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Different Amounts of Added Litter Do Not Affect Long-Term Carbon Mineralization and Stabilization in Topsoils and Subsoils

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ABSTRACT

Background: Over half of global soil organic carbon (SOC) stocks are stored in subsoil. A larger fraction of carbon (C) input may be retained and stabilized in subsoil as compared to topsoil. This retained fraction determines C sequestration efficiency, which may also depend on the amount of C input.

Aims: This study aimed to determine how litter decomposition and stabilization differ between topsoil and subsoil and how these processes respond to increasing amounts of added litter.

Methods: A field incubation experiment was initiated at three forest sites (Braunschweig, Hohes Holz and Krofdorf). At each site, ^{13}C-enriched pre-decomposed beech litter was added at doses of 2–64 g kg^{-1} of soil and buried in mesocosms in topsoils and subsoils. After 1, 2 and 4 years, samples were excavated and size-fractionated (<20 µm).

Results: Different amounts of substrate addition did not affect the mineralization of this substrate or the formation efficiency of new fine fraction carbon (OC_{fine}). Furthermore, soil depth had little impact, with 75% and 71% of the added substrate mineralized in the topsoils and subsoils, respectively, after 4 years. Substrate addition also did not significantly enhance the mineralization of native SOC. The lowest formation of new OC_{fine} was observed at the Braunschweig site characterized by sandy soils, suggesting a potential link between soil texture and OC_{fine} formation.

Conclusions: The SOC formation efficiency is decoupled from the amount of C added; nevertheless, increasing the amount of C input will lead to a continuous linear rise in SOC stocks.

1 | Introduction

Soil organic carbon (SOC), the largest terrestrial carbon (C) pool, stores approximately three times as much C as the atmosphere (Eglinton et al. 2021). The aim of strategies focused on conserving and accumulating SOC is to counteract rising atmospheric CO_2 levels and at the same time enhance soil fertility, contributing to improved soil functions and ensuring food security (Minasny et al. 2017). Improved understanding is therefore required of the complex, interrelated processes of organic matter (OM) decomposition along the entire continuum from fresh litter input to the formation of stabilized SOC (Prescott and Vesterdal 2021). As the decomposition process progresses over time, plant litter undergoes continuous alterations in size, mass and chem-

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ical composition, driven by microbial transformations. Various mechanisms have been proposed to enhance SOC stabilization, including the study of inherent chemical recalcitrance, the inaccessibility of C to decomposers due to the interaction of molecules with mineral surfaces, and the spatial separation of decomposer and substrate (Lehmann et al. 2020; Torn et al. 1997; Von Lützow et al. 2006). However, the early and advanced stages of decomposition have often been studied separately and in isolation, leading to a fragmented understanding that hampers the ability to predict C cycling, particularly in field conditions (Prescott and Vesterdal 2021).

It is crucial that the increasing significance of estimating SOC stocks and dynamics throughout the soil profile is recognised, as more than half of total global SOC stocks are located below 20 cm (Jobbágy and Jackson 2000; De Vos et al. 2015). Deeply stored C is presumed to play a substantial role in long-term SOC storage, as C age increases with depth, ranging from a few years to potentially several hundred years in the first metre of the soil profile (Balesdent et al. 2018). SOC turnover and mineralization decline with depth due to decreased microbial activity, low SOC content, low substrate density and limited nutrient and oxygen availability (Rumpel and Kögel-Knabner 2011; Don et al. 2013). Consequently, subsoil mineralization rates are expected to be lower. Additionally, a higher SOC stability in subsoil is supported by reduced disturbance from drying–rewetting cycles, freezing–thawing cycles and tillage (Rumpel and Kögel-Knabner 2011). The primary pathways of C entry into subsoil involve belowground litter, root exudates and dissolved and particulate OC transported through larger pores or via bioturbation (Rumpel and Kögel-Knabner 2011). The decomposition rates of fresh roots have been found to decrease linearly with increasing soil depth up to 100 cm (Gill and Burke 2002). Despite these findings, the mechanisms stabilizing C in deeper layers remain incompletely understood. However, several studies have investigated subsoil C cycling and stabilization, highlighting processes such as organo-mineral associations, physical protection within aggregates and microbial adaptations to low-energy environments (Angst et al. 2021; Kleber et al. 2007; Rumpel and Kögel-Knabner 2011). These contributions underline existing knowledge while emphasizing the continuing need for integrated research into deep SOC dynamics.

C sequestration in soils is generally achieved when the formation of new SOC exceeds the loss of native or old SOC via mineralization, leaching or erosion. In contrast, Fontaine et al. (2007) suggested that increasing fresh C inputs in deeper horizons might enhance the likelihood of native SOC mineralization. This process may occur when microbes, which are limited by available resources, gain access to additional energy sources. The application of additional C inputs, as shown by Kuzyakov et al. (2000), significantly influences the mineralization of native SOC, which, in turn, affects the overall balance of SOC and ecosystem functioning. The degree of this effect is likely influenced by the amount or dose of the added C input (Z. Liang et al. 2023). Additionally, the efficiency of microbes and their capacity to decompose OM may be enhanced by increasing substrate concentrations, as this boosts microbial activity and enzyme production. Exoenzymes are necessary in order to clip macromolecules into sizes that are permeable into microorganisms. The production of such exoenzymes by microorganisms may generate a higher return on investment in a C-rich environment (Ekschmitt et al. 2005). Don et al. (2013) observed that SOC mineralization declined as compost concentration decreased in a 6-month laboratory incubation experiment. This was attributed to increased mineral soil masses that reduced the spatial proximity of microbes and their exoenzymes to the substrate. An understanding of the relationship between additional C input doses and SOC mineralization is therefore essential for evaluating the effects of OM amendments on soil processes and potential C sequestration.

In recent years, the concept of dividing SOC into two operational pools, namely, mineral-associated organic carbon (MAOC) and particulate organic carbon (POC), has gained acceptance (Lavallee et al. 2020). POC, the younger and more labile portion, forms from litter residue fragments with turnover times of years to decades, whereas MAOC, characterized by longer turnover periods (decades to centuries) and lower C/N ratios, forms through sorption to mineral surfaces or microbial necromass (C. Liang et al. 2017). During physical fractionation, however, especially by size, it is impossible to obtain pure POC and MAOC fractions. Both size fractions tend to be slightly contaminated by some material from the other fraction (Leuthold et al. 2024; Six et al. 2024). To address this, the terms fine fraction carbon (OC_{fine}) and coarse fraction carbon (OC_{coarse}) will be used hereafter (Poeplau et al. 2024), noting that OC_{fine} is typically the slower cycling pool due to dominant organo-mineral interactions (Poeplau et al. 2018). The concept of SOC saturation, suggesting that a soil's ability to store additional OC_{fine} is constrained by fine fraction and mineral surfaces (Cotrufo et al. 2019; Hassink 1997), is still a subject of debate. It has also been challenged by recent studies (Begill et al. 2023; Salonen et al. 2023; Schweizer 2022) which indicate that biomass input, rather than available mineral surfaces, is the primary constraint on SOC build-up (Janzen et al. 2022). A crucial question that remains unanswered is whether the dose of C added to soils, referring to the quantity of C input, affects SOC formation efficiency, which is the ratio between C input to soils and SOC formation. Moreover, in SOC dynamics, it is important to understand how the mid- to long-term mineralization of old SOC responds to added substrate inputs at various depths, as interactions between existing and newly added C can significantly influence the decomposition, transformation and stabilization of SOC. Long-term DIRT (Detrital Input and Removal Treatments) experiments have demonstrated that sustained changes in plant litter inputs, including both additions and exclusions (e.g., litter or root removal), can significantly alter SOC stocks, turnover rates and stabilization processes over decadal timescales (Lajtha et al. 2018; Nadelhoffer et al. 2004). These findings highlight the strong influence of organic input quantity on both the formation and persistence of SOC across ecosystems. However, relatively few such experiments (Bowden et al. 2014; Fontaine et al. 2007; Lajtha et al. 2018) have been conducted under natural conditions with depth-resolved sampling and extended timeframes, leaving many aspects of these complex processes insufficiently understood.

In this study, the fate of ^{13}C-labelled pre-decomposed beech litter added at varying doses was studied in the topsoil (7–12 cm) and subsoil (60–70 cm) at three different temperate forest sites: Braunschweig, Hohes Holz and Krofdorf. These sites, distinguished by their contrasting soil textures (sandy, loamy and clayey, respectively), provided a valuable opportunity to

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TABLE 1 | Topsoil (7–12 cm) and subsoil (60–70 cm) properties of the Braunschweig, Hohes Holz and Krofdorf sites: pH (H_2O), electrical conductivity (EC, μS cm^{-1}), sand, silt and clay content (mass%), soil organic carbon (SOC) (g kg^{-1}), total nitrogen content (g kg^{-1}) and carbon-to-nitrogen ratio (C/N).

Sites	Depth	pH (H_2O)	EC (H_2O) μS cm^{-1}	Sand	Silt mass%	Clay	Native SOC content g kg^{-1}	Soil nitrogen content	C/N
Braunschweig	Topsoil	4.0	102	49	45	7	52.4	2.34	22.4
	Subsoil	4.7	22	81	16	3	0.9	0.05	17.6
Hohes Holz	Topsoil	4.0	73	2	89	10	21.1	1.01	20.9
	Subsoil	4.4	46	2	71	28	2.2	0.25	8.8
Krofdorf	Topsoil	4.5	46	12	73	15	16.3	0.89	18.3
	Subsoil	5.2	20	4	69	27	2.5	0.33	7.6

explore how variation in soil properties and site-specific climatic conditions influence the dynamics and decomposition of added litter. An assessment was undertaken of how decomposition differs in topsoil and subsoil with the same substrate and with increasing substrate doses, and how added C is recovered in different soil C fractions, that is, OC_{fine} and OC_{coarse}, and its impact on mineralization of native SOC. It was hypothesized that (1) higher substrate doses increase mineralization rates because more substrate provides more easily accessible resources for microbes, (2) subsoil has a larger proportion of stabilized substrate as OC_{fine} than topsoil due to more available mineral surfaces that enhance C stabilization, and (3) Braunschweig site show lower OC_{fine} formation from added substrate than Hohes Holz and Krofdorf site soils because its sandy texture provides fewer mineral surfaces being available for C stabilization.

2 | Materials and Methods

2.1 | Field Incubation Experiment

2.1.1 | Study Sites

A field incubation experiment was initiated in April 2018 to investigate the long-term (planned for 20 years) transformation and fate of OM substrate in different soils in field conditions. Three experimental forest sites with different soil textures were selected (Table 1). The first site, situated on the Thünen Institute campus in Braunschweig, Lower Saxony (52°17′00.48″ N 10°26′43.91″ E), features a sandy soil within a mixed forest of beech (*Fagus sylvatica* L.) and oak (*Quercus robur* L.). This site is classified as dystric Arenosol based on the World Reference Base for Soil Resources (WRB). The second site, Hohes Holz near the village of Eggenstedt in Saxony-Anhalt (52°05′11.42″ N 11°13′19.68″ E), has a silty texture and is classified as a haplic Luvisol (WRB) under a mixed beech forest. It serves as a monitoring station for the Integrated Carbon Observation System (ICOS; Heiskanen et al. 2022) and the Terrestrial Environmental Observatories (TERENO; Zacharias et al. 2011), supporting climate and ecosystem research. The third site, located in Krofdorf-Gleiberg in Central Hesse (50°40′56.77″ N 8°38′35.53″ E), is characterized by a higher clay content, is classified as stagnic Luvisol (WRB), is covered by a beech-dominated (*F. sylvatica* L.) vegetation and is a monitoring site of the intensive environmental forest monitoring (ICP Forests Level II; Fleck et al. 2016). As shown in Table 2, the abiotic characteristics of temperature, precipitation and soil moisture varied between the three sites during the experimental period from March 2018 to March 2022.

2.2 | General Soil Parameters

To determine the pH value, 5 mL dried fine soil was mixed with 25 mL distilled water in an overhead shaker at 20 rpm for 2 h in 50 mL centrifuge tubes. Subsequently, the suspension was not left to stand as per DIN ISO 10390 but was centrifuged for 10 min. The pH value was measured using a pH meter (ProLab 4000, SI ANALYTICS, Weilheim) in combination with a pH electrode (Blue Line 28 pH, SI ANALYTICS, Weilheim). For time and cost efficiency, a pH/EC measurement robot (SP2000, SKALAR, Breda) was used. Soil texture was assessed by separating the suspended fine particles through sieving and sedimentation, classifying the soil particles into clay (<2 μm), silt (2–63 μm) and sand (>63 μm but <2000 μm) fractions. SOC and total nitrogen (N) were analysed using dry combustion with an elemental analyser (Leco TruMac and RC612), on bulk soils only.

2.3 | Preparation of Labelled Substrate

In order to track the fate of OM under field conditions, isotopically labelled organic material was mixed with local soil material and introduced into the soil in mesocosms. The used substrate was derived from an earlier experiment in which highly labelled beech litter (^{13}C content of 10 at%), was mixed with non-labelled beech litter at a ratio of 1:10 and then spread on the forest floor at an experimental site. A net (2 cm mesh size) was installed on top of the litter layer to, first, prevent surface translocation by wind and, second, avoid dilution of the labelled litter over time by the seasonally fallen litter. For more details, see Liebmann et al. (2020). This litter was left in the forest for 2 years to undergo natural degradation. After 2 years of field incubation, the litter was collected, and dissolved OM was extracted from it by applying cold water extraction. Afterwards, the remaining decomposed litter was dried and homogenized to create a uniform size of <1 mm. It had a ^{13}C content of 1.44% ± 0.02% atom%, a

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TABLE 2 | Abiotic factors of the Braunschweig, Hohes Holz and Krofdorf experimental sites by depth: temperature (°C), precipitation (mm a^{-1}) and moisture (%) metrics for topsoil (7–12 cm) and subsoil (60–70 cm).

Site	Depth	Mean annual temperature °C	Mean annual precipitation mm y^{-1}	Mean soil temp incubation period	Max. temp period °C	Min. temp period	Mean soil moisture incubation period %	Max. mois-ture	Min. moisture
Braunschweig	Topsoil	9.2	599	11.4	22.1	1.6	10	20	6
	Subsoil			10.5	18.4	2.0	9	20	5
Hohes Holz	Topsoil	9.8	598	9.7	19.5	0.2	25	33	12
	Subsoil			9.6	15.5	2.5	22	32	16
Krofdorf	Topsoil	7.4	700	10.4	19.1	1.7	21	31	7
	Subsoil			9.7	16.0	3.3	31	39	18

Note: Soil temperature and moisture during the incubation period were measured using sensors installed at the experimental sites. Mean annual temperature and precipitation values were derived from nearby weather stations or previously published data (BMEL 2016; Landesanstalt für Umweltschutz Sachsen-Anhalt 2017; Müller 2017; Sauerbeck 2005). The Hohes Holz site also includes an Integrated Carbon Observation System (ICOS) eddy-covariance station, though site-specific meteorological data were not yet available at the time of this study.

total C content of 42.9% and a C/N ratio of 26.4 and was used as the substrate for the current study, allowing us to focus on later stages of decomposition rather than the initial phases. To prevent dilution of the applied ^{13}C-labelled material with fresh litterfall during the field phase, the substrate was later embedded below the surface in mesocosms installed at two depths (7–12 and 60–70 cm), as described in the following section.

2.4 | Mesocosm Preparation and Installation

Soil samples were collected from the three selected forest sites, that is, Braunschweig, Hohes Holz and Krofdorf, in March 2018 from two depth increments: topsoil (7–12 cm) and subsoil (60–70 cm). The soil samples were dried at 40°C and sieved through 2 mm. Cylindrical mesocosms with a height of 3.8 cm, a diameter of 8.0 cm and a volume of 191 cm^3 were prepared. The top and bottom ends were covered with fine-meshed net (with a mesh size of 530 µm, 50% open area) to prevent loss of material but to permit exchange with the environment, allowing fine root penetration, water and gas flow, as well as colonization by microorganisms (Figure S1). The soil used for each mesocosm was collected from the corresponding site and depth and then homogenized. Different quantities of substrates (dried) were added to the soils: 8, 16, 32 and 64 g substrate kg^{-1} soil were added to the topsoil samples (equalling a carbon addition of 3.4, 6.9, 13.7 and 27.5 g kg^{-1}), whereas the amounts applied to the subsoils were 2, 4, 8 and 16 g substrate kg^{-1} soil (equalling a carbon addition of 0.9, 1, 7, 3.4 and 6.9 g kg^{-1}). Adding more substrate significantly increased SOC content in both topsoil and subsoil. In the topsoil, applying up to 64 g kg^{-1} more than doubled SOC levels compared to the control, except in Braunschweig where SOC increased by 1.4 times. In the subsoil, the highest substrate addition of 16 g kg^{-1} resulted in SOC levels that were 8.7 times higher in Braunschweig, 4.1 times higher in Hohes Holz and 3.7 times higher in Krofdorf (Table S1). The repetition of 8 and 16 g kg^{-1} allowed a direct comparison between topsoil and subsoil in absolute terms of substrate doses. The 2 g kg^{-1} dose equals the average annual litterfall in a temperate deciduous forest per area size of the mesocosm if concentrated to the depth of the mesocosm of 3.8 cm (Brumme et al. 2021).

In order to mix the soil materials with substrate material, approximately 6–8 kg of soil from each depth and site was mixed in a bucket with the appropriate amount of substrate (Figure S1). This mixed sample was then used to fill 30 mesocosms. Each substrate-amended sample had three replications. In addition, three replicates of the control samples, none of which included the isotopically labelled substrate, were installed in both depths. Therefore, a total of 900 mesocosms (i.e., 300 mesocosms at each site) were buried at both depths in April 2018 for 20 years, with the intention being to conduct sampling 10 times at different intervals. The soil profile pit was excavated, and then lateral pockets were created at 60–70 cm in order to place the mesocosms in their respective locations with undisturbed soil on top of each mesocosm. The pit was then sealed (Figure S1). For the topsoil mesocosms, the uppermost 10 cm of soil was carefully removed, the mesocosms were installed, and the soil was replaced on top of the mesocosms. Two of the mesocosms in each pocket were marked with a red plastic string. The year of the planned sampling

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can be read from a sign at the end of the string on the surface. The pit was then sealed.

2.5 | Mesocosm Sampling and Sample Preparation

Mesocosm sampling was conducted on three dates: after 1 year (April 2019), 2 years (April 2020) and 4 years (April 2022). For sampling, the pockets in the upper and lower soil layers are dug up, but the entire pit remains closed (Figure S1). Fifteen mesocosms from the topsoil and 15 mesocosms from the subsoil were removed at each site (15 mesocosms × 2 depths × 3 sites × 3 years). The samples were weighed and dried at 40°C for at least 2 weeks. Gravimetric water content was calculated after weighing the dried soil samples. In order to analyse the samples for C and N contents as well as for stable C isotopes, dried fine soil was ground in a planetary mill with an agate vessel (<60 μm).

2.6 | SOC Fractionation and ^{13}C Isotopic Analysis

The particle-size fractionation method was employed to distinguish between the coarse fraction (OC_{coarse}), which is the operationally defined fraction similar to POC, and the fine fraction (OC_{fine}), which resembles MAOC (Lavallee et al. 2020). For the clayey and loamy soils, around 10 g of the soil sample was immersed in 150 mL distilled water and subjected to ultrasonic dispersion (100 J mL^{-1}) (Just et al. 2021). The dispersed soil suspension was wet-sieved through a 20 μm sieve by a continuous flush of distilled water with an aerosol pump sprayer to separate the fine (<20 μm) and coarse (>20 μm) fractions until the water passing through the sieve became transparent. The coarse fraction retained on the sieve was collected in a vessel and subsequently oven-dried at 60°C. Afterwards, the soil suspension that passed through the sieve was treated with 0.8 g L^{-1} $CaCl_2$, serving as a flocculation agent, and was then subjected to centrifugation at 4000 rpm for 20 min. After centrifugation, the supernatant was discarded, and the fine fraction (<20 μm) was transferred into a glass vessel for drying at 60°C. Separating liquid and solid phase prior to drying has the advantage that drying is much faster and a more complete recovery of the dried fine fraction can be achieved. For sandy soil (85%–90% sand content) with a low proportion of aggregated soil particles, the pre-treatment procedure differed slightly. Here, 10 g dried soil sample was immersed in 100 mL distilled water, and instead of applying ultrasonic dispersion, an overhead shaker was used for 20 min at 20 rpm. The rest of the procedure remained the same as that for the loamy and Krofdorf soils. Lastly, all the fractions were weighed and milled. Average mass recovery was 96% ± 7%.

In this study, the ^{13}C abundance, expressed as relative abundance (δ^{13}C) compared with the international Vienna Pee Dee Belemnite standard, was measured using an isotope ratio mass spectrometer (DeltaPlus, Thermo Fisher Scientific, Waltham, MA, USA) coupled to an elemental analyser (CE Instruments FLASH EA 1122 NA 1500, Wigan, UK). The procedure involved weighing the milled samples in tin capsules according to their expected C content and adjusting it according to the sensitivity of the device (30 μg C per sample). A two-pool mixing model was applied to calculate the fraction of new substrate-derived OC_{fine} and OC_{coarse} at time *i*, according to Balesdent et al. (1987), using atom%:

$$f\ (\text{substrate}) = \frac{\text{AT\%}\,(\text{fraction}) - \text{AT\%}\,(\text{control})}{\text{AT\%}\,(\text{substrate added}) - \text{AT\%}\,(\text{control})}, \quad (1)$$

where AT% (fraction) is the atom per cent of bulk soil or fractions (either OC_{coarse} or OC_{fine}) from substrate addition treatments, AT% (control) is the atom per cent of bulk soil or fractions with no substrate addition at the time of sampling, and AT% (substrate added) is the atom per cent of added substrate (1.44 ± 0.02 atom%, with a δ^{13}C value of 315‰) at the start of the experiment. Average C recovery (calculated as the sum of all four fractions divided by the SOC measured in the unfractionated bulk soil sample collected after mesocosm sampling and prior to the start of the fractionation process) of the topsoils and subsoils was 101% ± 9% and 102 ± 10, respectively. On the basis of the coarse and fine fraction contents, four different pools were calculated: native OC_{fine} and new OC_{fine} as well as native OC_{coarse} and new OC_{coarse}, with new and native referring to the added substrate-derived C and native C of the soils, respectively.

The absolute priming effect, that is, mineralization of native SOC induced due to substrate addition, was calculated as

$$\text{Additional native SOC mineralisation} = \Delta\text{SOC}_{\text{native}}^{\text{treatment}} - \Delta\text{SOC}_{\text{native}}^{\text{control}}, \quad (2)$$

where

$$\Delta\text{SOC}_{\text{native}}^{\text{treatment}} = \text{SOC}_{\text{native,initial}}^{\text{treatment}} - \text{SOC}_{\text{native,final}}^{\text{treatment}} \text{ and } \Delta\text{SOC}_{\text{native}}^{\text{control}} = \text{SOC}_{\text{native,initial}}^{\text{control}} - \text{SOC}_{\text{native,final}}^{\text{control}},$$

where $SOC_{initial}^{control}$ represents the initial SOC at the time of incubation without any substrate addition, $SOC_{final}^{control}$ represents the final SOC after 4 years without any substrate addition, and $SOC_{initial}^{treatment}$ and $SOC_{final}^{treatment}$ represent the initial and final amounts of native SOC with substrate addition. OC_{fine} formation efficiency was calculated as the percentage of substrate added, where new OC_{fine} represents the amount of OC_{fine} formed 4 years after the addition of varying quantities of substrate:

$$\text{OC}_{\text{fine}}\ \text{formation efficiency}\ (\%) = \frac{\text{new OC}_{\text{fine}}}{\text{Substrate added}} \times 100 \quad (3)$$

2.7 | Statistical Analysis

Statistical analysis was conducted using R version 4.1.1 (R Core Team 2020) with *ggplot2* (Wickham 2011) for data visualization. A two-way analysis of variance (ANOVA) was performed using the basic *stats* package (R Core Team 2020) to evaluate the effects of sites (Braunschweig, Hohes Holz and Krofdorf) and substrate addition (8, 16, 32 or 64 g substrate kg^{-1}) on SOC dynamics in topsoil and subsoil, which were analysed separately. The interaction between site and substrate addition was also examined. To assess depth differences, a comparison was made using only the 8 and 16 g substrate kg^{-1} treatments, as these were common across both depths. Prior to conducting ANOVA, Levene's test for homogeneity of variance groups was met. Post hoc pairwise was performed on the raw data using the *car*

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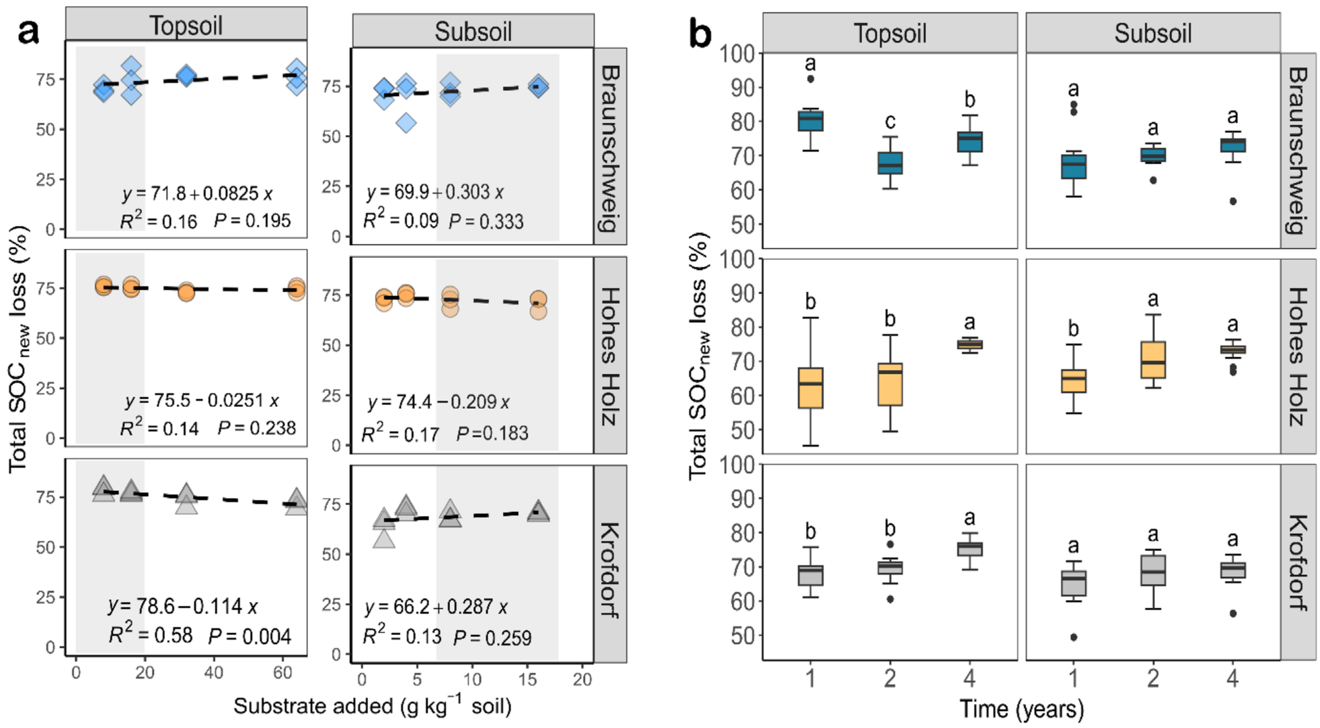

FIGURE 1 | Loss of added substrate (SOC_{new}) (% of initial SOC): (a) with varying doses of added substrate (g kg^{-1}) after 4 years. Each point represents one mesocosm ($n = 3$ replicates per treatment × 3 sites × 2 depths = 72 mesocosms). Shaded regions highlight comparisons between depths for the same substrate doses. Please note that the x-axis follows an exponential scale rather than a linear one; (b) the trend in loss of added substrate over a period of 4 years ($n = 12$ per boxplot and total $n = 216$). (*) This value seems to be an outlier due to non-comparable decomposition conditions on the edge of the experimental site where the mesocosms of Year 1 were buried. Different lowercase letters above bars (a–c) indicate significant differences between total SOC new loss with time, based on Tukey HSD tests at $\alpha = 0.05$. SOC, soil organic carbon.

package (Fox et al. 2012) to ensure that the assumption of equal variances across the comparisons was conducted using Tukey's honestly significant difference (HSD) test, available through the *multcomp* package (Hothorn et al. 2016), to identify significant differences between texture, substrate addition and depth. Statistical significance was determined at $\alpha = 0.05$. Data manipulation and preparation were carried out using *dplyr* (Wickham et al. 2023), with standard deviations reported alongside mean values to provide comprehensive error estimations and ensure clarity in the interpretation of results.

3 | Results

3.1 | SOC Loss Due To In Situ Incubation of Added Substrate in Topsoil and Subsoil

No significant differences were observed in the effect of increasing substrate doses on SOC_{new} loss in either the topsoils ($p = 0.8$) or subsoils ($p = 0.5$) after 4 years (Figure 1a, Table S4a,b). All the sites experienced a similar loss of newly added substrate-derived SOC in their topsoils ($p = 0.7$). However, at the different sites, significant differences in subsoil losses were observed, with Hohes Holz (loamy) showing the highest losses ($p = 0.03$), followed by Braunschweig (sandy) and Krofdorf sites. As each soil texture is represented by only one site, the observed differences reflect site-specific conditions rather than broad effects of soil texture alone. After 4 years, the overall loss of newly added substrate was significant across depths ($p = 0.001$) when the same amounts (8 and 16 g kg^{-1}) had been added to both topsoil and subsoil. However, this significant loss was observed in the Krofdorf site only, where the topsoils exhibited greater losses of substrate-derived SOC compared with the subsoils (Figure 1a, Table S4c). Site played a significant role in the decomposition rates, with the Braunschweig (74% ± 7% in topsoil and 67% ± 1% in subsoil) experiencing the highest substrate-derived SOC loss compared with the Hohes Holz (70 ± 8 in topsoil and 70% ± 6% in subsoil) and Krofdorf (69% ± 6% in topsoil and 67% ± 5% in subsoil) over 4 years. The Braunschweig site consistently experienced higher temperatures and lower moisture contents than the Hohes Holz and Krofdorf sites throughout the study (Table 2). Over time, the substrate loss increased after 4 years (Figure 1b, Table S3), except in the Braunschweig topsoils. After just 1 year, an average of 70% of the added substrate in topsoil and 66% in subsoil was already lost. Over 4 years, these numbers increased to losses of 74% in topsoil and 71% in subsoil (Figure 1b, Table S3).

3.2 | Transformation of Added Substrate Into OC_{fine} and OC_{coarse}

In absolute terms (Figure 2a), the formation of new OC_{fine} increased significantly and linearly with higher substrate doses ($p < 0.001$) in both topsoils and subsoils. Among the studied sites, Hohes Holz exhibited the highest new OC_{fine} formation ($p < 0.001$), significantly surpassing Krofdorf and Braunschweig. Notably, depth effects were evident at Hohes Holz for substrate

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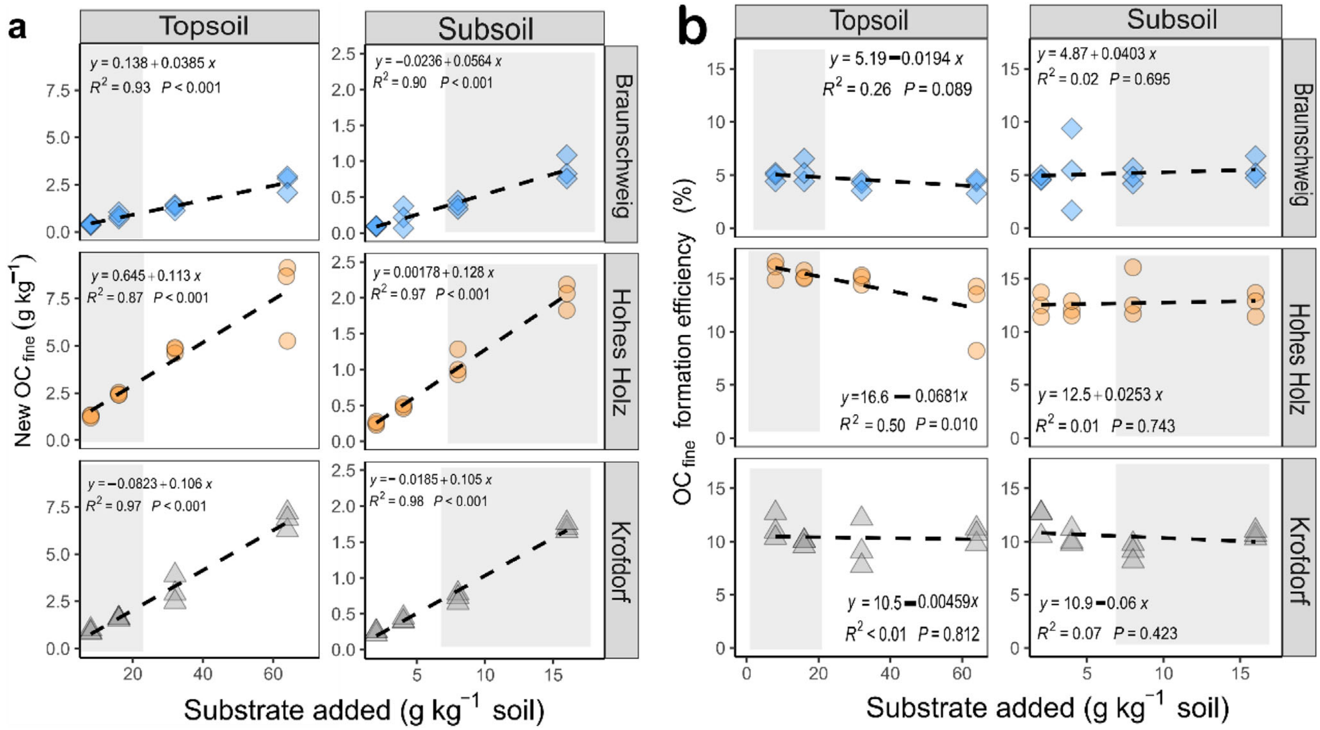

FIGURE 2 | (a) Newly formed fine fraction carbon (OC_{fine}) (g kg^{-1}) derived from varying doses of added substrate (g kg^{-1}) after 4 years in absolute terms; and (b) the efficiency of OC_{fine} formation (%), calculated as the ratio of new OC_{fine} to the substrate added, with different substrate doses added after 4 years (total $n = 72$). Each point represents one mesocosm ($n = 3$ replicates per treatment × 3 sites × 2 depths = 72 mesocosms). Shaded regions highlight comparisons between depths for the same substrate doses. Please note that the *x*-axis follows an exponential scale rather than a linear one and *y*-axis scales differ between topsoil and subsoil panels.

doses of 8 and 16 g kg^{-1}, with significant differences observed between topsoils and subsoils ($p = 0.009$). In contrast, no such depth-related differences were found at the Krofdorf and Braunschweig sites (refer to Table S6a–c).

When analysed in relative terms (Figure 2b), depth differences were significant only at Hohes Holz ($p = 0.004$), highlighting its distinct response compared to Braunschweig and Krofdorf, where no depth-related variation was observed. This indicates that the efficiency of new OC_{fine} formation in Braunschweig and Krofdorf soils was consistent across depths when the same substrate was applied. Interestingly, increasing substrate doses did not alter the efficiency of new OC_{fine} formation in either topsoils or subsoils ($p = 0.8$). Overall, Hohes Holz soils consistently demonstrated the highest formation efficiency, followed by Krofdorf, with Braunschweig soils performing the lowest in both absolute and relative terms (see Table S6a–c).

On average, 75% ± 3% and 71% ± 4% of the added substrate was lost (either respired or mobilized as dissolved organic carbon) from the topsoils and subsoils, respectively, after 4 years (Figure 3a). A significant ($p = 0.002$) decrease in SOC_{new} loss with increasing substrate doses was observed only in the Krofdorf topsoil (Figure 3a). However, only 10% ± 4% and 9.5% ± 4% of the added substrate were quantified into new OC_{fine} in topsoils and subsoils, respectively. The Hohes Holz site showed the highest new OC_{fine} (14.5% ± 2% in topsoils and 12.6% ± 1% in subsoils), followed by Krofdorf (10.3% ± 1% in topsoils and 10.5% ± 1% in subsoils), and with the lowest percentage observed in the Braunschweig soils (4.6% ± 0.8% in topsoils and 5.1% ± 1.7% in subsoils) (Figure 3a,b). Hohes Holz soils also had the highest OC_{fine}/OC_{coarse} ratio of the total recovered new C (Figure 3b).

New OC_{coarse} was recovered at a rate of 15% ± 4% and 19% ± 6% in the topsoils and subsoils, respectively. The highest recovery of added substrate as new OC_{coarse} was found at the Braunschweig site (21% ± 4% in topsoils and 22% ± 6% in subsoils), followed by the Krofdorf site (14% ± 3% in topsoils and 21% ± 4% in subsoils), and the lowest was found in the Hohes Holz (11% ± 2% in topsoils and 14% ± 3% in subsoils) (Figure 3a,b). Increasing the doses of substrate did not significantly affect the recovery of OC_{coarse} in either topsoil ($p = 0.1$) or subsoil ($p = 0.6$) (Figure 3). However, the depth differences were significant at the Krofdorf and Hohes Holz sites ($p = 0.0002$), whereas Braunschweig showed recovered almost similar amounts of OC_{coarse}.

3.3 | Mineralization of Native SOC Due To Substrate Addition

The additional loss of native SOC resulting from substrate addition was generally minimal across all substrate doses except in the topsoils at the Braunschweig site (Figure 4). The Braunschweig topsoils were identified as outliers due to the significant spatial variability observed over the years (Figure S2). Therefore, the results for the Braunschweig soils should be interpreted with caution and not overemphasized, as decomposition conditions at the edges of the experimental site might have influenced

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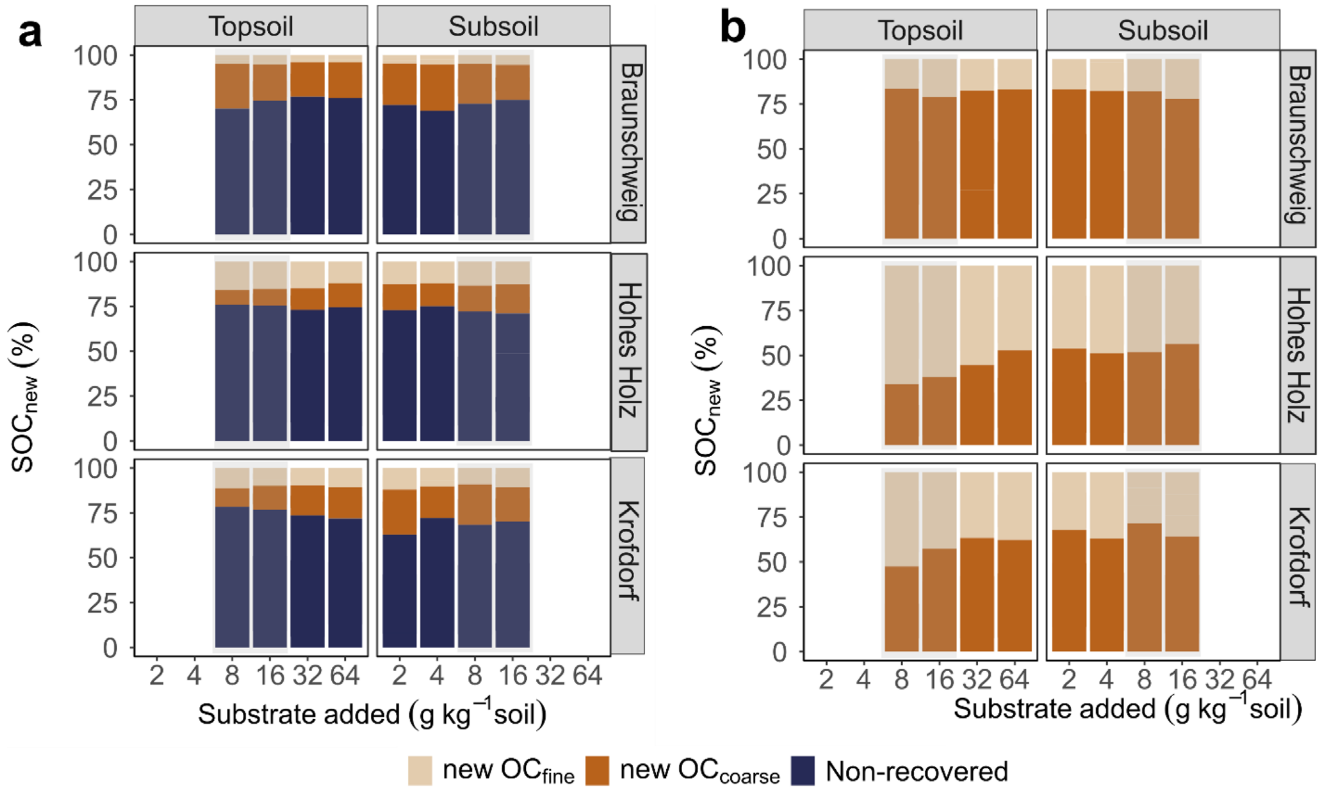

FIGURE 3 | Mass balance-derived proportions of added substrate that were (a) recovered as fine fraction carbon (OC_{fine}) or as coarse fraction carbon (OC_{fine}) or non-recovered (lost via respiration and leaching), and (b) recovered as fine fraction carbon (OC_{fine}) or coarse fraction carbon (OC_{fine}) after 4 years of field incubation. Please note that the *x*-axis follows an exponential scale rather than a linear one. SOC, soil organic carbon.

these findings. After 4 years, the increased loss of native SOC completely offset the effects of substrate addition, probably due to the heterogeneous site characteristics, which may include slightly higher temperatures compared with the other soil types.

Across all sites, increasing the dose from 8 to 64 g kg^{-1} did not significantly ($p = 0.7$) affect the loss/mineralization of native SOC in the topsoil. In contrast, in the subsoils, increasing the substrate dose from 2 to 8 g kg^{-1} ($p = 0.03$) and from 2 to 16 g kg^{-1} ($p < 0.001$) significantly decreased the extent of native SOC loss. With regard to soil depth, there were no significant differences within the Hohes Holz or Krofdorf sites ($p = 0.9$). Both SOC_{new} (the change in SOC specifically due to substrate addition) and SOC_{total} (the overall change resulting from substrate addition) exhibited a significant ($p < 0.001$) positive response to increasing substrate addition, showing similar trends across all treatments in terms of depth and texture, except for Braunschweig soils. This suggests that additional loss of native SOC did not substantially offset the positive impact of substrate addition (Figure 4).

4 | Discussion

4.1 | No Effect of Doses on Substrate Decomposition or SOC Formation

Despite a substantial gradient in the amount of substrate addition, no general effect of dose on substrate decomposition was observed after 4 years. Across all substrate doses, 70%–75% of the added substrate had already been respired after the first year (Figure 1b). This temporal dynamic in litter decomposition was expected and is in line with Gregorich et al. (2023), for example, who found a 75% loss of ^{13}C-labelled barley litter after 1 year. Although pre-decomposed litter used in this study had undergone dissolved organic carbon extraction in a prior step to remove labile components, it still exhibited substantial decomposability. This may be due to residual labile compounds or the effect of homogenization, which could have released additional easily decomposable and soluble materials, contributing to the high decomposition rates observed during the first incubation year (Bird and Torn 2006). What was unexpected, however, was the absence of any effect of different doses on decomposition. Only the minimum dose of 2 g kg^{-1} was within the range of leaf litter C input that can be expected in temperate forests if directly incorporated into mineral soil (Brumme et al. 2021). The highest dose was thus at least 32 times above the maximum leaf litter input that is common in temperate forest systems and is constrained by net primary production (NPP). Poeplau et al. (2024) elucidated NPP as the ultimate constraint for SOC formation and concluded that the ability of soils to stabilize SOC is not a limiting factor but depends on soil texture, which modifies the fraction of NPP that is retained and stabilized as SOC. This new conceptual view is supported by the present results that show that even with an unrealistically high input of litter substrate, the soil's ability to "digest" and retain this substrate and transform it into a stabilized fraction is not exceeded. This is consistent with a short-term laboratory experiment with less extreme C application rates in which it was observed that substrate decomposition was not affected by

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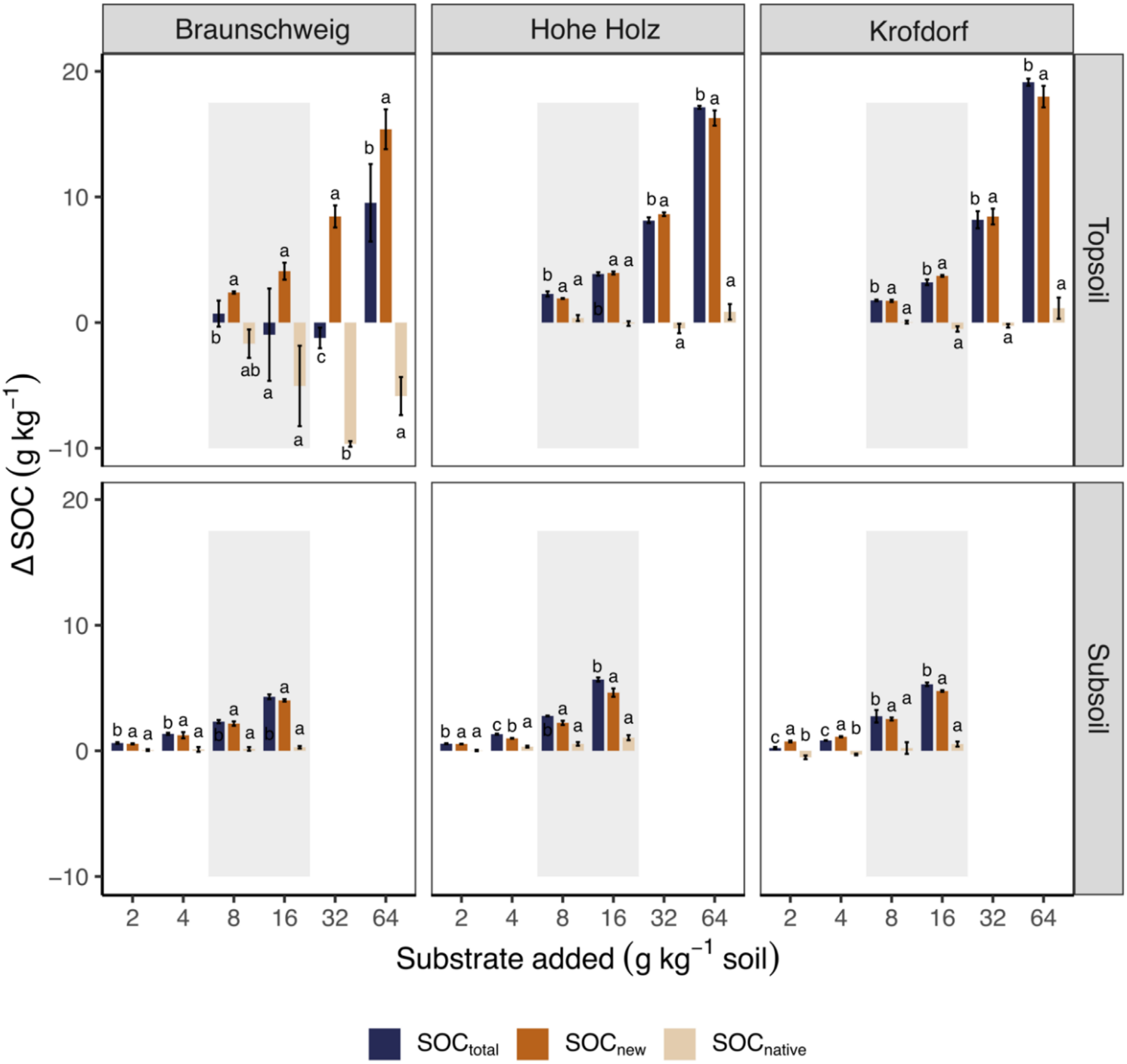

FIGURE 4 | Mass balance-derived proportions of added substrate that were recovered as SOC_{new} (the change in SOC specifically due to substrate addition) and SOC_{total} (the overall change resulting from substrate addition) along with SOC_{native} over 4 years ($n = 72$) (see Equation 2). SOC_{native} values indicate the extent of mineralization of native SOC, with negative values representing increased mineralization of SOC_{native} and positive values indicating reduced mineralization, leading to SOC_{native} stabilization. The term $\Delta SOC_{treatment} - \Delta SOC_{control}$ (g kg^{-1}) represents the difference in SOC change between treated and control samples over the 4-year period. (*) SOC_{native} mineralization in sandy topsoils seems to be an outlier due to non-comparable decomposition conditions at the edge of the experimental site where mesocosms were buried. Shaded regions highlight comparisons between depths for the same substrate doses. Please note that the *x*-axis follows an exponential scale rather than a linear one. Different lowercase letters above bars (a–c) indicate significant differences between SOC fractions within each substrate level, based on Tukey HSD tests at $\alpha = 0.05$. SOC, soil organic carbon.

six varying application rates of maize residues (0–6 g kg^{-1}) in a 70-day soil mesocosm experiment, and likewise for ryegrass in a 90-day soil incubation experiment (Mendoza et al. 2022). This indicates that, under conditions of this study, the similar proportion of added substrate was retained in the soil relative to the added quantity. However, this pattern may not generalize to all soil types or environments, particularly those with extreme conditions such as permafrost or water-logged conditions, where SOC dynamics differ substantially. Additionally, OC_{fine} was formed linearly with substrate addition amounts in absolute terms (Figure 2a). However, when examined relative to the added substrate, the efficiency to form OC_{fine} derived from substrate addition was almost the same for all doses. This suggests that the formation of OC_{fine} was not influenced by varying substrate doses, highlighting a constant C sequestration efficiency across all doses of substrate addition (Figure 2b). C sequestration efficiency is the ratio between C input and retained or stabilized SOC. One possible explanation is that microbial communities adjusted to

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the different input levels. Although microbial properties were not measured directly, previous studies have shown that increased C inputs can enhance microbial activity and enzyme production, supporting consistent decomposition and transformation of SOC (Allison and Vitousek 2005; Blagodatskaya and Kuzyakov 2008; Cotrufo et al. 2013; Fontaine et al. 2003).

4.2 | Minimal Influence of Added Substrate on SOC Dynamics

The addition of new substrate did not strongly affect the overall SOC dynamics in most soils, showing a minimal impact across all substrate doses. This could be because microbes preferred newly added labile carbon to existing SOC. As shown in Figure 4, the changes in SOC_{new} and SOC_{total} were almost identical, indicating that the added substrate did not play a major role in altering SOC dynamics. Furthermore, findings from Xiao et al. (2015) demonstrated a non-linear response when fresh plant litter was added to grasslands under field conditions, with declining efficiency at higher litter inputs. They proposed two mechanisms for this non-linear response: (1) an increase in microbial growth and extracellular enzyme synthesis, leading to higher mineralization of both new and native SOC, and (2) a microbial preference for newly added labile plant litter over existing SOC. These results suggest that, under our experimental conditions, microbes preferentially mineralized the added pre-decomposed substrate over native SOC, except in Braunschweig topsoils. Despite being partially decomposed, the substrate retained labile components that were rapidly respired, consistent with microbial preference for more accessible C sources. In contrast, some studies, such as those by Mendoza et al. (2022) and Shahbaz et al. (2017), found strong interactions at higher C input levels, where increased microbial activity was linked to enhanced mineralization of native SOC. For instance, a 90-day soil incubation experiment with ^{13}C-labelled ryegrass concluded that additional C at higher doses stimulates microbial activity, leading to enhanced decomposition of native SOC through co-metabolism (Kuzyakov et al. 2000). Overall, these findings highlight the complexity of SOC dynamics and underscore the important roles played by microbial preferences, along with environmental conditions and experimental setups, in SOC dynamics.

The results from the Braunschweig topsoil should be interpreted cautiously. After 1 and 2 years, native SOC loss due to substrate addition was minimal, but a sudden increase in SOC loss occurred in some treatments after 4 years. This sharp shift, combined with the lack of significant SOC loss in the subsoil at the same site and at other sites, suggests the observed effect may be an artefact. The Braunschweig soils typically show high variability in SOC because they contain a large proportion of particulate OM, which complicates calculations based on mass balance approaches using different samples.

4.3 | Small Differences in SOC Dynamics Between Topsoils and Subsoils

Mineralization of SOC_{new} appeared to be similar at both investigated depths, with only slightly lower decomposition rates observed in the Krofdorf subsoil compared with the Krofdorf topsoil (Figure 1a). This finding is in agreement with that of Solly et al. (2015), who in a litterbag experiment reported that the decomposition of root litter did not vary with depth after 2 years. In their experiment, the root litter was buried at 5, 20 and 35 cm depths in three German beech forest plots. These findings indicate that there is similar potential in both topsoil and subsoil to mineralize the newly added C. Besides a lower OC density and a time lag for microbial biomass to adapt to high C inputs in subsoils, it is mainly the missing continuous high input of bioavailable C that is low in subsoils compared with topsoils. Such reduced bioavailable C was found to be the main factor that hampered the decomposition of subsoil SOC (Kirschbaum et al. 2021). Also for root litter, similar results were reported by Sanaullah et al. (2011), who buried litterbags with ^{13}C and ^{15}N-labelled wheat root material at depths of 30, 60 and 90 cm in loamy agricultural soil for 3 years and found that the remaining root-derived C and N were similar across all soil depths after 3 years. In the present study, no significant differences in the native SOC loss due to added substrate were found between topsoil and subsoil except for the Braunschweig soil. This contrasts with Shahzad et al. (2019), who reported a faster turnover of deep soil C in response to fresh inputs. Additionally, Fontaine et al. (2007) and Pries et al. (2018) attributed the stability of C in subsoils to the absence of fresh C inputs. In contrast, Salome et al. (2010) indicated that C mineralization in subsoils is more constrained by the physical separation between the substrate and decomposers. Salome et al. (2010) also observed that the proportion of total C mineralized was similar or even increased with depth, suggesting that abiotic conditions in the subsoil may even be more favourable in subsoil with stable moisture and temperature conditions.

In constant abiotic conditions, Wordell-Dietrich et al. (2017) found that added root litter decomposed faster in their topsoil samples than in their subsoil samples. Soil abiotic conditions such as temperature and water content (60% WHC) were standardized in their experiment. In the annual data on temperature and moisture that applied in the present study (Table 2), a greater variability was observed in topsoils than in subsoils, where temperatures were more stable. This reduced temperature variability in subsoils marginally slows decomposition rates, leading to a slight increase in C stocks (Kirschbaum et al. 2021). Therefore, significant gains in SOC through C burial via abiotic effects are unlikely. Similarly, soil moisture levels could play a role, with both excessive dryness and excessive wetness potentially impeding decomposition processes (Chen et al. 2000). At the sites in the present study, the maximum soil moisture was similar across different depths, with the Braunschweig topsoils experiencing a range of 6%–20% and Braunschweig subsoils ranging from 5% to 20%. In the Hohes Holz soils, topsoils had moisture levels between 12% and 33%, whereas subsoils ranged from 16% to 32%. In Krofdorf soils, subsoils exhibited higher moisture contents (18%–39%) than topsoils (7%–31%), most probably due to the stagnic conditions at the site. Slightly lower decomposition rates in Krofdorf subsoils as compared to topsoils may be explained by oxygen deficiency under stagnic conditions. Despite these variations, periods of restricted mineralization due to low moisture may be compensated by subsequent periods with adequate moisture. The interplay between temperature and moisture further complicates predictions, making it challenging to determine optimal conditions for C storage at depth. Nonetheless, the present data indicate that abiotic limitations for mineralization in subsoils

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are comparable to those in topsoils, with only minor differences observed across soil types (Table 2).

It should be noted that this mesocosm experiment partly isolated the treated soils from their surroundings, allowing mesofauna activity but excluding larger macrofauna. This setup provides insights into potential processes but cannot fully capture the complexities of natural soil systems in which C input is derived from belowground and aboveground litter and is also leached litter from the forest floor. In natural conditions, C inputs to subsoils and topsoils differ significantly in type, quantity and frequency, influencing decomposition dynamics and hampering comparability between subsoils and topsoils with regard to their impact on decomposition rates. Thus, this rather artificial experimental setup with a standardized unique substrate was needed to elucidate the impact of soil depth on decomposition dynamics. However, absolute numbers of decomposition rates may differ from actual litter decomposition rates in forest systems.

4.4 | Site-Specific Differences in SOC Formation Efficiency

This study demonstrated clear site-specific differences in the fate of substrate-derived C, with the Braunschweig site losing more of the added substrate compared with the Hohes Holz and Krofdorf sites (Figure 1b). A likely explanation for this difference could be the contrast in soil texture: The Braunschweig site was the most coarse-textured site with the highest sand content. This would well align with previous findings (Angst et al. 2021; Poeplau et al. 2015), revealing that Braunschweig sites (sandy soils), with fewer reactive surface areas, are less capable of retaining new C from decomposing plant material. Rumpel et al. (2004 also studied the fate of organic compounds by examining the distribution and composition of OM in soil horizons of different soil types under forest cover and demonstrated an increase in SOC stabilization in fine-textured soils, particularly in deeper soil layers, highlighting a strong association of organic C with the clay fraction (<0.63 μm). This OC_{fine} is considered the more stabilized fraction of SOC (Lavallee et al. 2020). Research indicates that soil texture, especially the presence of fine particles (<20 μm), limits SOC stabilization in the OC_{fine} fraction by providing mineral surfaces for OC accumulation and serving as nuclei for new micro-aggregate formation (Lehmann and Kleber 2015; Totsche et al. 2018). Accordingly, the formation of OC_{fine} was lowest in the Braunschweig soil. It is important to note that this should not be interpreted as C saturation, as the Braunschweig topsoil and subsoil had different initial C contents but similar OC_{fine} formation rates (Table 1). Moreover, the highest amounts of OC_{coarse} were observed in the Braunschweig soil, indicating that a higher proportion of the added C remained unaltered (new OC_{coarse}) after 4 years. Previous studies have also found that the OC_{coarse}/OC_{fine} ratio is highest in Braunschweig soils, underscoring the role of silt and clay particles in driving OC_{fine} formation rates (Begill et al. 2023). Although the Braunschweig soils in the present study showed higher amounts of new OC_{coarse} compared with the Krofdorf and Hohes Holz soils, suggesting a similar overall recovery of substrate-derived SOC across all soil textures, the OC_{coarse}/OC_{fine} ratio varied significantly. Despite this variation, the difference in this ratio did not notably affect the amount of respired C. This intriguing finding suggests that OC_{coarse} and OC_{fine} may have shared similar stability characteristics in this study, warranting further investigation of their respective roles in SOC dynamics.

5 | Conclusions

This study provides new insights into the dynamics of SOC stabilization in response to varying substrate input rates across different soil depths. Its findings show that after 4 years, on average, 75% of the substrate added to topsoils and 71% of that added to subsoils was lost (either respired or leached). This result suggests a surprisingly consistent rate of C turnover across different soil depths, except for the Krofdorf soils, and thus a steady potential for the stabilization of new C. Notably, OC_{fine} formation efficiency was unaffected by the dose or amount of substrate applied, implying that increasing C application rates will lead to a proportional rise in SOC stocks. This could have important implications for C management and C sequestration measures if it is also valid for other ecosystems. There seems to be no overload that reduces C sequestration efficiency, that is, the fraction of added C that is stabilized. The addition of substrates had minimal impact on native SOC, showing little effect on overall SOC dynamics. The Braunschweig site exhibited the lowest formation of fine fraction carbon (OC_{fine}), most likely underscoring the significant role played by soil texture in SOC stabilization. This result emphasises the overarching importance of the amount of OM that enters the soil: the more, the better. This sheds new light on the huge potential of soils to "digest" C inputs and thus contributes to improving understanding of controlling mechanisms for SOC formation.

Author Contributions

The experiment was designed by Axel Don. Laboratory work was performed by Neha Begill. Data analysis was conducted by Neha Begill, Christopher Poeplau, and Axel Don. The initial draft of the manuscript was prepared by Neha Begill. All co-authors, including Christopher Poeplau, Henning Meesenburg, Corinna Rebmann, and Axel Don, critically reviewed the manuscript and approved the final version.

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Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article. Additionally, the data that support the findings of this study are openly available in Zenodo.

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