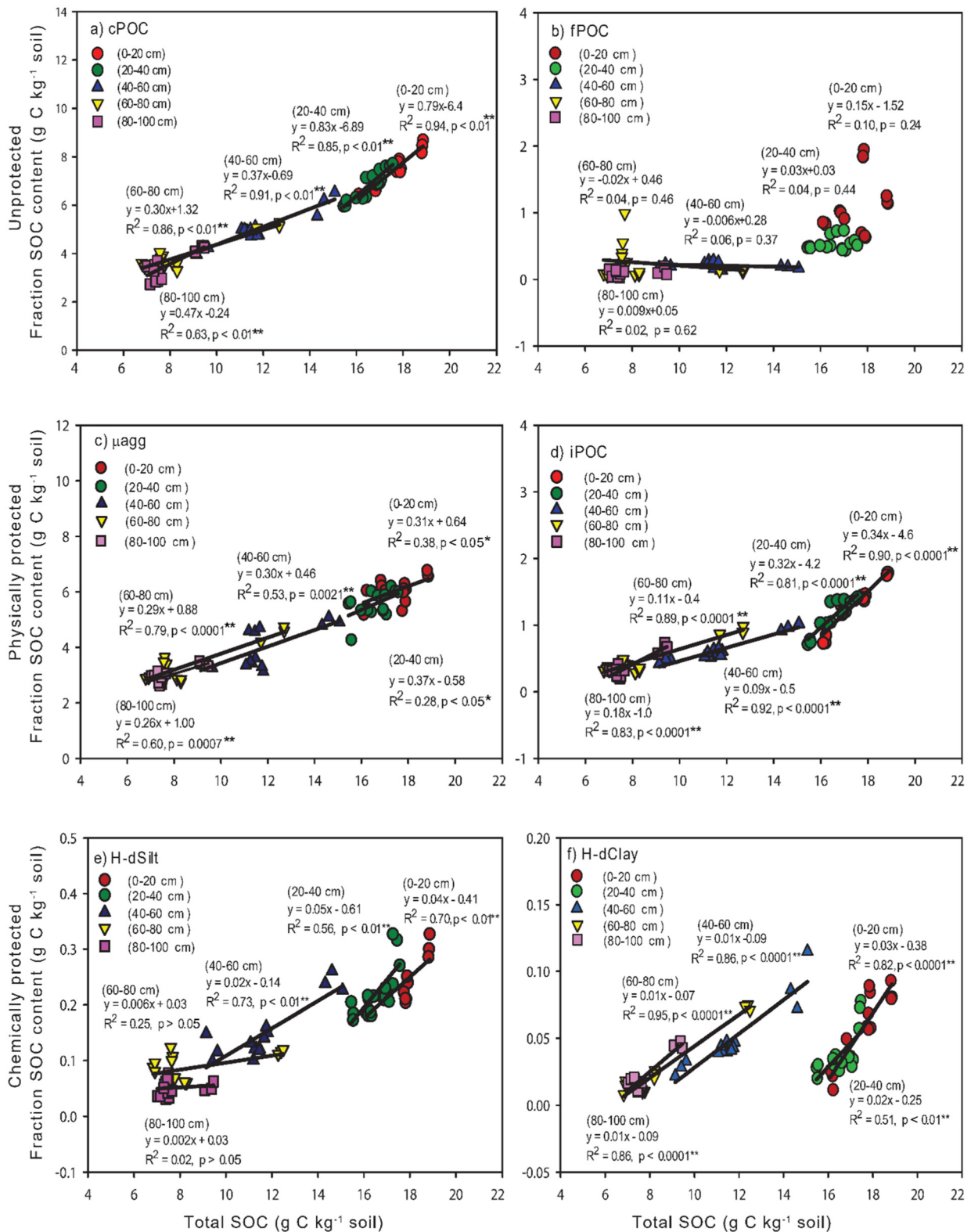
Fig. 4. Relationship between the total soil organic carbon and its constituent fractions across soil profile (g C kg^{-1} fraction) under long-term fertilization.

may be attributed to the continuous supply of easily decomposed fresh C material (Li and Han, 2016; Yang et al., 2018).

4.2.2. Physically protected

The MNPK and M treatments increased the SOC content as iPOC throughout the soil profile (MNPK in the topsoil and M in the subsoil), which is similar to many earlier reports (Sleutel et al., 2006; Tian

et al., 2017). SOC can act as a gluing agent for aggregation that could help in the formation of micro-aggregates (Six et al., 2004), which may be a reason for the increased iPOC (Tisdall and Oades, 1982). In our study, increased micro-aggregation decreased the rate of SOM turnover and enhanced the SOC content of microaggregate-derived iPOC after manure application (Su et al., 2006; Tian et al., 2017). Our study also confirmed that the application of MNPK notably increased the

iPOC in the topsoil (by 128% and 93% in the 0–20 cm and 20–40 cm, respectively) while M significantly enhanced the SOC content of the iPOC in the subsoil (by 147%, 85% and 84% in 40–60 cm, 60–80 cm, and 80–100 cm, respectively) (Hai et al., 2010; Marriott and Wander, 2006). Compared to unbalanced fertilization (PK), balanced fertilization (NPK and MNPK) significantly increased ($p < 0.05$) the proportion of iPOC in the topsoil compared with the control (CK) (Table 1) and resulted in the highest SOC content in the iPOC under NPK in the topsoil (Fig. 2d). Balanced NPK fertilizer could strongly stimulate crop growth more than SOC decomposition, which results in an overall build-up of plant remnants in the soil matrix, thus enhancing the C content of the iPOC (He et al., 2015). Moreover, SOC stabilization in micro-aggregates as a result of OM application is an indicator of the physical protection of SOC that reduces turnover rate (Six et al., 2002). Therefore, the MNPK treatment promoted not only SOC sequestration but also SOC stabilization (Kou et al., 2012; Six et al., 2000a, 2000b). The C stabilized in iPOC ranged from 16.5 to 38.8% of the total microaggregate fraction (Fig. 2d), and similar proportions of iPOC content (12–30%) in the total μagg C fraction were also reported by Stewart et al. (2008).

4.2.3. Chemically protected

Significant differences among the fertilization treatments were observed regarding the C fractions with silt and clay obtained after the acid hydrolysis (H-dSilt and H-dClay). Our findings contradict those reported by Xu et al. (2016), who noted no considerable differences among the proportions of silt and clay-sized particles under different fertilization regimes in brown-earth soil. The increase in organic C of H-dSilt, H-dClay and H-μSilt across soil profile and H-μSilt in the topsoil (Figs. 2e, f; 3c) suggested that physico-chemical and chemical mechanisms played a vital role in terms of SOC protection. However, a significant proportion of SOC stabilized in the H-μSilt indicated the importance of physico-chemical protection mechanism in manure plus chemical fertilization (Fig. 3c).

4.2.4. Biochemically protected

The biochemical protection of SOC acquired through condensation and complexation reactions during decomposition in soil on a time-scale basis depends on the residue quality (Six et al., 2002). Maize residues have greater portions of lignin, tannin and smaller proportions of cellulose and hemicellulose (Yan et al., 2013) and high C/N ratio (Reis et al., 2011); hence, its decomposition is generally slower than other crops, such as soybean and wheat (Rahn et al., 1999; Scheffer, 2002). The augmentation of OC in the NH-dSilt with increases in the total SOC, especially in both topsoil layers and the first layer of the subsoil, might have been related to the greater portion of soil mass in the respective biochemically protected fractions. Compared to the CK, manure and mineral fertilization significantly enhanced the OC in the biochemically protected silt and clay by 19% and 100%, respectively (Fig. 3a, b), and this effect can be attributed to the reduced soil dry mass of fractions conjugated with biochemical protection. Additionally, fertilization could expedite the turnover of the fractions and SOC contents of NH-dSilt and NH-dClay under biochemical protection.

The aggregate-associated chemically (H-μSilt, H-μClay) and biochemically protected fractions (NH-μSilt and NH-μClay) were stabilized after occlusion or association with mineral particles within micro-aggregates and are generally referred to as stable fractions. Stewart et al. (2009) observed that free silt and clay particles respond faster to C inputs than aggregate-derived silt and clay particles (μSilt and μClay). In the present study, we noted that OC accretion in fractions under physico-chemical and physico-biochemical protection in both top- and subsoil was mainly attributed to the application of MNPK and M treatments (Fig. 3c, d and e, f). In contrast, no clear increase was noted in chemically protected silt and biochemically protected silt in 60–80 cm and 80–100 cm, respectively (Figs. 2e, 3a), implying that different protection mechanisms might be associated with aggregated and non-aggregated mineral fractions subjected to long-term fertilization.

As previously reported by Kiem and Kögel-Knabner (2003), we found greater SOC proportions in the silt-sized fractions pertinent to biochemical and chemical protection compared to the clay-sized fractions in terms of respective protection. This difference may be associated with the differential susceptibility of the respective fractions towards hydrolysability as well as the greater lignin and lesser carbohydrates concentrations in the silt-sized fraction. Additionally, Plante et al. (2006a, 2006b) considered that the stability of C associated with silt-sized particles is comparatively higher than that associated with clay-sized particles.

4.3. Relationship between fraction SOC and total SOC

4.3.1. Non-protection

Based on the current findings, the SOC content of the cPOC fraction was significantly increased under the MNPK and manure alone (M) treatments in the topsoil and subsoil, respectively. The highest rate of C accumulation (83%) was found in the 0–20 cm layer while the lowest C accumulation (30%) was found in the 60–80 cm layer (Fig. 4a), and these findings can be used to assess the rate of C accumulation with increasing total SOC. A linear relationship ($p < 0.01$) between the total SOC and the unprotected cPOC fraction across the soil profile, i.e., higher slope values, was found for the topsoil compared to the subsoil, implying the non-saturation behaviour of this fraction towards the increase in total SOC content. Stewart et al. (2008) determined a linear best fit model for unprotected cPOC fractions. Generally, the unprotected fraction is of plant origin and mostly consists of plant debris, seeds and spores and rarely black carbon or charcoal (Six et al., 2002). Hence, the unprotected fraction may be the major fraction for OC sequestration in black soils. One possible explanation may be the incorporation of fPOC in the microaggregates or association with minerals at high concentrations of OC (Stewart et al., 2008). Furthermore, the negative correlations between OC stabilized in fPOC and the total SOC could have led to the positive effect imposed by priming, i.e., the microbes consumed the OC of this fraction as a result of fertilizer application (Blagodatskaya and Kuzyakov, 2008) because the POC fraction is an important energy source for soil microbes; thus, the OC content of this fraction can be linked directly with soil fertility (Lehmann and Kleber, 2015). Furthermore, Six et al. (2002) proposed that SOC in the light fraction (fPOC in our study) showed no increment with increasing C input, which may be explained by the saturation behaviour of the non-protected fraction, which is controlled by the balance between the C input and decomposition rate of the fraction. Moreover, a saturation state of the non-protected fraction might limit different factors, such as soil temperature, hydration, and substrate biodegradation etc. (Stewart et al., 2008); hence, the regular supply of easily metabolized C ensured the increase in cPOC fraction under MNPK and M treatment in the topsoil and subsoil, respectively.

4.3.2. Physical protection

OC occlusion within micro-aggregates in subsoils is a C storage mechanism that was previously overlooked (Lorenz and Lal, 2005; Lützow et al., 2006), with more studies focusing on SOC storage by the silt- and clay-sized mineral fractions (Angst et al., 2018) through organo-mineral protection. In our study, micro-aggregation was notably found in the subsoil layers (Fig. 4c), with the highest accumulation tendency (y = 0.30) in the 40–60 cm layer. The effect of high C input was more prominent in the subsoil (having less C content) than that in the topsoil due to the well aggregation in topsoil under high OC content and presence of gluing agents (Baumert et al. (2018). Similarly, a positive linear correlation of the iPOC with increases in the total SOC was found not only in the topsoil but also in the subsoil (Fig. 4d), which is not in line with Six et al. (2002), who reported the limited capacity of iPOC because of the clay type and content available for protection in this fraction. In our study, we observed a significant amount of SOC stabilized in the iPOC, which may be due to the type and amount of clay

within microaggregates, thus indicating that the maximum SOC stabilization occurred in the iPOC (Stewart et al., 2008). Furthermore, a curvilinear relationship was found between the iPOC and clay content (Kölbl and Kögel-Knabner, 2004), which indicated that the physically protected fraction (POC) contained the maximum amount of organic carbon (Stewart et al., 2008).

Six and Paustian (2014) concluded that POC stabilization within microaggregates (iPOC in our study) is a key C stabilization mechanism. Our results are consistent with these outcomes and indicated that long-term NPK combined with organic manure in the topsoil and the application of manure in the subsoil are imperative to enhancing SOC stabilization in the iPOC. Thus, OC accretion in the iPOC showed that long-term
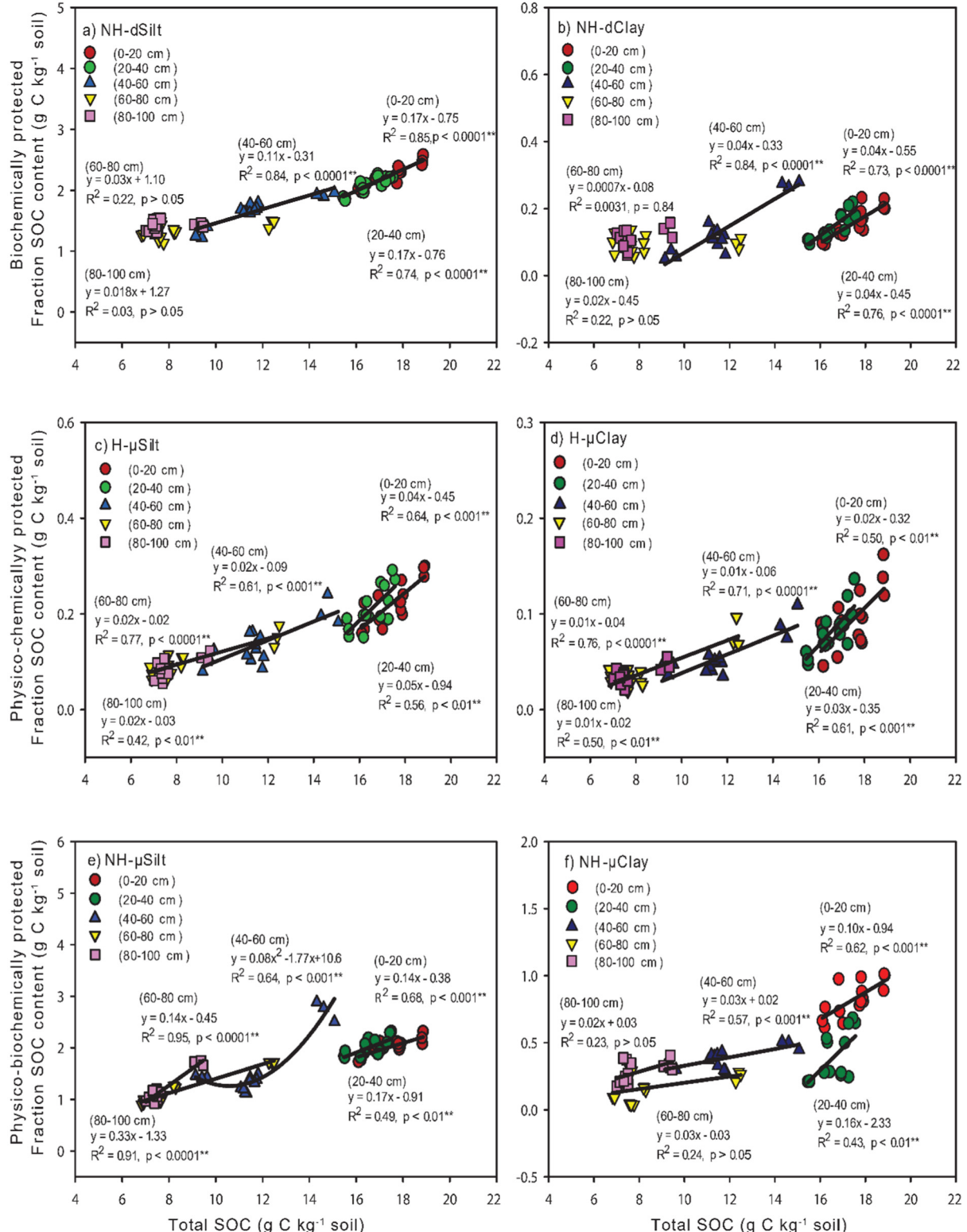

Fig. 5. Relationship between the total soil organic carbon and its constituent fractions across soil profile (g C kg^{-1} fraction) under long-term fertilization.

manure application alone or in combination with chemical fertilizer enhanced the SOC stock. Physically protected iPOC declined with the depth increment from 0 to 20 cm down to 40–60 cm and then started to increase from 40 to 60 cm down to the last layer (80–100 cm). One possible explanation could be the change of texture (from silty clay loam in the topsoil to clay loam in the subsoil). In the topsoil, we observed an accretion in the organic C content of the iPOC fraction with an increase in total organic C in the subsoil.

4.3.3. Chemical protection

The higher SOC content with increases in total SOC in the H-dSilt, H-dClay, H-µSilt, and H-µClay was only observed in the topsoil layers (Figs. 4e, f and 5c, d). However, there were variable accumulation tendencies found for all of those chemically protected fractions in the subsoil. In H-µSilt and H-µClay, a higher SOC accumulation tendency was found in both layers of topsoil than subsoil, which may be attributed to the difference in aggregation mechanisms, i.e., biological processes in top- compared to (physical processes) in the subsoil (Lorenz and Lal, 2005; Von Lützow et al., 2006). We observed significant differences among most of the treatments except MNPK in the case of C fractions associated with free silt and clay particles. Additionally, these fractions were highly linked to SOC content, implying that C fractions under chemical protection have not yet reached saturation. Furthermore, texture and mineralogy dictated the saturation of this pool (Hassink, 1997). Six et al. (2002) concluded that C associated with silt and clay at 2:1 soil was notably higher than that at 1:1 soil due to the type of clay.

4.3.4. Biochemical protection

Both of the non-hydrolysable (silt and clay) fractions were clearly ($p < 0.01$) responsive to the increase in total SOC in the topsoil and first layer of subsoil (0–40 cm and 40–60 cm, respectively) (Fig. 5a, b). This shows that these fractions were either saturated or were near saturation because their slower C turnover in comparison to unprotected (cPOC) fractions (Chung et al., 2008). However, the C accumulation tendency of the physico-biochemically protected NH-µSilt was relatively higher throughout the profile in comparison to the rest of the biochemically protected fractions in most cases (Fig. 5e), implying the importance of the NH-µSilt fraction for SOC stabilization. Biochemical stabilization or protection of SOC was attributed to the complexity of the inherent chemical nature and composition of organic materials (Six et al., 2002) which may have been associated with the reduced supply of fresh OM in the subsoil (Fontaine et al., 2007). In addition to the plant and microbe-derived nature, SOC also consists of black C, which is quite stable in nature (Roth et al., 2012). Biochemically resistant materials such as charcoal are not driven by saturation since they may not be affected by the association with silt or clay particles (Six et al., 2002; Stewart et al., 2008). Fractions under biochemical protection exhibited indications of C saturation in some layers, (Fig. 5g, h), suggesting that biochemically recalcitrant SOC may be plant-derived instead of dominated by charcoal at those layers. More organic C could be integrated within silt than clay-sized fraction, and clay particles may have preferentially remained as a part of the silt-sized microaggregates. Plante et al. (2006b) proposed that the non-hydrolysable silt fraction contains more C than the clay fraction, which may be related to the greater lignin and lower carbohydrate concentrations in the silt fraction. Furthermore, the negatively charged basal surfaces of clay minerals can serve as strong binding sites for organic matter (Haberhauer et al., 2001).

5. Conclusion

Organic manure and chemical fertilizer applications caused the accumulation of SOC contents in topsoil and subsoil, with a clear difference in the stabilization mechanisms throughout the soil profile. Most of the SOC was stabilized in non-protected and micro-aggregate derived physically protected fractions.

The low treatment effects under physico-chemical protection mechanisms and lack of treatment effect in the biochemical and physico-biochemical fractions in the subsoil layers (60–80 and 80–100) indicated the near or complete saturation of these fractions. These results demonstrate the effectiveness of manure application to sequester C, especially in subsoil for a long period of time in the black soil of China. Future studies should focus on impact assessments of variable amounts of manure on the protection mechanisms and explore the drivers behind C sequestration.

CRediT authorship contribution statement

Muhammad Mohsin Abrar: Conceptualization, Data curation, Investigation, Writing - original draft. **Minggang Xu:** Conceptualization, Resources, Writing - review & editing. **Syed Atizaz Ali Shah:** Data curation, Investigation, Methodology. **Muhammad Wajahat Aslam:** Formal analysis. **Tariq Aziz:** Methodology, Writing - review & editing. **Adnan Mustafa:** Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Muhammad Nadeem Ashraf:** Formal analysis. **Baoku Zhou:** Investigation, Resources. **Xingzhu Ma:** Resources.

Declaration of competing interest

The authors declared that there is no conflict of interest either financially or otherwise.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.138181.

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