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DECADAL NITROGEN FERTILIZATION DECREASES MINERAL-ASSOCIATED AND SUBSOIL CARBON: A 32-YEAR STUDY

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ABSTRACT

Crop residues and manure are important sources of carbon (C) for soil organic matter (SOM) formation. Crop residue return increases by nitrogen (N) fertilization because of higher plant productivity, but this often results only in minor increases of SOM. In our study, we show how N fertilization and organic C additions affected SOM and its fractions within a 32-year-long field-experiment at Puch, Germany. Five organic additions, no-addition (control), manure, slurry, straw and straw + slurry, were combined with three mineral N fertilization rates (no, medium and high fertilization), which resulted in 1·17–4·86 Mg C-input ha^{-1} y^{-1}. Topsoil (0–25 cm) SOM content increased with N fertilization, mainly because of the C in free light fraction (f-LF). In contrast, subsoil (25–60 cm) SOM decreased with N fertilization, probably because of roots' relocation in Ap horizon with N fertilization at the surface. Despite high inputs, straw contributed little to f-LF but prevented C losses from the mineral-associated SOM fraction ($\rho > 1{\cdot}6\ \mathrm{g\,cm^{-3}}$) with N fertilization, which was observed without straw addition. Above (straw) and belowground (roots) residues had opposite effects on SOM fractions. Root C retained longer in the light-fractions and was responsible for SOM increase with N fertilization. Straw decomposed rapidly (from f-LF) and fueled the mineral-associated SOM fraction. We conclude that SOM content and composition depended not only on residue quantity, which can be managed by the additions and N fertilization, but also on the quality of organics. This should be considered for maintaining the SOM level, C sequestration, and soil fertility.

KEY WORDS: soil organic matter; density fractionation; nitrogen fertilizer; manure and straw slurry; cropland soil

INTRODUCTION

Improving and maintaining soil organic matter (SOM) levels is necessary for the functioning of physicochemical and biological properties of soils (Keesstra *et al.,* 2016; Laudicina *et al.,* 2015). Poor soil physicochemical functioning can lead to land and nutrients degradations such as due to erosions (Auerswald *et al.,* 2009; Novara *et al.,* 2013; Rodrigo Comino *et al.,* 2016a). Crop residue return is important for soil conservation practices because it serve as a major carbon (C) source for improving SOM levels (Cerdà *et al.,* 2016), which is vital for nutrients conservation and soil structural development (Brevik *et al.,* 2015; Withers *et al.,* 2007).

The pool size of SOM depends on its formation from plant residues and its mineralization to CO_2 (Cotrufo *et al.,* 2015). Generally, it is assumed that increasing amounts of C inputs to soil improve SOM levels. Several field studies, however, show that increasing C inputs did not always increase SOM levels (Heitkamp *et al.,* 2012a; Novara *et al.,* 2016; Stewart *et al.,* 2008). Such phenomena of SOM change are often linked with C storage capacity of various SOM fractions (Six *et al.,* 2002).

The SOM fractions are mainly distinguished according to their protection mechanism and decomposition stage (Schrumpf *et al.,* 2013). Mostly, SOM fractions with various protection mechanisms are separated based on density and their association with soil silt and clay particles (Gunina & Kuzyakov, 2014). The physically unprotected fraction of SOM was represented by the free light fraction (f-LF), which is strongly affected by recent C inputs. Within soil aggregates, SOM is physically protected by spatial separation from decomposing microorganisms (i.e., their extracellular enzymes) and by low oxygen diffusion into aggregates, which slows its decomposition (Six *et al.,* 2002). The aggregates protected SOM fraction often termed the occluded light fraction (o-LF). Degradation of light fractions (and microbial turnover) leads to the formation of highly decomposed residues which mostly are sorbed to the silt and clay size particles that forms strongly bonded mineral-associated heavy SOM fraction (HF) (Schrumpf *et al.,* 2013). This physicochemical stabilization (after microbial substrate degradation) substantially reduces the turnover of SOM in HF. The SOM increase because of large C additions (such as crop residue) is mostly explained by C accumulation in HF. However, because of the limited physical or physicochemical protection capacity, large C additions

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may cause only minor increase of bulk SOM, especially in high-C soils (Six *et al.,* 2002; Shahbaz *et al.,* 2016). This indicates that C-input driven by, for example, high crop residue return, therefore, would not be directly beneficial for SOM.

Crop residue return is frequently increased by nitrogen (N) fertilization; however, the effect of the N-fertilization-triggered increase of C addition is not always certain (Dou *et al.,* 2016; Zhang *et al.,* 2016). This is because the stable SOM fraction (mineral-associated HF) is not mainly input-driven but also depends on residue decomposability (Barbera *et al.,* 2010; García-Orenes *et al.,* 2016). Recent views suggest that stable SOM formation is mainly related to the conversion of residue C input into microbial residues that make up most of the C associated with HF (Cotrufo *et al.,* 2013; Gleixner, 2013; Lehmann & Kleber, 2015). N-fertilization improves aboveground residue quality and decreases C/N ratio. Due to high decomposability residues with lower C/N ratios support high microbial-residues formation compared with low quality (e.g. roots), which decomposes slowly (Cotrufo *et al.,* 2013). However, in contrast to low quality, accelerated decomposition of high-quality residues (e.g., under high N availability) can promote C losses (as CO_2 emissions or leaching of dissolved C) more than stabilization within SOM (De Almeida *et al.,* 2016; Pabst *et al.,* 2016). The soil N availability and residue decomposability (with contrasting quality) can, therefore, affect the partitioning of C within SOM fractions and its distribution along soil depths.

The importance of SOM has mostly been considered for topsoil (0–25 cm, plough layer); information for subsoil is scarce (Gregory *et al.,* 2014; Ogle *et al.,* 2005). Subsoil may contains a large fraction of total organic C and is sensitive, for instance, to land use changes (Rumpel & Kögel-Knabner, 2011). The subsoil SOM stabilization is primarily affected by root growth (its exudations) and dissolved C leaching from topsoil (Don *et al.,* 2009; Rumpel & Kögel-Knabner, 2011). In general, because of relatively less exposure to environmental extreme events subsoil SOM is assumed to be more stable than topsoil (Cerdà *et al.,* 2010; Rumpel & Kögel-Knabner, 2011). Subsoil mostly had relatively high clay contents and thus subsoil SOM stabilization can be affected by the factors affecting C accumulation in topsoil mineral-associated HF (Stewart *et al.,* 2008; Hobley & Wilson, 2016). Nonetheless, no clear information is available on the long-term management effects, on total SOM change that can be explained by C stabilization in its fractions, and we know little about the ultimate effects on subsoil C.

The present study was therefore designed to explain and compare the integrated long-term impacts of C inputs (varies in quality and quantity) and N fertilization rates on topsoil (0–25 cm) SOM and its fractions, and to estimate the effects on subsoil (25–60 cm) SOM contents. Density fractionation approach was used to separate C in topsoil SOM fractions. We assumed that C storage in SOM fractions will reflect the total SOM change, but their response rate can differ. In particular, the specific goals of this study were as follows: i) to estimate and compare the changes in topsoil SOM levels due to C inputs (variable organics) and N fertilization over the study period, that is, 32 years; ii) to analyze the effects of topsoil managements on SOM accumulation in subsoil; and iii) to quantify and compare the effects of C inputs and N fertilization on partitioning of C among topsoil SOM fractions (f-LF, o-LF, and HF), and overall impact of these fractions on SOM formation.

MATERIALS AND METHODS

Site Description

The long-term (well designed and documented) field experiment (48°11′37·85″ N, 9°13′04·55″ E) is located at Puch, a village close to Munich, Germany. The study site represents a common soil type in Central Europe and covers a wide range of management options in a widespread, cereal-based crop rotation. The soil was classified as Luvisol (Parabraunerden; IUSS-WRB, 2015), derived from loess sediments with silt-loam texture (sand: 9%, silt: 73%, clay: 18%) overlying moraine deposits of the Riss glaciation. The mean annual precipitation and temperature since 1983 were 868 mm y^{-1} and 8·4°C, respectively (Heitkamp *et al.,* 2012a). Prior to the experiment, the site was used as cropland for decades or even centuries, and we, therefore, assume no major disequilibrium of C contents due to land use changes. In the plough layer (0–25 cm, maintained since 1983), the pH decreased from 6·4 to 6·1 in the studied period (1983–2015). The pH and a test with 10% hydrochloric acid indicated the absence of carbonates. To estimate the changes in SOM contents during the study period, starting conditions of SOM (in 1983) were analyzed using topsoil samples (0–25 cm). Soil samples were taken for plots receiving various organic additions but bulked across replicates and N fertilization rates (Figure 1). Therefore, in 1983, different amounts of SOM contents (g C kg^{-1}) were calculated for different organic additions that ranged 11·2 (no-addition control), 10·6 (Slurry as well as for Straw), and 10·8 (Manure and Straw + Slurry; Figure 4a); see further details in Heitkamp *et al.* (2012a).

Experimental Design

The crop rotation is silage maize (*Zea mays* L.) – winter wheat (*Triticum aestivum* L.) – winter barley (*Hordeum vulgare* L.). The experiment was laid out as a full factorial strip design with two factors ($n = 3$; Figure 1). Organic additions were considered as the first factor and N fertilization rate (three levels) as the second factor (Figure 2, Table I). In all organic additions, application of phosphorus (P) and potassium (K) fertilization was equal but varied between years according to crop needs (Hege & Offenberger, 2006). Five organic addition levels were selected for factor one: i) Control (no addition, straw removed); ii) Manure (straw removed, cattle farmyard manure applied every third year); iii) Slurry (cattle slurry application, straw removed); iv) Straw (alone straw incorporated); and v) Straw + Slurry (straw incorporated combined with slurry application).

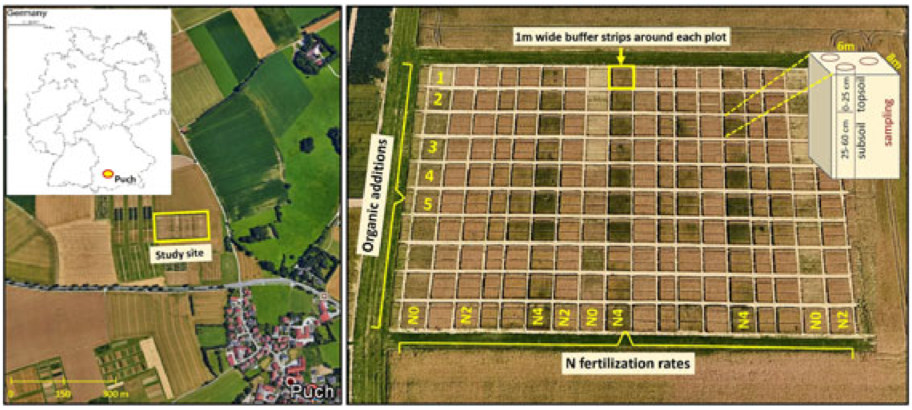

Figure 1. Aerial view of the study site (48°11′37·85″ N, 9°13′04·55″ E), located at Puch close to Munich (Germany), showing the field experimental design which consists of two factors: organic additions (1. control, 2. manure, 3. straw, 4. slurry, 5. straw + slurry) and N fertilization rates (N0, N2, and N4 represent no, medium, and high N fertilization, respectively). The expanded box shows random soil sampling points (three samples, which were bulked) for both topsoil and subsoil of individual plot. [Colour figure can be viewed at wileyonlinelibrary.com]

In August and April, before the maize crop, slurry (on average 7·6% dry matter, 5·8% OM, 4·4 kg N m^{-3}, 2·8 kg NH_4 N m^{-3}) was applied at rates of 30 m^3 ha^{-1} (corresponds to the regional practices). Since 1999, the slurry application was changed to account for more recent management of the region (Table I): to maize, two applications of 25 m^3 (each at sowing time) and additionally (in spring) to winter wheat or winter barley (before sowing) 25 m^3 ha^{-1} were applied each time. The manure was spread every third year in August before the maize crop. From 1983 to 1998, manure was applied on the fresh mass basis of 30 Mg ha^{-1} (on average 23% dry matter, 17% OM, 5·5 kg N Mg^{-1}), and, from 1998 on, the application rate was increased to 40 Mg ha^{-1}.

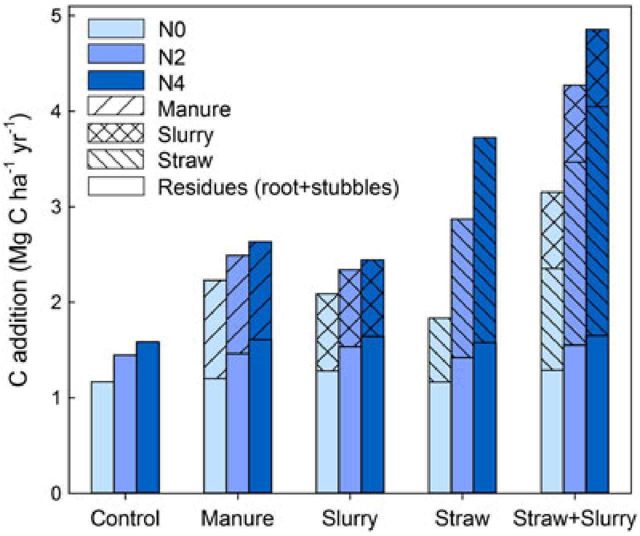

Figure 2. The contribution of organic carbon (C) sources to total annual C additions (Mg C ha^{-1}) starting from 1984. The C input by manure, slurry, straw, and crop roots (stubbles) was measured and calculated (see detail Heitkamp *et al.,* 2012a). N0, N2, and N4 represent no, medium, and high N fertilization, respectively. Control: without organic additions; Manure: straw removed, farmyard manure applied every third year; Slurry: cattle slurry application, straw removed; Straw: straw incorporated; Straw + Slurry: straw incorporated combined with slurry application. [Colour figure can be viewed at wileyonlinelibrary.com]

The second factor, N fertilization (three levels, no (N0), medium (N2), and high (N4) fertilization), varied between crops. The amount of N fertilization rates given to winter wheat and barley was different, because of the specific nutrient demands of the crops (Table I).

Until 1993, straw yield was measured in all organic addition plots, and thereafter only for organic additions with straw removal. A C content of 45% was considered for straw, and the straw yield was estimated based on the mean harvest index (grain to aboveground biomass ratio), which was remarkably stable (0·49 ± 0·01) through time and among treatments. The straw was incorporated directly into the plot of its origin. This provided realistic on-farm conditions because the amount and chemistry of straw may be directly influenced by the respective treatments. Consequently, the amount of incorporated straw increased with N fertilizer rate (Figure 2). The amount of manure and slurry was fixed and measured before addition, and their C contents were calculated by dividing organic matter by 1·92 (Larney *et al.,* 2005). The fraction of crop residues in soil added by roots and crop-stubbles was estimated as described by Heitkamp *et al.* (2012a). The used regression model of yields with crop residues does not separate between roots and stubbles. Nevertheless, it is reasonable to assume that a major part of the estimated C input of residues stems from roots. According to estimates of Bolinder *et al.* (2007), we

Table I. Mineral N fertilization rates

N-fertilization (kg N ha^{-1} y^{-1})	N0	N2	N4
1984–1998			
barley	0	60	80/40
wheat	0	50/30*	70/50/40
maize	0	100	120/80
since 1999			
barley	0	50/30	80/40/40
wheat	0	50/20/30	80/60/60
maize	0	100	120/80

*N-amounts divided by slash indicate split applications.

calculated that ca. 75% of our estimates may be contributed by roots. Therefore, we argue that the C input by crop residues in treatments without straw incorporation is clearly dominated by roots. Overall, mean annual C input for all organic additions ranged from 1·17 to 4·86 Mg C ha^{-1} y^{-1} and increased with N fertilization rate (Figure 2). For detailed information about the experiment (i.e., C input estimation and calculations, crop yields, N balances), see Hege & Offenberger (2006) and Heitkamp *et al.* (2012a).

Soil Sampling

Following the wheat harvest in August 2015, soil samples were taken from a depth of 0–25 cm (topsoil) and 25–60 cm (subsoil) with the help of a soil auger. The sampled topsoil (0–25 cm) represents the plough horizon (Ap), which is annually mixed by tillage since 1983. The subsoil (25–60 cm) was sampled to estimate the SOM accumulation, which is affected because of topsoil managements. For each organic addition, the soil was sampled in three field replicates of each selected N fertilizer rate. Within each selected N field replicate plot, three random sampling (for both topsoil and subsoil) was done, and thereafter, soil was bulked and represented one composite field replicate of each selected N fertilization level (Figure 1). Three levels of N fertilization were selected: N0, N2, and N4 (Table I). The soil samples were air dried at room temperature, sieved (<2 mm), and visible parts of large crop residue (e.g., mixed during sampling from recent crop harvest) removed. Additionally, we sampled soil with 100 cm^3 cylinders (three replicates per plot, 10–15 cm depths) to determine bulk density. The samples were oven dried (105°C), left for cooling in a desiccator, and weighed.

Density Fractionation

The SOM density fractionation approach was applied to both topsoil (0–25 cm) and subsoil (25–60 cm), but the yield of f-LF and o-LF from subsoil was too small to carry out precise measurements. Therefore, the density fractionation was only presented for topsoil, and we assumed the subsoil SOM was mainly HF-C.

To isolate the density fractions of topsoil SOM, 20 g of air-dried soil portioned into two replicates (i.e., 10 g each) was placed into a centrifugation tube. A 30 mL of sodium polytungstate solution with a density of 1·6 g cm^{-3} was added to each soil portion in the tube (Cerli *et al.,* 2012). The tube was then gently turned several times by hand; the solution was centrifuged at 4000 rpm for 40 min, and the supernatant with floating material ($\rho > 1{\cdot}6\,g\,cm^{-3}$) was filtered (cellulose acetate filter, 0·45 μm; Sartorius, Germany) and washed with ca. 1 L distilled water to obtain a salt-free f-LF. To isolate o-LF, a similar amount of sodium polytungstate solution ($\rho = 1{\cdot}6\,g\,cm^{-3}$) was added to the remaining sample after removing of f-LF. The sample was mixed with sodium polytungstate, and then the soil aggregates were dispersed by ultrasound (Retesch, Germany) with a calibrated input energy at 440 J ml^{-1}. After dispersion, the suspension was left to settle overnight and centrifuged for 40 min at 4000 rpm. The supernatant (consist of o-LF) was filtered and washed as described before. To separate sand particles (>53 μm) from the remaining sample after the separation of f-LF and o-LF, wet sieving was carried out with 53 μm mesh size. The measured organic C contents of sand fraction were very low (<0·01%), and therefore, fraction <53 μm (silt plus clay) was considered as the HF (Breulmann *et al.,* 2016). The HF was washed with distilled water, and suspended particles were precipitated by adding few drops of 0·5 M $AlCl_3$. The clear supernatant was removed, and the precipitated HF was collected. All the density fractions were dried (at 40°C, to constant weight) and weighed.

Analysis of Total Carbon Contents

Before analysis, all density fractions and topsoil and subsoil samples were dried (40°C). The soil samples were ball milled (MM2, Fa Retsch, Germany), while density fractions of SOM were homogenously manually ground by using mortar and pestle. The C contents were measured using an elemental analyzer (Vario EL II, Germany). The soil was carbonate-free; therefore, we consider total soil C as organic C. Further, we reported C measurements only as contents instead of calculating stocks because there were no significant effects of the tested factors on topsoil bulk density (organic addition $p = 0{\cdot}310$, N fertilization $p = 0{\cdot}788$, and their interactions, organic addition × N fertilization $p = 0{\cdot}209$).

The ΔSOM contents (%C) of topsoil, in relation to its initial and final SOM values, were calculated (Johnson *et al.,* 2014) as follows:

$$\Delta SOM = [(SOM_n - SOM_i)/SOM_i]*100$$

where SOM_n is the total SOM concentration ($g\,C\,kg^{-1}$) at our sampling time (2015), and SOM_i is the initial SOM contents in 1983.

Statistics

Statistical analyses were performed using R (version 2·11·1). A linear mixed model was used to test the effect of organic additions (factor 1, five levels) and N fertilization (factor 2, three rates) on top-soil and subsoil SOM and topsoil density fractions. The main two factors were used as fixed effects, while the spatial structure (strip design) was introduced as a random factor. Results are presented as means ($n = 3$) ± standard error.

To quantify changes of C contents since the beginning of the experiment, replicate values of ΔSOM were related to the annual C inputs (Mg C ha^{-1} y^{-1}; Halvorson & Schlegel, 2012; Johnson *et al.,* 2014). An exponential relationship showed the best fit according to the highest adjusted R^2 values. The effect of C additions on density fractions was additionally quantified. Linear regressions between density fractions and C inputs were applied to the whole dataset ($n = 45$) and within each organic addition among N fertilizer rates ($n = 9$). Results different at $p < 0{\cdot}01$ level are considered as significant.

RESULTS

Soil Organic Matter Contents Depending on C Inputs and N Fertilization

After 32 years of C input (1·17 to 4·86 Mg C ha^{-1} y^{-1}), the relation of changes in topsoil SOM contents (% change compared with initial SOM values) against total C inputs was best described by an exponential model (Figure 3). The total SOM contents in topsoil were significantly affected by both organic addition and N fertilizer rates (Figure 4a). The topsoil SOM contents ranged from 9·6 to 12·6 g C kg^{-1} and generally increased with N fertilization rate. Compared with initial values in 1983, the highest positive change (up to 17%) of SOM along N fertilization rate over 32 years was recorded under slurry addition, either with straw removal or incorporation (Figure 3). SOM contents remained stable under manure additions, which represent the traditional practice in the region. Highest losses of C (12–14%) were recorded when N fertilizer was applied alone (control) and or with straw (2–8%) incorporation.

The subsoil SOM contents were also affected by N fertilization and organic additions (Figure 4a). Contrary to topsoil, however, the subsoil SOM contents decreased with increasing N fertilization rate. The total SOM content in the subsoil ranged from 4·1 to 5·5 g C kg^{-1} soil. Compared with other organic additions, the control had the highest amount of subsoil SOM at each N fertilization rate (Figure 4). Note that starting conditions in 1983 were not measured for subsoil. The measured C contents in topsoil SOM were highest in the control, and therefore, we assume that the high C contents in this treatment reflect the heritage of the starting conditions. That this restriction also applies for the N fertilization is highly unlikely: while organic additions were arranged in strips, plots receiving N fertilizer rates were randomized. Thus, the highly significant effect of N fertilization ($p \leq 0{\cdot}001$) strongly indicates a real treatment effect and cannot be explained by spatial heterogeneity.

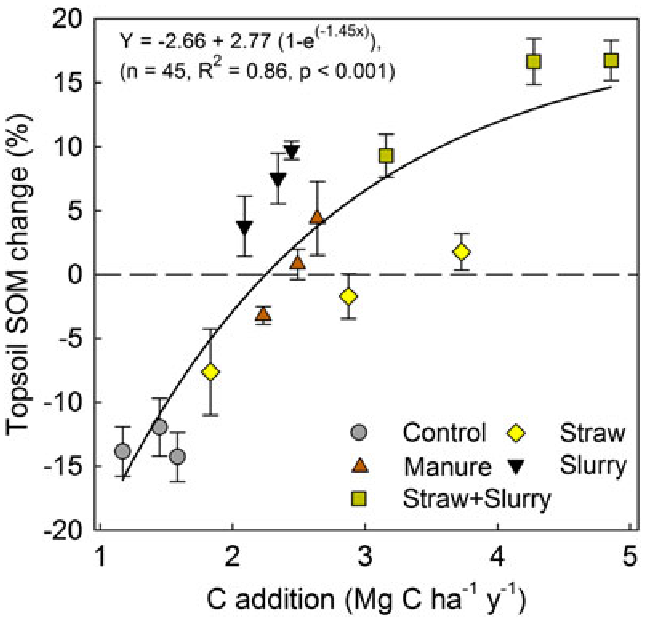

Figure 3. The curve represents the exponential relationship between the mean annual C additions and changes of topsoil soil organic matter (SOM) contents (%, between 1983 and 2015) over 32 years. The 0-line corresponds to C content in soil at the start of the experiment (32 years ago). Control: without organic additions; Manure: straw removed, farmyard manure applied every third year; Slurry: cattle slurry application, straw removed; Straw: straw incorporated; Straw + Slurry: straw incorporated combined with slurry application. Bars represent the ±standard error of the mean ($n = 3$). The probability levels of the linear mixed model describing the effects (C addition, N fertilization, interaction) for accepting the null hypothesis that the factors have no effect for the change of SOM contents (%) are as follows: C addition ≤ 0·001; N fertilization ≤ 0·001; and interactions: C addition × N fertilization = 0·409. [Colour figure can be viewed at wileyonlinelibrary.com]

Effect of C Inputs and N Fertilization on SOM Fractions

The total sample recovery after soil density fractionation was ca. 97%. Total C contents in f-LF ($p < 1{\cdot}6$) ranged from 0·26 to 0·74 g C kg^{-1} and in o-LF from 0·35 to 0·54 g C kg^{-1} of soil (Figure 5a,c). Therefore, both fractions together comprised up to 20% of total SOM. The C content of the f-LF was affected by both N fertilization and organic addition. C contents of the o-LF depended mainly on organic additions. The highest occlusion was found when slurry (alone or in combination with straw) or manure was applied. The effect of N fertilization on o-LF varied depending on the organic addition (Figure 5c,d), indicated no response (control, slurry), increased (manure, straw), and decreased (slurry + straw).

The C contents of the HF ranged from 6·5 to 8·7 g C kg^{-1}, thus constituting up to 80% of total SOM contents. The HF was affected by organic addition, with the highest C content under slurry + straw and the lowest C content in the control (Figure 5e,f). Despite the increasing C additions with increasing N fertilization, the C contents in HF decreased across N fertilizer rates, when straw was removed (Figures 2 and 5e,f). Nonetheless, upon straw incorporation, non-significant slopes (across N rates) indicated that the C associated with HF at least did not decrease with increasing N fertilization rate (Figure 5f).

DISCUSSION

Effect of C Inputs and N Fertilization on Topsoil and Subsoil SOM

Compared with the initial SOM values, slurry application (alone or with straw) increased topsoil SOM contents over 32 years (Figures 3 and 4a). This indicated that slurry contained either relatively stable C and thus was retained in soil (Shahbaz *et al.,* 2014; Weyman-Kaczmarkowa & Politycka, 2002), or that slurry improved root growth (Kandeler *et al.,* 1994). The total SOM changes remained unaffected under manure addition, presumably because this management is closest to the traditional practice, and SOM contents were therefore in dynamic equilibrium. The more labile nature of straw explains why its addition did not maintain the SOM contents at a similar level as manure or slurry. Other researchers have reported that substantial amounts of

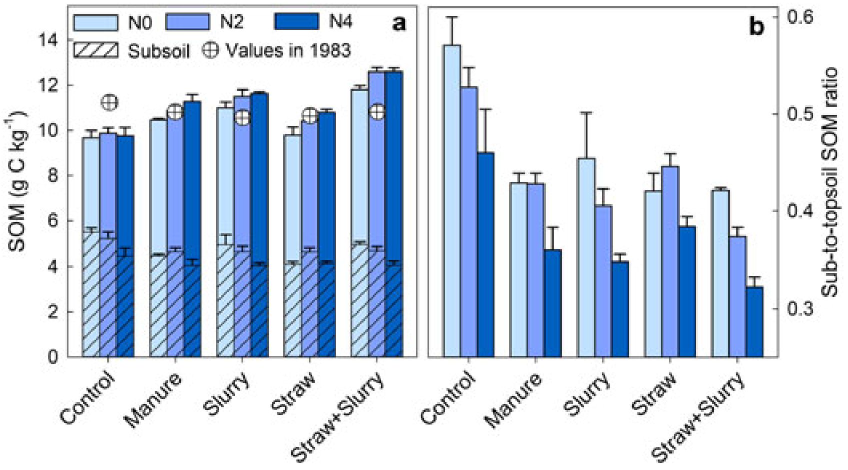

Figure 4. **(a)** Contents of soil organic matter (SOM) in topsoil (0–25 cm), subsoil (25–60 cm), and initial topsoil (32 years ago, crossed circle); and **(b)** the ratio of subsoil to topsoil SOM contents. N0, N2, and N4 represent no, medium, and high N fertilization, respectively. Control: without organic additions; Manure: straw removed, farmyard manure applied every third year; Slurry: cattle slurry application, straw removed; Straw: straw incorporated; Straw + Slurry: straw incorporated combined with slurry application. Bars represent the ±standard error of the mean ($n = 3$). The probability levels of the linear mixed model for accepting the null hypothesis that the factors have no effect are as follows: topsoil SOM (organic addition ≤ 0·001; N fertilization ≤ 0·001; and interactions: organic addition × N fertilization = 0·750), subsoil SOM (organic addition = 0·002; N fertilization ≤ 0·001; and interactions: organic addition × N fertilization = 0·323), sub-to- topsoil SOM ratio (organic addition ≤ 0·001; N fertilization ≤ 0·001; and interactions: organic addition × N-fertilization = 0·691). [Colour figure can be viewed at wileyonlinelibrary.com]

straw incorporation did not have marked effects on total SOM contents (Powlson *et al.,* 2011; Poeplau *et al.,* 2015; Novara *et al.,* 2016). SOM contents increasing with N fertilizer rates in soils without straw incorporation can be explained by the increasing crop residue return by stubbles and roots. Because straw was removed, roots with their slower decomposition were mainly responsible for the SOM increases (Heitkamp *et al.,* 2012b; Rasse *et al.,* 2005).

The exponential relationship between change in topsoil SOM and C inputs was especially evident with straw incorporation (alone or combined with slurry). This shows a decreasing overall efficiency of C accumulation with increasing amounts of aboveground biomass. One reason is a closer C/N ratio of straw with increasing N fertilizer rates because litter with closer C/N ratios decomposes faster (Ogle *et al.,* 2005). Faster decomposition, however, should also increase the amount of microbial residues, which form a major part of stable SOM (Cotrufo *et al.,* 2013; Gleixner, 2013). Recently, Castellano *et al.* (2015) linked the stabilization efficiency of the labile litter with the concept of "C saturation." That concept proposes that effective stabilization of microbial residues occurs only when mineral surfaces have free capacity for sorption. This explanation does not fit to our dataset. Although the shape of the relationship between ΔSOM and C additions (Figure 3) does fit the "C saturation" concept, the data from the subsoil and density fractions contradict this hypothesis. The subsoil is characterized by low C contents, and the SOM stabilization capacity should be high, resulting in linear relationships with SOM input. Instead, we show that C decreased in subsoils receiving more C through increased biomass by N fertilization (Figure 4).

Other studies have shown that the supply of fresh C and high N fertilization can destabilize subsoil C because of priming effects (Fontaine *et al.,* 2007; Khan *et al.,* 2007; da Silva Oliveira *et al.,* 2016). Recent findings also indicate that microbial community composition can strongly change with N fertilization (Kuslien *et al.,* 2014; Fanin *et al.,* 2015) and that microbial activity governs the integration of new C-input into the soil (Lange *et al.,* 2015). Regardless of the specific mechanism involved, the results show that subsoil SOM drops as N fertilizer rates increase (Figure 4). A similar finding was recently reported by Steinmann *et al.* (2016) on a large sample set ($n = 268$, for the Cologne-Bonn region, Germany), where despite increasing topsoil SOM contents, the subsoil SOM contents declined over 8 years. This was in contrast to the general notion that subsoil SOM is highly stabilized and insensitive to management. Moreover, fractions that are supposed to be stabilized – such as the organo-mineral HF – can also clearly be affected by management (Hobley *et al.,* 2016).

Distribution of Organic Matter in Density Fractions

The C contents of f-LF increased with N fertilizer rates and therefore with C additions (Figure 5). However, this increase of C in f-LF was much stronger when straw was removed (in control, manure, and slurry), indicating the importance of root-derived C (Figure 5a). The amount of manure and slurry application in our experiments was fixed, and thus any changes in f-LF must be due to residue C originating mainly from roots (and to a lower degree from crop stubbles). Strongly increasing C contents in the f-LF under straw removal (Figure 5) reflect an enrichment of root C (Schrumpf *et al.,* 2013) because of its slower mineralization rates compared with straw (Rasse *et al.,* 2005; Shahbaz *et al.,* 2016). f-LF is known to be most responsive to C input, especially derived by cattle manure additions (Gregorich *et al.,* 2006; Yagüe *et al.,* 2016). Nonetheless, under straw incorporation (dominant aboveground biomass), the low response of f-LF contents to C input indicated that

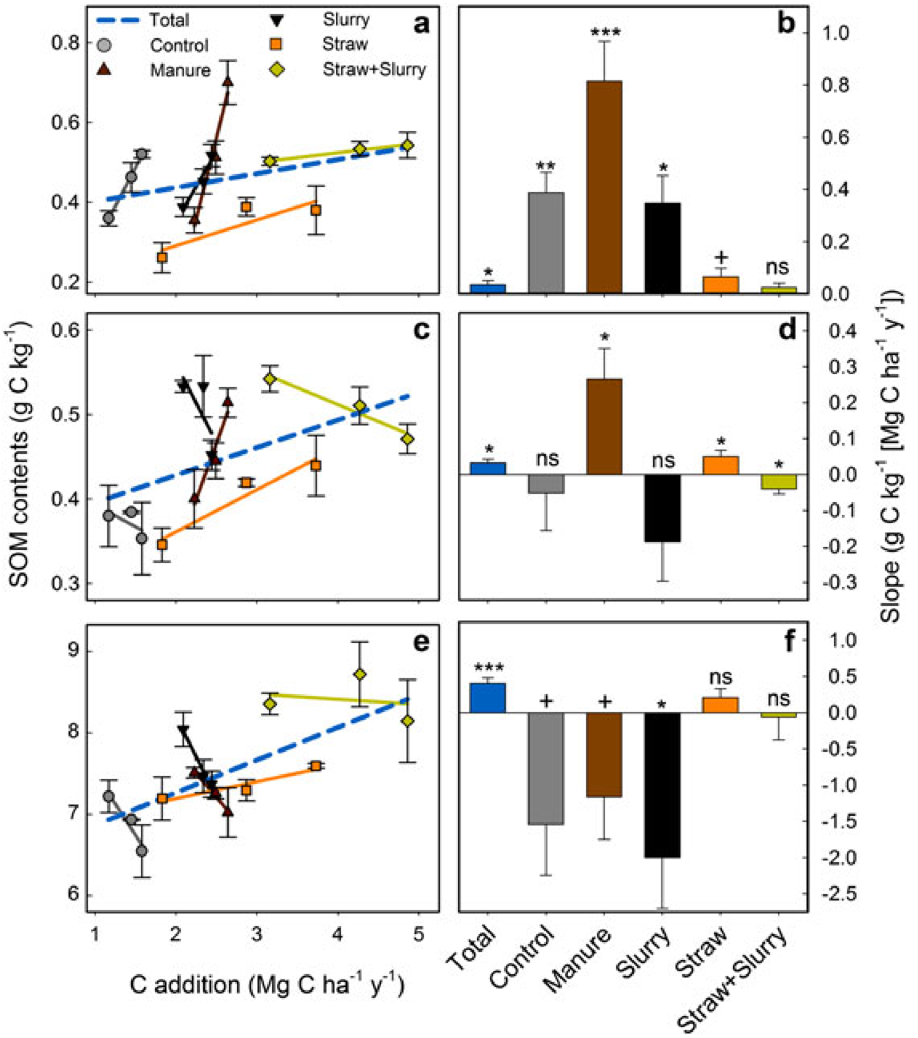

Figure 5. Soil organic matter (SOM) contents and their relationships with mean annual C addition over 32 years. The slopes of the linear regressions (±standard errors) either of the total dataset ($n = 45$) or within individual treatment (organic additions n = 9) along N fertilization rates are given. **(a)** free light fraction (f-LF) and **(b)** their slopes, **(c)** occluded light fractions (o-LF) and **(d)** their slopes**, (e)** mineral-associated heavy fraction (HF) and **(f)** their slopes. Control: without organic additions; Manure: straw removed, farmyard manure applied every third year; Slurry: cattle slurry application, straw removed; Straw: straw incorporated; Straw + Slurry: straw incorporated combined with slurry application. ***: $p \leq 0{\cdot}001$; **: $p \leq 0{\cdot}01$;*: $p \leq 0{\cdot}05$; +: $p \leq 0{\cdot}1$; ns: $p > 0{\cdot}1$. The probability levels of the linear mixed model for accepting the null hypothesis that the factors have no effect on SOM contents are as follows: f-LF (C addition ≤ 0·001; N fertilization ≤ 0·001; and interaction: C addition × N fertilization = 0·010), o-LF (C addition ≤ 0·001; N fertilization = 0·428; and interaction: C addition × N fertilization = 0·005), and HF (C addition ≤ 0·001; N fertilization = 0·0109; and interaction: C addition × N fertilization = 0·326). [Colour figure can be viewed at wileyonlinelibrary.com]

straw was rapidly decomposed. The retention of roots and the fast decomposition of straw in the f-LF may explain the behavior of the HF.

The HF-C decreased with increasing C additions and N fertilization when straw was removed. This means that root-derived C was unable to increase, or even sustain, C contents in the HF under high N fertilization (Figures 5 and 6). In a meadow ecosystem, N fertilization increased plant biomass production without changing bulk SOM or its fractions (Neff *et al.,* 2002). This was explained by a substantially increased C turnover in plant material and f-LF, followed by replacement of C in the HF by microbial residues derived from labile substances (Cotrufo *et al.,* 2013; Gleixner, 2013; Gunina & Kuzyakov, 2014). However, roots or low-quality residue inputs (high C/N) can also contribute markedly to SOM storage (Rasse *et al.,* 2005; Barbera *et al.,* 2010). Therefore, we propose that roots and straw fulfilled different functions with regards to SOM storage. On the one hand, root-derived C was retained in the f-LF and thus directly reflected the amount of C input and explained the often observed minor increase of SOM with N fertilization in cropland soils (Lu *et al.,* 2011). On the other hand, straw, exhibiting (relative to roots) faster decomposition, fueled mineral-associated SOM with microbial residues. The decreasing C contents in the HF without straw incorporation indicate that the stimulating effect of N on SOM turnover (Neff *et al.,* 2002; Qiao *et al.,* 2016) could not be counteracted by remaining C inputs. Importantly, this discussion centers on the effect of N fertilization solely within one type of organic addition. Accordingly, our finding is *sensu strictu* valid only for C inputs, which are stimulated by N fertilization.

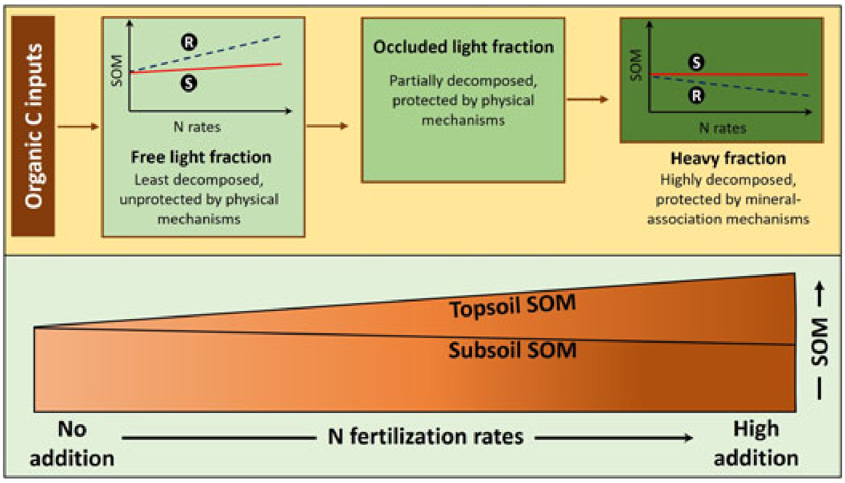

Figure 6. The stabilization of topsoil and subsoil soil organic matter (SOM) under long-term organic C inputs and N fertilization rates (lower part). The upper part represents the partitioning and stabilization (by different mechanisms) of added organic C into topsoil SOM fractions. The inset on free light fraction and heavy fraction shows the contribution of root-dominated (R in circle) or straw-dominated (S in circle) C inputs in C storage within SOM fraction along N fertilization rates. [Colour figure can be viewed at wileyonlinelibrary.com]

The C contents in o-LF showed inconsistent results and were mainly affected by organic additions (Figure 5c,d). The increase of C contents in o-LF with manure additions is consistent with other findings, where high aggregates formation to animal manure additions attributed to the increase of occlusion. The addition of manure along N fertilization can increase favorably root biomass (Hati *et al.,* 2006), which are important for aggregate formation during growth (Denef *et al.,* 2002) and decomposition (Majumder & Kuzyakov, 2010; Shahbaz *et al.,* 2016). The results, however, indicated that straw incorporation resists, while slurry addition favored the decreases of occluded C under increasing N fertilizer rates.

Overall, the SOM fractions (f-LF, o-LF, and HF) are a sensitive indicator for evaluating changes in the soil quality, because of their vital role in nutrient cycling, SOM formation, and soil structural development (Brevik *et al.,* 2015; Schrumpf *et al.,* 2013; Six *et al.,* 2002). In our study, the C content of f-LF increased and of HF decreased with N fertilization, particularly when straw was absent (Figures 5 and 6). In contrast, straw incorporation with N fertilization did not improve C contents of f-LF but prevented the loss of C in HF, which is less responsive to environmental changes. HF is considered the most important principal component for long-term SOM stabilization, and it plays a pivotal role in soil structural development because of its strong bindings effect (Six *et al.,* 2002). It has been shown that soil mineral particles (HF) with low SOM content exhibit faster dispersal in soil water compared with SOM-rich mineral fractions (Dexter & Czyz, 2000; Schjønning *et al.,* 2009). Under low HF-associated SOM contents (as without straw additions, Figure 5e), therefore, the prevalence of erosion-induced land degradation may increase possibly due to decrease of soil stability (Jiménez *et al.,* 2016; Keesstra *et al.,* 2016; Schjønning *et al.,* 2009). In addition to the positive impact on mineral-associated SOM fraction, straw incorporation also provides soil physical protection against raindrop impact, resulting in reduced sediment detachment (Auerswald *et al.,* 2009; Cerdà *et al.,* 2016; Prosdocimi *et al.,* 2016). Although the overall effect of straw removal on bulk SOM (at our low-erosion study site) was minor, our findings, however, support the potential benefits of straw incorporation (to protect decadal mineral-associated SOM from loss, and so may improve soil quality, e.g., by mulching effect and improving soil structure) at exposed site suffering from high rates of erosion (Auerswald *et al.,* 2009; Rodrigo Comino *et al.,* 2016a, 2016b; Prosdocimi *et al.,* 2016).

CONCLUSIONS

Nitrogen fertilization substantially increased C input by roots and straw into the soil because of higher plant productivity. Therefore, total SOM increased with N fertilizer rates during the 32 years in the Ap horizon (0–25 cm). Subsoil (25–60 cm) SOM, however, decreased with increasing C additions and N fertilization, probably because roots are relocated to the topsoil as the N supply increased. The increase of total SOM in the topsoil was driven mainly by the light fraction. Mineral-associated C, however, decreased with increasing C input induced by N fertilization. Straw contributed little to the f-LF but prevented C losses from the mineral-associated HF. We ascribe this finding to different functions of roots (dominating crop residue input when straw was removed) and straw: whereas under root-dominated residue input, light SOM fraction increased linearly with N fertilization; more easily, decomposable straw was transformed (from f-LF) by microorganisms and stabilized on minerals thereafter. Accordingly, the often described minor increase of SOM with N fertilization reflects the opposite response of functionally variable SOM fractions to root and aboveground residues. This calls for caution when recommending removal of aboveground crop residues, such as straw, for example, for bioenergy or other purposes. Although the overall effect of straw removals on bulk SOM can be minor, the SOM stabilized on mineral fractions, which are less responsive to environmental changes, could be lost over decades. In conclusion, organic residues increase the SOM level, but their effects strongly depend not only on their quantity (e.g., regulated by management and N fertilization) but also on the quality and functions of plant residues remaining and added on and in soil.

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