

Priming effects decrease with the quantity of cover crop residues – Potential implications for soil carbon sequestration

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ABSTRACT

Meta-analyses suggest a global potential of cover crops to increase soil organic carbon (SOC) stocks, yet with a large variation across studies, which underlines the need to understand the effect of cover crops on carbon (C) sequestration under specific soil and climate conditions. We studied the C sequestration potential from cover crops, based on a Danish long-term field experiment (LTE) initiated in 1997, where SOC and C in the fractions of particulate organic matter (POM) and mineral associated organic matter (MAOM) was measured to 1-m depth. Next, we performed a mesocosm study where the fate of ¹⁴C-labeled cover crop residues (fodder radish, *Raphanus sativus* L.) and SOC priming were traced in two texturally similar soils from the LTE with different SOC concentrations (2.0 vs. 2.6% SOC). The results showed that cover cropping for up two decades had negligible effect on SOC in POM and MAOM fractions. Yet, the mesocosm study showed considerable overall SOC increases (20–25% of added C) when the cover crop C input exceeded rates of 0.2–0.3 mg C g⁻¹ in the two soils. This was due to a combination of new SOC formation and priming effects shifting from positive to negative. The input rates of 0.2–0.3 mg C g⁻¹ correspond to the C input from cover crops with an aboveground yield of approximately 0.7–1.1 Mg dry matter ha⁻¹, which is a level not always achieved at the field site. The combined observations from the field and mesocosm study suggest that SOC buildup was not constrained by soil C saturation, but rather by low cover crop productivity and/or positive priming effects. Therefore, agricultural management practices (e.g., species choice and sowing time) should be adopted to achieve a sufficient cover crop C input to secure that the positive priming effect is not exceeding the rate of SOC formation.

1. Introduction

Cultivation of cover crops to replace bare fallow in autumn and winter is an established agricultural practice to prevent nitrogen leaching and eutrophication of aquatic ecosystems (Thorup-Kristensen et al., 2003; Christianson et al., 2021). More recently, it has been suggested to exploit cover crops also to sequester soil organic carbon (SOC) and thereby mitigate climate change by reducing atmospheric carbon dioxide (CO₂) concentrations (Kaye and Quemada, 2017; Abdalla et al., 2019). Various meta-analyses have suggested a global potential of cover crops to increase SOC stocks in topsoils (typically defined as the upper 20 or 30 cm soil layer) at rates of 0.3–0.6 Mg carbon (C) ha⁻¹ yr⁻¹ (Poeplau and Don, 2015; Abdalla et al., 2019; Jian et al., 2020). The effect in subsoils remains uncertain due to scarcity of data (Poeplau and Don, 2015; Tautges et al., 2019; Jian et al., 2020). It is recognized that

multiple environmental factors, such as soil properties and climate, can influence the effect of cover crops on the SOC stock (Poeplau and Don, 2015; Abdalla et al., 2019; Jian et al., 2020; McClelland et al., 2021), thus stressing the need to quantify cover crop C sequestration potential under specific farming conditions and with the subsoil being included (Qin et al., 2023).

Despite the general potential of cover crops to improve SOC stocks, some studies reported that cover cropping had no effect or even reduced SOC (Poeplau and Don, 2015; Abdalla et al., 2019; Jian et al., 2020). Such results can be influenced by uncertainties related to spatial heterogeneity of SOC at the sampling sites combined with only few years duration of the cover cropping practice (Poeplau and Don, 2015; Prommer et al., 2020). However, based on a long-term crop rotation field experiment (LTE) initiated in 1997 (Olesen et al., 2000), De Notaris et al. (2021) also reported negligible changes in topsoil SOC contents

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after implementing cover crops for up to 23 years. As the global cover crop area is increasing, e.g., from about 4 to 6 million ha during 2012–2017 in the US (Christianson et al., 2021), it is important to understand and potentially manage the factors that control the effect of cover crops on C sequestration (Qin et al., 2023). Modelling studies have suggested that managing cover crops for higher biomass production and thus C input to the soil (e.g., via increasing the growth period) is an effective way towards higher SOC sequestration (McClelland et al., 2021; Qin et al., 2023), but knowledge is still lacking on the pathway(s) linking cover crop C inputs to dynamics and stabilization of SOC.

The effect of cover crops on SOC stocks is determined by the balance between the contribution to formation of new SOC and depletion of existing (native) SOC (Bradford et al., 2008; Liang et al., 2017). The C derived from cover crops can exist in soils as relatively short-lived particulate organic matter (POM) as well as long-lived mineral-associated organic matter (MAOM) after microbial modification and/or transformation (Cotrufo et al., 2013; Liang et al., 2017; Lavallee et al., 2020). However, according to current hypotheses, the accumulation of MAOM can be restricted by C saturation of mineral surfaces (e.g., silt and clay fractions) for physico-chemical C stabilization of MAOM (Castellano et al., 2015; Cotrufo et al., 2019). At the same time, the cover crop C inputs may enhance microbial activity and accelerate mineralization of native SOC by positive priming effects (Kuzyakov et al., 2000; Kim et al., 2020; Prommer et al., 2020). This implies that overall C sequestration via cover cropping will only occur when stabilization of new SOC exceeds the positive priming effect or when the cover crop C input leads to negative priming (Liang et al., 2017). Depending on, e.g., previous main crop species and weather conditions, the cover crop C inputs show wide inter-annual variation (Komainda et al., 2016; De Notaris et al., 2018; Seita et al., 2022), which may affect the intensity and direction of priming effects with a consequence on the overall C sequestration (Bradford et al., 2008; Blagodatskaya and Kuzyakov, 2008). In particular, low C inputs of cover crops could lead to positive priming, where the depletion of native SOC exceeds the formation of new SOC from cover crops (Bradford et al., 2008; Blagodatskaya and Kuzyakov, 2008; Liang et al., 2017). Yet, there is lack of empirical data to evaluate the importance of priming effects for low doses of cover crops C input, which may often occur in agroecosystems, constrained by, e.g., climate and length of the growing period (Poeplau and Don, 2015; Mendoza et al., 2022; Qin et al., 2023). This knowledge gap hampers a comprehensive understanding of the potential of cover crops to increase SOC stocks.

To evaluate the overall effect of cover cropping on SOC storage, we quantified SOC to 1-m soil depth in a LTE (Olesen et al., 2000), where previous studies showed non-significant cover crop effect in the topsoil (Hu et al., 2018; De Notaris et al., 2021). We fractionated the SOC into POM and MAOM to investigate the C saturation status of the soils, and to test whether the lack of SOC increase after long-term cover cropping was due to topsoil C saturation (Castellano et al., 2015; Lavallee et al., 2020), or rather was due to an insufficient annual cover crop C input, potentially in combination with a positive priming effect (Bradford et al., 2008; Blagodatskaya and Kuzyakov, 2008; Hu et al., 2018). To further explore this dichotomy, we performed a mesocosm study with different doses of ^{14}C labeled cover crop residue added to topsoils from the LTE with different initial organic C (OC) concentrations (i.e., 20 vs. 26 g OC kg^{-1} soil, hereafter referred as 2.0 and 2.6% SOC), but otherwise similar texture and mineralogy. Thereby, at least the 2.0% SOC soil should hold an unsaturated capacity for MAOM stabilization, i.e., with 2.6% SOC being attainable as a minimum. We hypothesized that an overall SOC increase could be achieved by sufficient cover crop C inputs, i.e., when the formation of new SOC in the form of MAOM (Cotrufo et al., 2013; Castellano et al., 2015) exceeded the priming effect (Bradford et al., 2008; Blagodatskaya and Kuzyakov, 2008).

2. Materials and methods

2.1. Field study of long-term effects of cover cropping on SOC

2.1.1. Experimental design and soil sampling

The present study was part of the LTE on organic and conventional rotations with cover crops, initiated in 1997 (Olesen et al., 2000) at Foulum, Aarhus University, Denmark (56°30' N, 9°34'E). The site has an Atlantic climate with a mean annual air temperature of 8.3 °C and precipitation of 746 mm (Cappelen, 2019). The Foulum soil is a typical moraine deposit, classified as Mollic Luvisol according to FAO world reference base (IUSS Working Group WRB, 2015; De Notaris et al., 2021), with an upper Ap horizon (0–40 cm) of sandy loam with approximately 8% clay (<2 μm), 30% silt (2–63 μm) and 59% sand (63–2000 μm).

The LTE had a factorial randomized block design based on two blocks with distinct initial SOC concentrations (2.0% vs. 2.6% SOC), which was likely due to management history (cropland vs. pasture) prior to the establishment of the LTE (Djurhuus and Olesen, 2000; De Notaris et al., 2021). Four selected long-term managements were studied, previously designated as OGL+CC, OGL–CC, CGL+CC and CGL–CC (Liang et al., 2022), where OGL and CGL refers to organic and conventional treatments, respectively, including grain legumes (GL), and the CC notation refers to treatments with (+CC) and without (–CC) cover crops. The OGL and CGL systems were introduced in 1997 and 2005, respectively, and the two systems had the same 4-yr crop rotation, but were fertilized with animal manure and mineral fertilizer, respectively (Hu et al., 2018; De Notaris et al., 2021).

We studied the LTE in the first year of the sixth crop rotation cycle (2019–2022), where the recent crop was spring barley (*Hordeum vulgare* L.). The crop sequence during the preceding four years (2015–2018) was spring barley, faba bean (*Vicia faba* L.), spring wheat (*Triticum aestivum* L.) and spring oats (*Avena sativa* L.). The cover crops in the organic system consisted of a mixture of perennial ryegrass (*Lolium perenne* L.), chicory (*Cichorium intybus* L.), white clover (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.), which were undersown in spring (May). The cover crop in the conventional treatment was either perennial ryegrass undersown in May or a mixture of fodder radish (*Raphanus sativus* var. *oleiformis* L.) and winter rye (*Secale cereale* L.) sown after harvest of the main crop (De Notaris et al., 2021). All cover crops were overwintering and terminated by harrowing in spring, which was followed by ploughing to a depth of ~20 cm before sowing the main crop.

Soils were sampled in November 2019, i.e., after continuous cover cropping (or not) for 23 years in the OGL and 15 years in the CGL system. For each treatment, topsoils (~2 kg) from each of four field replicated plots (6 × 3 m) were sampled by pooling 8 soil cores (0–25 cm, 2 cm diameter) taken in a W-shaped pattern across the fields. Subsoils from 25–50 and 50–100 cm depth were sampled using a 1-m long soil auger (4-cm diameter) mounted on a Gator Utility Vehicle (John Deere). Two of these larger soil cores were retrieved from each plot and divided into the desired depth intervals. In total, the sampling procedure resulted in 48 soil samples that were carefully mixed and sieved (2 mm) before subsamples (~0.3 kg) were air-dried for analysis of total OC and OC in different physical fractions (described in section 2.1.2). For the topsoil (0–25 cm), field-moist subsamples (~0.7 kg) was stored at –18 °C for analyses of microbial biomass C (MBC) and β -glucosidase activity.

2.1.2. Density and size fractionation

Soil samples were separated into three organic matter fractions according to Haddix et al. (2020) representing (i) the free POM (fPOM), (ii) the coarse heavy fraction (63–2000 μm) with occluded POM and sand (oPOM + sand), and (iii) the fine fraction (<63 μm) with MAOM. Briefly, 10 g air-dried soil samples were gently shaken in 35 mL of 1.8 g cm^{-3} sodium polytungstate (SPT) and centrifuged (3260 g, 30 min). The floating fPOM in the supernatant was transferred with a cut-off 3-mL disposable pipette to a pre-weighed cellulose filter (1.6 μm) in a

funnel and rinsed with deionized water four times to remove any residual SPT. Next, the supernatant was discarded and the soil pellet was dispersed in 35 mL of 0.5% sodium hexametaphosphate by reciprocal shaking (100 rev min⁻¹) for 18 h to break macro- and microaggregates. Dispersed samples were rinsed with deionized water in a 63- μ m sieve to separate the coarse fraction (oPOM + sand) from the fine fraction (MAOM). All fractions (fPOM, oPOM + sand and MAOM) were dried at 50 °C for four days and ball-milled for determination of OC concentrations using a Thermo Flash 2000 NC Analyzer (Thermo Fisher Scientific, Delft, The Netherlands). To verify the C recovery of the fractionation procedure, original samples of the 2-mm sieved air dry soil were analysed concurrently for SOC.

2.1.3. Microbial biomass C and β -glucosidase activity

Substrate-induced respiration (SIR) was measured as a proxy for active microbial biomass (West and Sparling, 1986). Briefly, samples of 12 g moist soil (10 g dry weight) were weighed into 100 mL flasks and pre-incubated at 35% water holding capacity (WHC) for 5 days at 20 °C. The flasks were covered by punctured parafilm to prevent water loss, but allow for aeration. After pre-incubation, soils were amended with glucose solution to 7.5 mg C g⁻¹ soil and a final water content of 60% WHC (West and Sparling, 1986; Lin and Brookes, 1999). The flasks were closed with butyl stoppers and headspace gas (2 mL) was sampled hourly for 6 h. The 2-mL gas samples were transferred to 6-mL helium-filled Exetainer vials and concentrations of CO₂ were determined by gas chromatography as described in detail by Petersen et al. (2012). Microbial biomass C was calculated from the SIR rates according to Anderson and Domsch (1978).

To test for potential legacy effects of increased microbial C mineralization in the cover crop treatments, we measured the β -glucosidase activity, which is the most abundant of the enzymes involved in cellulose degradation, mediating hydrolysis of cellobiose to glucose (Almeida et al., 2015; Kim et al., 2020). The β -glucosidase activity was measured using 1 g soil amended with 4 mL of modified universal buffer (pH 6) and 1 mL of *p*-nitrophenyl- β -D-glucoside (25 mM) as the substrate (Eivazi and Tabatabai, 1988). After 2 h of incubation at 20 °C with shaking (150 rev min⁻¹), substrate hydrolysis was inhibited by addition of 4 mL of TRIS buffer (pH 12) and 1 mL of CaCl₂ (0.5 M). The samples were centrifuged (4000 g, 10 min) and the concentration of *p*-nitrophenol was measured colorimetrically at 400 nm. For each soil sample, the β -glucosidase activity was calculated from two technical replicates and one control for background correction (Eivazi and Tabatabai, 1988).

2.2. Mesocosm study of the fate and dose effects of cover crop C

2.2.1. Soils and ¹⁴C-labeled cover crop residues

Soils for mesocosm experiments were sampled from the two blocks of OGL+CC treatment in the LTE, which had different SOC levels of 2.0% and 2.6%. The SOC difference between blocks was an unintended factor in the LTE (Djurhuus and Olesen, 2000; De Notaris et al., 2021), but was exploited here to allow for evaluation of SOC saturation effects since the soils had similar texture and had been under the same management for 23 years. Soils (~15 kg) from each block were sampled in March 2021 by pooling approximately 60 soil cores (0–20 cm depth, 4 cm diameter) taken in a W-shaped pattern across the fields. The soil was sieved (<2 mm), homogenized and stored at 2 °C for less than one week prior to the mesocosm experiment. Chemical, physical and biological properties of the two soils (Table S1) were measured as previously described (Liang et al., 2018, 2019) or as described in section 2.1.

To produce ¹⁴C-labeled cover crop material for mineralization studies, fodder radish (FR) was grown in the field during September to November 2020 in eight PVC cylinders (diameter, 29.5 cm; height, 25 cm) inserted to 25 cm soil depth. Fodder radish was chosen since it is often grown in the LTE and in terms of C composition it is similar to other cover crops grown in the study area, e.g., with low concentrations of fiber (<100 mg g⁻¹) and lignin (<10 mg g⁻¹) as well as C:N ratios of

9–15 (De Notaris et al., 2020). The plants were exposed to ¹⁴CO₂ twice a week during the growth period as described previously (Rasmussen et al., 2008). Briefly, transparent plastic bags were fastened to the cylinders and alkaline ¹⁴C-labeled (99 atom%) Na₂CO₃ solution inside the bags was acidified with excess HCl to create a ¹⁴CO₂ enriched atmosphere. The bags were removed after labeling for 1–3 h, depending on the weather conditions. Labeled FR samples, including shoots and roots (after rinsing with water), were oven-dried (50 °C) and mixed. Prior to soil amendment, the FR residue was ball-milled to ensure homogeneity of ¹⁴C in the added plant residue (which is important for quantification of priming effects) and to allow for representative amendment of low doses of FR (such as approximately 37.5 mg FR per soil mesocosm). The resulting ¹⁴C-labeled FR residue had 37.6% C and 2.8% N, as determined by combustion (Thermo Flash 2000 NC Analyzer), and specific ¹⁴C activity of 61 Bq mg⁻¹, as determined by liquid scintillation counting (Wallac 1414, PerkinElmer) after oxidation and capture of the produced ¹⁴CO₂ in scintillation fluid (Hidex 600 OX Oxidizer, Hidex Oy, Finland).

2.2.2. Initial distribution of FR carbon in soil fractions

The initial distribution of FR carbon in different soil fractions (i.e., after FR amendment but prior to incubation) was quantified (Table S2) by adding FR residue (1 mg C g⁻¹ soil) to ~10 g autoclaved soils (triplicates), and measuring ¹⁴C in different soil fractions (fPOM, oPOM + sand and MAOM) as described in section 2.1.2. Due to the high solubility of FR carbon, ¹⁴C in the dissolved organic matter (DOM) fraction was included in this analysis. Thus, prior to extraction of fPOM, soil samples were shaken gently in deionized water (35 mL, 15 min, 100 rev min⁻¹), centrifuged (3260 g, 30 min), and supernatants were collected after filtration (cellulose filter, 1.6 μ m) for analyses of ¹⁴C in DOM.

2.2.3. Fate and priming effects of ¹⁴C-labeled cover crop residues

Mesocosms for studying FR residue mineralization and priming effects were prepared with 132-g soil samples (on dry weight basis) that were thoroughly mixed with cover crop residue, and repacked in 100-cm³ stainless steel rings (height, 3.5 cm; diameter, 6.1 cm), i.e., to a bulk density (BD) of 1.32 g cm⁻³, similar to the original BD in the field (Table S1). To ensure homogeneous distribution, the added cover crop residue was first mixed into 5 g soil with a spoon (2 min), and then this soil portion was mixed into the rest of the soil for another 2 min. Hereafter, the mixed soil and cover crop residue was packed into the 100-cm³ stainless steel ring using a piston. To achieve an even density throughout the soil cores, each soil sample of 132 g was divided into three portions of 33, 33, and 66 g that were packed sequentially into one, two and four fourths of the height of the steel rings. Treatments were prepared in triplicates with different inputs of cover crops, corresponding to doses of zero (C₀, reference), 0.1 (C_{0.1}), 0.2 (C_{0.2}), 0.3 (C_{0.3}), 0.6 (C_{0.6}), 1.0 (C_{1.0}), and 1.6 (C_{1.6}) mg C g⁻¹ soil. These C doses was estimated according to Table S3, reflecting C inputs from cover crops with 0.4–5.9 Mg aboveground dry matter ha⁻¹, which is a realistic cover crop yield at the LTE site (De Notaris et al., 2018, Fig. S1). The mesocosms were amended with demineralized water to 75% water-filled pore space (WFPS) and incubated individually in closed 2-L glass jars for 88 days in the dark at 15 °C. The relatively high water content reflected the upper limit of soil moisture under field condition (Li et al., 2015), which could result in partially anaerobic conditions that may also occur in hotspots of mineralization of intact cover crop residues under field conditions (Li et al., 2016). Cumulative CO₂ and ¹⁴CO₂ efflux was determined by placing a vial with CO₂ trap solution (20 mL of 0.3 M NaOH) and a vial with water in each glass jar (to maintain soil moisture). Four jars without soil mesocosms were included as references for atmospheric CO₂. The jars were opened and the CO₂ trap solution was replaced after 3, 6, 15, 25, 32, 39, 53, 72, and 88 days. Mesocosms were weighed when the jars were open and water spray was used to maintain constant WFPS.

After 88 days, the CO₂ loss induced by added FR residues dropped to a constant low level, and the biological stability of the remaining FR-

derived C in the soil was tested by adding glucose (0.1 mg C g⁻¹ soil), i. e., to test if the FR-derived C would be susceptible to increased microbial activity from the new C input (Kallenbach et al., 2016). Glucose was also added to the reference treatments (i.e., C₀) in order to calculate the priming effect related to added FR residues (see section 2.2.4). Briefly, 1 mL of glucose solution was added dropwise to the soil at day 88 and water spray was used to fully restore the soil moisture to 75% WPFS. The mesocosms were incubated for additionally 77 days (i.e., 165 days in total) with sampling of trapped CO₂ and ¹⁴CO₂ after 4, 11, 28, 56 and 77 days. After terminating the incubation, soil samples were destructively sampled for measurement of ¹⁴C in different soil fractions (DOM, fPOM, oPOM + sand and MAOM) as described above (section 2.1.2 and 2.2.2).

2.2.4. Analyses and calculations of total and ¹⁴C-labeled CO₂ emissions

Base traps removed from the glass jars were sealed and analysed within one week. The amount of CO₂ trapped was determined with 5 mL aliquots by titrating the remaining NaOH after carbonate precipitation with BaCl₂, using an automated titrator (Metrohm 848 Titrino plus, Switzerland) with stepwise (2 µL) addition of 0.5 M HCl. The cumulative CO₂ production (µg CO₂-C g⁻¹ soil) induced by FR residues (i.e., from mineralization of both FR and primed SOC) was calculated as the difference between the CO₂-C trapped in mesocosms with and without residue amendment (reference, C₀). The cumulative CO₂-C production induced by FR residues was expressed as percentage of the FR carbon initially added (and could therefore exceed 100% in the case of positive priming effects).

The ¹⁴CO₂ efflux was determined by liquid scintillation counting with 5 mL aliquots of trap solution mixed thoroughly with 15 mL of Optiphase HiSafe scintillation cocktail (PerkinElmer). The percentage of FR carbon lost during incubation from day *i* to day *j* (% C_{FR-loss, i-j}) was calculated as:

$$\% C_{FR-loss, i-j} = 100 \times \left({}^{14}CO_2-C_{FR, i-j} - {}^{14}CO_2-C_{reference, i-j} \right) \times \left({}^{14}C_{FR-added} \right)^{-1} \quad (\text{Eq. 1})$$

where ¹⁴CO₂-C_{FR, i-j} and ¹⁴CO₂-C_{reference, i-j} are the cumulative ¹⁴CO₂-C activities in the base trap solutions from mesocosms with and without FR residues, respectively, during the incubation period from day *i* to day *j*, and ¹⁴C_{FR-added} is the total ¹⁴C activity in the added FR cover crop residues. The mass of C loss from the FR residue during the same period (mass C_{FR-loss, i-j}) was then calculated as:

$$\text{Mass } C_{FR-loss, i-j} = \% C_{FR-loss, i-j} \times C_{FR-added} \quad (\text{Eq. 2})$$

where % C_{FR-loss, i-j} is from Eq. (1) and C_{FR-added} is the mass of added FR carbon (0.0–1.6 mg C g⁻¹ soil).

The relative rate of FR carbon loss during day *i* to *j* (R_{FR-loss, i-j}; µg CO₂-C mg⁻¹ C_{FR, i} d⁻¹) was calculated based on the amount of FR carbon remaining in the soil on day *i* (C_{FR, i}) and the CO₂-C loss from FR during day *i* to *j*:

$$R_{FR-loss, i-j} = \text{mass } C_{FR-loss, i-j} \times \text{mass } C_{FR, i}^{-1} \times (j - i)^{-1} \quad (\text{Eq. 3})$$

where

$$\text{Mass } C_{FR, i} = (1 - \% C_{FR-loss, 0-i} / 100) \times C_{FR-added} \quad (\text{Eq. 4})$$

and % C_{FR-loss, 0-i} and mass C_{FR-loss, 0-i} can be calculated from Eq. (1) and Eq. (2), respectively.

Cumulative priming effects (C_{PE}; µg C g⁻¹ soil) induced by added FR was calculated as:

$$C_{PE} = CO_2-C_{total} - C_{FR-loss} - C_{ref} \quad (\text{Eq. 5})$$

where CO₂-C_{total} is the cumulative CO₂-C production from the FR amended soil, C_{FR-loss} is the CO₂-C loss from added FR, and C_{ref} is the CO₂-C loss from the soil without added FR.

2.3. Statistical analysis

Statistical analyses were performed using R version 3.6.3 (R Core Team, 2020). For the LTE study, the main effects of farming systems (OGL and CGL) and cover cropping (+CC and -CC) on microbial biomass, enzyme activity and SOC contents (in bulk soils and different fractions) were tested by two-way analysis of variance (ANOVA) with farming system and cover cropping as fixed factors and block as random factor. For the mesocosm study, two-way ANOVA was used to test the effects of added FR dose and SOC content (fixed factors) on (i) C losses from FR after 88 and 165 days, (ii) FR carbon in MAOM fractions after 165 days, and (iii) net C losses induced by added FR residues after 165 days. The relationships between added FR carbon and priming effects were analysed using the *lm* function in R. The assumptions of variance homogeneity and normal distribution were checked by visual examination of the residuals against fitted values, histogram of residuals and the Shapiro-Wilk test. Significant ANOVA tests (*P* < 0.05) were followed by post hoc pairwise comparisons using the *emmeans* function with Tukey-adjusted *P*-values (Zar, 2010; Lenth, 2016). Measures of central tendency and dispersion are presented as means and standard error with *n* = 4 for LTE and *n* = 3 for mesocosm data, unless otherwise indicated.

3. Results

3.1. Effects of two decades of cover cropping on SOC concentrations and microbial activity

The topsoil SOC concentration in the LTE treatments was not significantly affected by cover cropping (*P* = 0.33) neither in conventional or organic cropping systems (Fig. 1a). The SOC concentrations consistently decreased with depth, but there was no significant effect of cover crops at individual depths (25–50 cm, *P* = 0.26; 50–100 cm, *P* = 0.33), although the OGL + CC system had a high mean SOC concentration at 50–100 cm depth (Fig. 1a). Cover cropping also had negligible effects on the distribution of SOC in the fPOM, oPOM and MAOM fractions in the topsoil and subsoils (Fig. 1b).

Microbial biomass C and β-glucosidase activity in topsoils were consistently highest in the organic farming system with cover crops and lowest on the conventional system without cover crops (Fig. 2). Across the two farming systems, cover cropping increased MBC by 40% and β-glucosidase activity by 21% (*P* < 0.01; Fig. 2).

3.2. Fate of cover crop C in mesocosms

Carbon losses from FR occurred immediately after the incorporation to soil (Fig. S2), and mineralization rates were highest (93–107 µg CO₂-C mg⁻¹ C_{FR, i} d⁻¹) during the initial stage of incubation with an exponential decrease to 5–9 µg CO₂-C mg⁻¹ C_{FR, i} d⁻¹ within 30 days (Fig. 3). Adding glucose (at day 88) slightly stimulated the mineralization of remaining FR carbon, yet loss rates always decreased to a constant level (2–5 µg C mg⁻¹ C_{FR, i} d⁻¹) within few days (Fig. 3, inserts). As a result, 74–82% of added FR carbon was lost across treatments after incubation for 88 d, which further increased to 80–90% with prolonged incubation until day 165 (Fig. 4). There were no systematic trends in the cumulative losses in response to the dose of added FR residues (Fig. 4).

The sum of FR carbon measured in different soil fractions at the end of the experiment represented 9–19% of the initially added FR carbon, which together with the measured CO₂-C loss resulted in a total FR carbon recovery of 95–107% (Table 1). The MAOM fraction stored the main part of (77–93%) of the FR carbon that remained in the soil (Fig. 5). The proportions of FR carbon in MAOM were similar regardless of soil SOC level and FR application doses (Fig. 5), with only one contradicting observation (at 0.3 mg FR C g⁻¹ in 2.0% SOC soil).

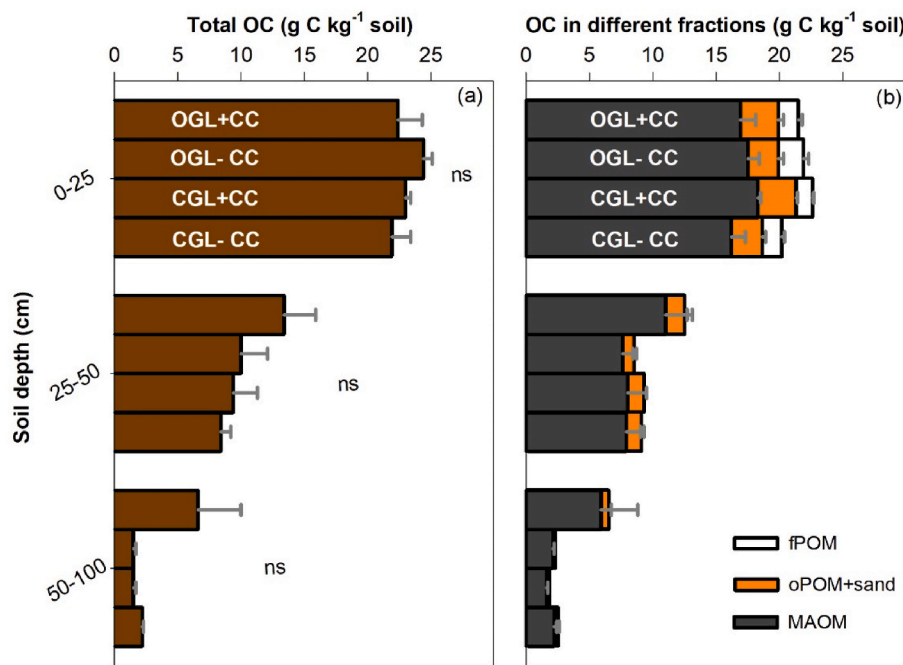


Fig. 1. (a) Total organic carbon (OC) and (b) OC in different fractions at 0–100 cm depth in soils from long-term field trials in organic (OGL) and conventional (CGL) farming systems with (+CC) or without (-CC) cover crops. Fractions: fPOM, free particulate organic matter (OM); oPOM + sand, occluded POM and sand; MAOM, mineral associated OM. Data are mean \pm standard error ($n = 4$). Differences between treatments in OC contents at individual soil depths were non-significant (ns, $P > 0.05$).

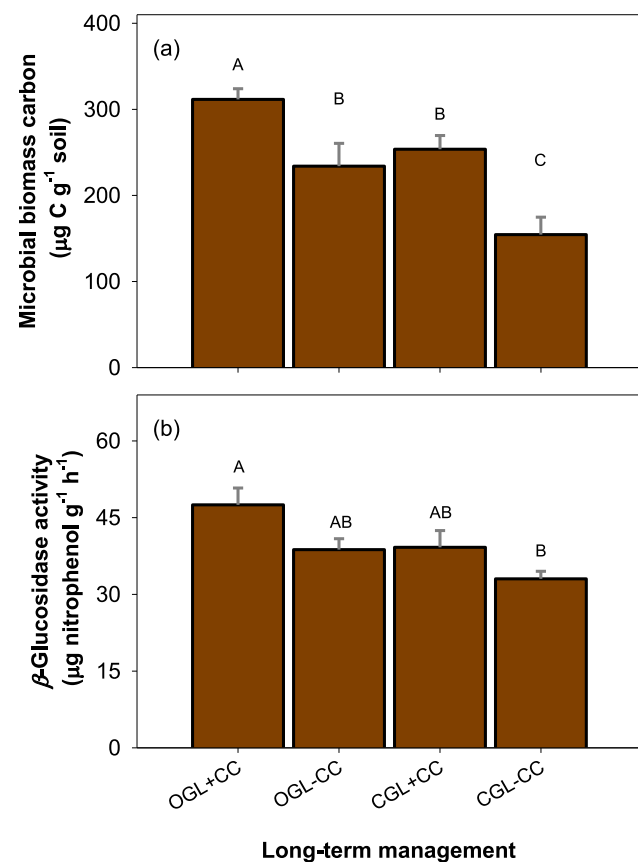


Fig. 2. (a) Microbial biomass carbon and (b) β -glucosidase activity at 0–25 cm depth in soils from long-term field trials in organic (OGL) and conventional (CGL) farming systems with (+CC) or without (-CC) cover crops. Data are means \pm standard error ($n = 4$). Different letters indicate significant differences ($P < 0.05$) across treatment.

3.3. Priming effects induced by cover crop C in the mesocosm study

The cumulative CO_2 -C loss induced by the added FR residue (i.e., the sum of C losses from the FR residue and primed SOC) were similar across most treatments (75–94% of the added C), yet with the highest value (150% of the added C) seen for 2.0% SOC soil at the FR dose of 0.1 mg C g^{-1} (Fig. 6). More importantly, the resulting relationship between the FR carbon dose and the induced C loss indicated both positive and negative priming effects with negative priming increasing at higher FR doses (Fig. 7).

The cumulative positive priming effects after incubation for 88 days (Fig. 7a and b) were highest in 2.0% SOC soil with the FR dose of 0.1 mg C g^{-1} ($39.8 \mu\text{g C g}^{-1} \text{ soil}$) and in 2.6% SOC soil with the FR dose of 0.2 mg C g^{-1} ($24.3 \mu\text{g C g}^{-1} \text{ soil}$). Based on the correlations between the dose of FR carbon and the cumulative priming effect, the cumulative priming effect by added FR shifted from positive to increasingly negative when C inputs were >0.2 – 0.3 mg C g^{-1} in these two soils (Fig. 7a and b). Adding glucose (at day 88) generally resulted in a positive priming effect in 2.0% SOC soil except for treatments with the highest cover crop C input, whereas in 2.6% SOC soil, the priming effect was generally near zero or negative (Fig. 7c and d).

4. Discussion

4.1. Two decades of cover cropping had negligible effect on SOC concentrations to 1-m depth

The LTE results showed that cover cropping for 15–23 years had negligible effect on the total SOC and SOC in POM and MAOM fractions to 1-m soil depth. Mineral associated OM represented 70–72% and 79–85% of total SOC in the topsoil and subsoil, respectively, which is similar to the range of MAOM fractions found for arable soils across Europe (Lugato et al., 2021).

Subsoils showed no presence of fPOM regardless of LTE managements, and also no response of POM to cover cropping (Fig. 1b). This was expected due to the limited cover crop C input to the subsoil layer, which was previously estimated to 90 and $170 \text{ kg C ha}^{-1} \text{ yr}^{-1}$ at 25–100 cm depth in the OGL and CGL systems, respectively (Liang et al., 2022). Based on data presented by Liang et al. (2019), the subsoil in the study

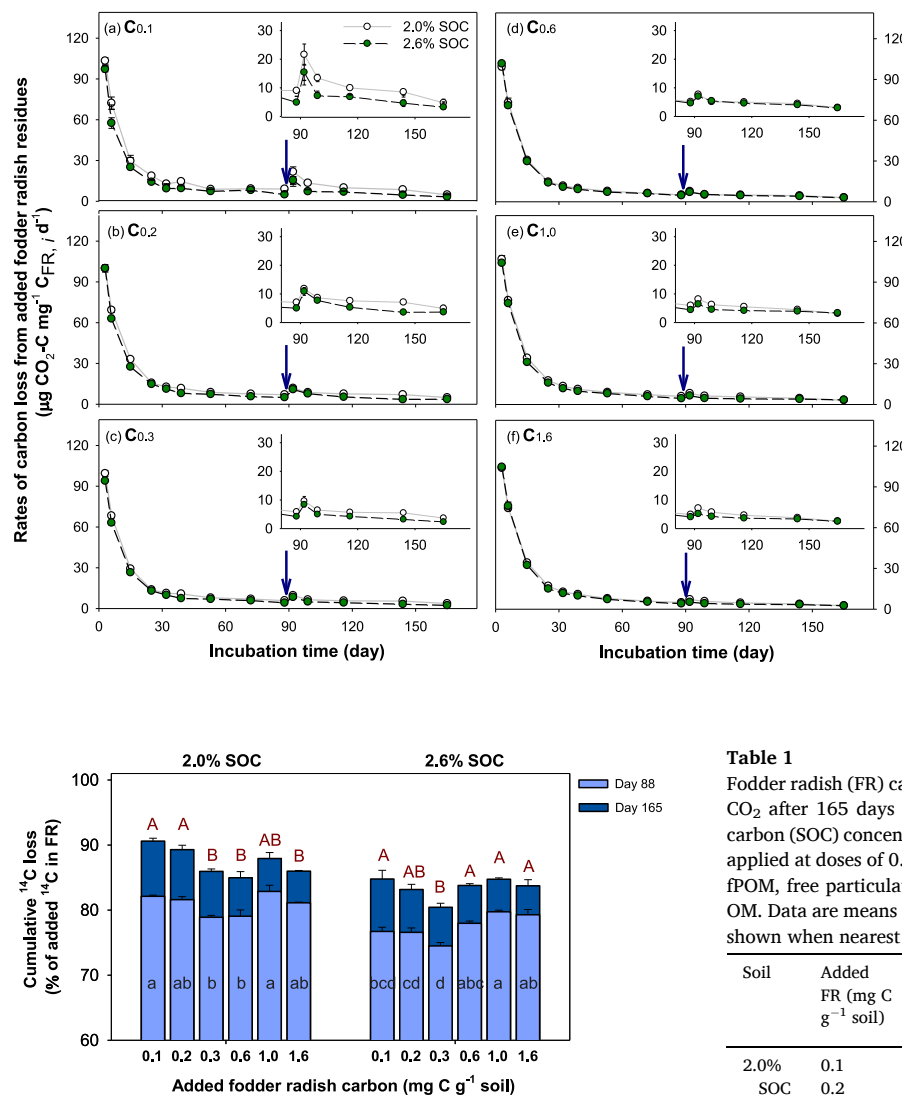


Fig. 3. Rates of carbon (C) loss from added fodder radish (FR) residues during incubation for 165 days in texturally similar soils with 2.0% or 2.6% soil organic carbon (SOC). Fodder radish residues were applied at increasing doses of 0.1 ($C_{0.1}$) to 1.6 ($C_{1.6}$) mg C g^{-1} soil (panels a–f). Insets show the dynamics after 88 days when glucose was added to test the biological stability of the remaining FR-derived C in the soil (indicated by arrows). Rates of C loss from FR were calculated as $^{14}CO_2-C$ loss between consecutive measurement days (i to j) relative to the FR carbon pool remaining in the soil on day i ($C_{FR, i}$). The unit refers to CO_2 mineralization relative to the amount of remaining FR carbon, $C_{FR, i}$, at incubation day i . Data are means \pm standard error ($n = 3$).

Fig. 4. Cumulative carbon (C) losses of fodder radish (FR) residues, expressed as % of added, after incubation for 88 and 165 days in texturally similar soils with 2.0% and 2.6% soil organic carbon (SOC). Fodder radish residues were applied at increasing doses of 0.1–1.6 mg C g^{-1} soil. Data are shown as means with standard error bars ($n = 3$), some of which are too small to visualize. Lowercase (88 days) and uppercase (165 days) letters indicate significant differences ($P < 0.05$) in FR carbon losses for each soil (2.0% and 2.6% SOC). Overall, C losses were higher in the soil with 2.0% SOC after incubation for both 88 and 165 days ($P < 0.001$).

area can be assumed to have a BD of 1.7 g cm^{-3} and generally retain ~30% of the added C input, after biotic and abiotic transformation. Based on these approximate, but realistic assumptions, the corresponding SOC increase would be 0.05 and 0.06 g C kg^{-1} soil after cover cropping in the OGL (23 years) and CGL system (15 years), respectively (given the mentioned subsoil cover crop inputs of 90 and 170 kg C ha^{-1} yr^{-1}). Such low SOC increases would be challenging (or impossible) to detect both analytically and particularly due to the spatial variation of SOC in subsoils (Poeplau and Don, 2015; Prommer et al., 2020).

In the topsoil, the amount of OC in the POM fraction was similar across the LTE managements, despite the recurrent input of cover crop residues in the +CC treatments (Fig. 1b). However, the +CC treatments showed increased microbial biomass and β -glucosidase activity (Fig. 2), as also found in a global meta-analysis, where microbial activity increased by 22% as the result of cover cropping (Kim et al., 2020). Microbial activity can promote the formation of fPOM and oPOM from

Table 1

Fodder radish (FR) carbon (% of added) in different fractions and mineralized to CO_2 after 165 days in two texturally similar soils with different soil organic carbon (SOC) concentrations (2.0% and 2.6% SOC). Fodder radish residues were applied at doses of 0.1–1.6 mg C g^{-1} soil. DOM, dissolved organic matter (OM); fPOM, free particulate OM; oPOM, occluded POM; MAOM, mineral associated OM. Data are means ($n = 3$). For all means, standard errors were calculated and shown when nearest integer was ≥ 1 .

Soil	Added FR (mg C g^{-1} soil)	Fate of FR carbon (% of added)					Total recovery (%)
		DOM	fPOM	oPOM + sand	MAOM	CO_2	
2.0% SOC	0.1	0	0	1	15 \pm 1	91	107
	0.2	0	0	2 \pm 1	15	89	106 \pm 1
	0.3	0	0	3	13 \pm 1	86	103 \pm 1
	0.6	0	0	3	16 \pm 1	85	104 \pm 1
	1.0	0	0	2	16 \pm 1	88	106 \pm 2
	1.6	0	0	2	15	86	103
2.6% SOC	0.1	1	0	0	8	86	95 \pm 1
	0.2	0	0	3 \pm 1	14 \pm 2	83	100 \pm 2
	0.3	1	0	3 \pm 1	15 \pm 1	80	99 \pm 2
	0.6	0	0	3	15	84	102 \pm 1
	1.0	0	0	3	15	85	103 \pm 1
	1.6	0	0	3	16	84	103 \pm 1

cover crop via fragmentation and *ex vivo* modification (Cotrufo et al., 2013; Liang et al., 2017; Lavallee et al., 2020), but the accumulation of newly formed POM may be transient, due to transformation into other soil pools, such as MAOM. This can explain the lack of increase of C in the POM fraction, yet concurrently, it would be expected to find that C stored in the topsoil MAOM increased over time in the +CC treatments. Yet, this was not observed in the present study.

Carbon inputs from cover crops (the sum from above- and below-ground) to topsoil was previously estimated to be about 1.3 and 0.7 Mg C ha^{-1} yr^{-1} in the OGL and CGL system, respectively (Hu et al., 2018). If we assume the topsoil to have a BD of 1.3 g cm^{-3} and to retain 10–20%

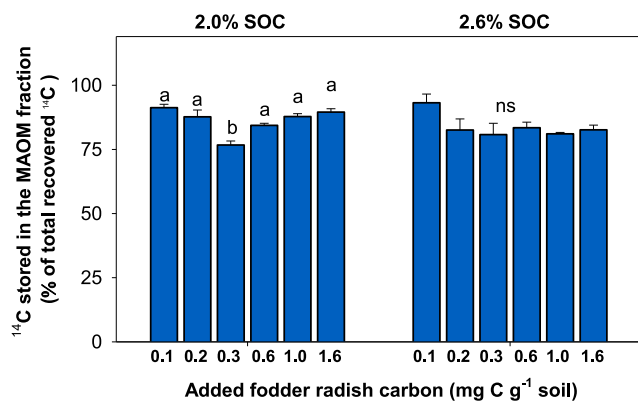


Fig. 5. Fodder radish (FR) carbon (C) stored in the mineral associated organic matter (MAOM) fraction after incubation for 165 days in texturally similar soils with 2.0% and 2.6% soil organic carbon (SOC). Fodder radish residues were applied at increasing doses of 0.1–1.6 mg C g⁻¹ soil. Data are shown as means with standard error bars ($n = 3$). Fodder radish C stored in MAOM was similar in two soils ($P > 0.05$). Lowercase letters indicate significant differences ($P < 0.05$) in MAOM distribution for each soil. ns, not significant ($P > 0.05$).

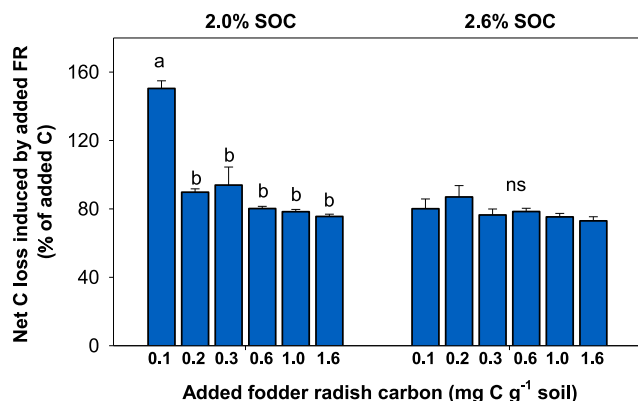


Fig. 6. Cumulative carbon (C) losses induced by added fodder radish (FR) residues after incubation for 165 days in texturally similar soils with 2.0% and 2.6% soil organic carbon (SOC). Fodder radish residues were applied at increasing doses of 0.1–1.6 mg C g⁻¹ soil. The cumulative C loss induced by the added FR residue was calculated as the difference between CO₂-C evolved in mesocosms with and without added residues. Data are shown as means with standard error bars ($n = 3$). Lowercase letters indicate significant differences ($P < 0.05$) in the induced C losses for each soil (2.0% and 2.6% SOC). Overall, the induced C losses were higher in the soil with 2.0% SOC ($P < 0.001$). ns, not significant ($P > 0.05$).

of the crop C input in MAOM after microbial transformation (Hu et al., 2018; Liang et al., 2019), then the corresponding MAOM increase by cover crops would be 0.9–1.8 g C kg⁻¹ soil in the OGL (23 years) and 0.5–1.0 g C kg⁻¹ soil in the CGL system (15 years). Thus, the low amount of cover crop input in itself makes it challenging to observe changes to the SOC pool even at scales of 15–23 years. The results therefore indicate that higher cover crop C inputs might be needed to facilitate significant SOC increases, but still it is uncertain if other factors are limiting long-term SOC sequestration in the form of MAOM from cover crops.

4.2. Mechanisms of C saturation fail to explain the lack of SOC sequestration from cover crops

Lack of SOC sequestration (in MAOM) from cover crops could in principle be related to the mechanism of soil C saturation due to limited

availability of mineral surfaces for physico-chemical stabilization (Castellano et al., 2015; Cotrufo et al., 2019; Lavallee et al., 2020). Yet, the concept of SOC saturation is debated (Vogel et al., 2014; Drexler et al., 2022), and at least cannot fully explain our LTE results, where cover cropping did not increase SOC accumulation across two field blocks with the same mineralogical composition (Fig. S3), but with SOC content varying from 2.0 to 2.6% SOC as influenced by land use history prior to establishment of the LTE. Indeed, C storage in MAOM was independent of cover cropping, but showed a strong positive correlation to the total SOC (Fig. S3), thus indicating that C saturation in general was not a limiting factor for SOC accumulation. At least, it was unlikely that the lack of SOC increase in the 2.0% SOC soil was due to C saturation, as soil with the same mineral composition could hold 2.6% SOC.

The mesocosm study with ¹⁴C-labeled FR residues was performed to trace the fate of cover crop C and the SOC stabilization potential in the selected LTE soils with 2.0 and 2.6% SOC. The results showed a rapid depletion of added FR carbon, which confirmed a typical pattern for turnover of labile C in arable topsoils in the study area (Liang et al., 2018, 2019) and also corresponded to the results of a comparable incubation study performed at 55% WFPS with the same soils as used in the present experiments (Z. Liang, unpublished results). The rapid turnover can be attributed to the high concentration of labile C in the added FR residue (Table S2), but the controlled incubation procedure, e. g., with relatively high WFPS (75%) and use of ball-milled plant material, makes direct comparisons to field conditions difficult. In addition, the ball-milling may also reduce the stabilization potential of added FR through fragmentation and physical mechanisms (Cotrufo et al., 2015), thus partly contributing to the absence of FR derived C remaining in the fPOM by the end of the incubation (Table 1). Nevertheless, comparison of processes, such as priming effects and stabilization of C in the MAOM fraction, in response to different doses of added cover crop C likely reflects an ecosystem response that could also prevail under field conditions.

After incubation for 88 days, the biological stability of remaining FR carbon was tested by adding glucose (0.1 mg C g⁻¹ soil) as a model compound of labile root-derived C that could contribute to increased positive priming effects (Kallenbach et al., 2016; Hu et al., 2018; Pausch and Kuzyakov, 2018). Thus, although more complex and continual sources of labile C would exist in plant/soil systems, the main purpose here was to test the susceptibility of the remaining FR carbon to biological decomposition. The resulting (slightly) accelerated mineralization of residual FR carbon dropped to a constant low level within few days (Fig. 3). As compared to the FR carbon remaining on day 88, it was found that 53–79% still remained after 165 days (Fig. S4), i.e., indicating that the majority of the remaining C on day 88 was biologically stable, most likely in the form of microbial products (Liang et al., 2017), which was consistent with previous studies (Plante et al., 2011; Kallenbach et al., 2016). The mesocosm results further showed that MAOM was the major pool of recovered FR carbon (Fig. 5), substantiating the potential of the LTE soils for SOC storage in the form of MAOM. These observations supported our interpretation of the results from the LTE field study, i.e., that the lack of SOC sequestration from cover cropping was not explained by limited SOC storage capacity and C saturation.

4.3. Sufficient cover crop C inputs contribute to SOC formation and negative priming

The mesocosms showed an overall C loss at the lowest FR dose (0.1 mg C g⁻¹) in 2.0% SOC soil, which was due to positive priming effects (Figs. 6 and 7). The positive priming at this low FR dose lasted for months (Figs. S5a and b), thus suggesting real (rather than apparent) priming effects (Conde et al., 2005; Blagodatskaya and Kuzyakov, 2008). Since the incubated FR residue had a C:N ratio of 13, we assume that the primed SOC was due to accelerated SOC metabolism by stimulated microbial activity, rather than an N-mining effect (Craine et al., 2007). This could represent a typical response of the LTE soils to low

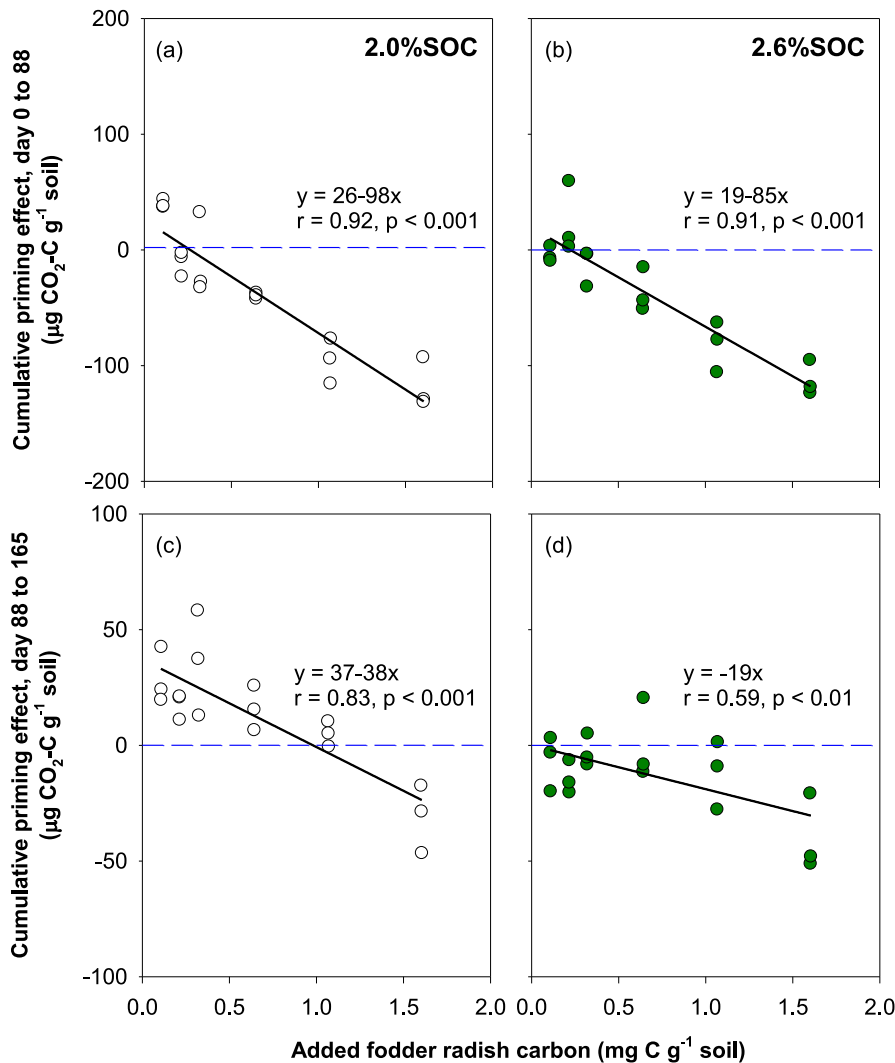


Fig. 7. Correlations between the dose of added fodder radish carbon (C) and the cumulative priming effect after incubation for 0–88 days (a,b) and 88–165 days (c,d) in texturally similar soils with 2.0% (a,c) and 2.6% (b,d) soil organic carbon (SOC). Glucose was added at 88 days to test the biological stability of the remaining FR-derived C in the soil. Values below and above zero (dashed line) indicate positive and negative priming of SOC, respectively. Pearson correlation coefficients (r) and significance (P) of correlations are shown in the panels.

doses of cover crop C input, and reflect an underlying priming mechanism, since cover crops at the field site generally have a narrow C:N ratio of 9–15 (De Notaris et al., 2020).

Adding glucose (at day 88) generally resulted in a positive priming effect in 2.0% SOC soil except for treatments with the highest cover crop C input, whereas in 2.6% SOC soil, the priming effect was generally near zero or negative (Fig. 7c and d). These results suggested that the 2.0% SOC soil was more vulnerable to priming from C inputs at low doses (both in the form of cover crop residue or glucose), with a risk of C depletion instead of C sequestration. As the specific basal soil respiration (i.e., CO₂-C per mg SOC in soils without added FR residues) was higher in 2.0% than in 2.6% SOC soil (Fig. S6), it is likely that the quality of native SOC differed between the two soils with relatively less accessible C in the 2.6% SOC soil. It can thus be speculated that the difference in priming effects between the two soils may reflect a greater complexity and stability of SOC in the 2.6% SOC soil, maybe due to the prehistory of pasture, with little effect of the absolute content of SOC itself (Slessarev et al., 2023; Su et al., 2023).

The cumulative priming effect of added FR shifted from positive to increasingly negative when C inputs were >0.2–0.3 mg C g⁻¹ in these two soils (Fig. 7a and b). Similar findings were reported by Bradford et al. (2008) in a one-year study where sucrose added at a low dose (150 g C m⁻² yr⁻¹) strongly and positively primed the native SOC, whereas

negligible priming effect was seen when sucrose was added at a high dose (800 g C m⁻² yr⁻¹). Furthermore, this pattern was consistent with the meta-analysis by Blagodatskaya and Kuzyakov (2008) suggesting that zero or negative priming occurred when the added C exceeded 2–5 times the amount of MBC. The initial MBC in the present study was about 0.12 mg C g⁻¹ for the two soils (Table S1), and the tipping point of priming effects at >0.2–0.3 mg g⁻¹ was thus just in the range of >2 times of MBC. Likely, only added C above this range was sufficient to trigger microbial growth (cf. Blagodatskaya and Kuzyakov, 2008), during which we speculate that microbial *r*-strategists, preferentially utilizing labile OM, could outcompete microbial *K*-strategists typically involved in SOC mineralization (Kuzyakov et al., 2000; Fontaine et al., 2003). Thereby, microbial metabolism may have shifted from decomposition of relatively recalcitrant SOC to more accessible FR residues, with a resulting negative priming effect that lasted for the first two weeks of the incubation study (Fig. S5) until the exhaustion of the most labile C input (Blagodatskaya and Kuzyakov, 2008).

4.4. Lack of SOC sequestration from cover crops is constrained by low productivity and C input

The lack of increase in SOC to 1-m depth despite cover crop C inputs for 15–23 years, indicates a limited C sequestration potential within the

studied LTE. However, the mesocosm experiment showed a possible net C increase equivalent to 4–25% of the added C on short term (5.5 months). In particular, overall C increase equivalent to 20–25% of the added C was achieved, when the cover crop C input was $>0.2\text{--}0.3\text{ mg C g}^{-1}$ in the studied soils. These threshold values would correspond to the C input from cover crops with an aboveground yield of about $0.7\text{--}1.1\text{ Mg dry matter ha}^{-1}$ (Table S3). However, cover crop yields below this threshold are common at the LTE (De Notaris et al., 2018, Fig. S1), thereby corroborating the absence of any detectable increase in SOC. Combining the observation from the LTE and mesocosm studies suggest that buildup of SOC stock was not essentially constrained by soil C saturation, but rather by the low productivity and C input from cover crops. Since the production of cover crops is highly controlled by the effective growth period and climate conditions, this might also define the climatic region of successful SOC sequestration via cover cropping (Seita et al., 2022), although the conclusions should be confirmed for other soil and input types (Poeplau et al., 2015a, 2015b), e.g., using more intact cover crop residues and species with higher C:N ratio than FR. In general, however, the present study suggests that agricultural management practices should be adopted (e.g., species choice and sowing time) that achieve an annual cover crop C input in excess of a threshold, which may vary among agroecosystems (De Notaris et al., 2019), but which should be large enough to result in de novo formation of SOC that exceeds the C losses from positive priming.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2023.109110>.

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