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Crop Rotation With Ley and Nitrogen Fertilisation Reduced Soil Carbon Loss in Three Swedish Long-Term Field Experiments

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ABSTRACT

Increasing soil organic carbon (SOC) stocks by improving cropland management practices has great potential to mitigate climate change. Long-term field experiments (LTEs) are valuable to study management effects on soil properties and crop yield. Yet most LTE studies are limited to the topsoil, and farming systems integrating multiple management strategies are often not assessed. This study used three Swedish LTEs to assess the effects of crop rotations and fertilisation on SOC changes. One arable rotation with only annual crops and a ley rotation with annuals, perennial ley and receiving manure were investigated at different application rates of mineral fertilisers. We analysed changes in SOC content and the distribution of SOC content and stocks at multiple soil depths, calculated C inputs and used phospholipid fatty acids (PLFAs) to evaluate how management practices affected SOC in relation to C inputs and microbial communities. Both systems lost carbon in the 0–20 cm topsoil from 1966 to 2019 across sites, but the sandy site lost more than the clayey sites. The ley rotation and nitrogen (N) fertilisation reduced carbon losses. In 2019, SOC stocks in the top 25 cm soil were 3.3 ± 1.6 Mg C ha^{-1} higher in the ley rotation compared with arable rotation and 2.9 ± 1.6 Mg C ha^{-1} higher with N fertilisation at the highest rate compared with no N fertilisation. However, the positive effects decreased with depth and became negative at some depths. As a result, differences in SOC stocks to an equivalent depth of 60 cm declined to 0.6 ± 2.4 Mg C ha^{-1} for rotations and to 1.0 ± 2.4 Mg C ha^{-1} for N fertilisation. The ley rotation had significantly higher belowground C inputs than the arable rotation, and belowground C inputs were highly associated with changes in SOC. Compared with the arable rotation, total PLFAs, bacterial PLFAs and the ratio of bacteria to fungi in topsoil were significantly higher in the ley rotation, partly attributed to manure application. Our study supports the beneficial effects of leys and manure amendments on SOC compared with systems with only annual crops. It also highlights the risk of losing SOC in the subsoil, especially under mineral N fertilisation. Site characteristics helped to explain the large variation, which must be considered when developing local strategies for SOC accrual in cropland.

Summary

- Management effects on subsoil organic carbon and microbial communities are insufficiently studied.
- Subsoil layers were densely sampled along profiles in contrasting farming systems.
- Topsoil C gains due to leys and N fertilisation were partly negated in the subsoil.
- Ley rotation and N fertilisation slowed down soil organic C loss due to higher belowground C input.

1 | Introduction

Agricultural soils are considered to have great potential to sequester carbon and mitigate climate change despite different estimations of the magnitude. The top 1 m agricultural soils globally are estimated to potentially sequester 1.9–3.1 Gt C in the '4 per mille initiative: Soils for Food Security and Climate', offsetting 20%–35% of global greenhouse gas (GHG) emissions (Minasny et al. 2017). Adoption of soil organic carbon (SOC) enhancing management in croplands worldwide could attain SOC sequestration between 0.90 and 1.85 Pg C year^{-1} for over 20 years in the top 30 cm soils alone, that is, 26%–53% of the target of the '4p1000 initiative' (Zomer et al. 2017). Many management practices have been recommended to tap into the potential of SOC sequestration, such as crop rotations, tillage practices, cover crops, ley farming, agroforestry, use of manure and biosolids (organic amendments), nitrogen (N) fertilisation, precision farming and irrigation, and so forth (Amelung et al. 2020; Jarecki and Lal 2003; Kätterer and Bolinder 2022; Paustian et al. 2016).

Long-term field experiments (LTEs) or chronosequence studies are invaluable in identifying the long-term effect of management practices because the changes in soil carbon stocks and other properties are slow and hard to detect in the short term (Dignac et al. 2017; Grosse et al. 2020). In general, the amount of carbon stored in soils is the balance between carbon inputs through aboveground post-harvest plant residues, roots, rhizodeposition and exogenous organic amendments and outputs through decomposition, erosion and leaching. A positive balance with increasing SOC storage implies sequestration and negative C emissions, while a less negative balance results in reduced SOC losses (C loss mitigation), both of which have a mitigating effect on climate change (Don et al. 2024).

The inclusion of more perennials in crop rotations and N fertilisation has been shown to enhance soil carbon storage in arable land (Bolinder et al. 2020). Although total assimilation of C may be similar in crops and perennial grasses (Pausch and Kuzyakov 2018), perennial forage allocates more C to roots and rhizodeposits. For example, the total root biomass of perennial forage crops is often at least three times that of small grain cereal crops (Bolinder et al. 2012). Moreover, a larger fraction of belowground compared with aboveground crop residues is stabilised in soil (Kätterer et al. 2011; Rasse et al. 2005). As a result, more belowground C inputs have been shown to increase SOC in rotations incorporating perennial leys compared with rotation with only annuals (Börjesson et al. 2018). Fertilisation with organic amendments such as manure application not only adds exogenous C inputs to soils but also enhances soil health and plant productivity, further increasing C inputs from plant growth and reinforcing the positive effect on SOC (Beillouin et al. 2023; Maillard and Angers 2014). Mineral fertilisation is considered a main contributor to SOC sequestration in croplands by Lessmann et al. (2022), especially in low-fertility soils, but this positive effect was much smaller in the global synthesis by Beillouin et al. (2023), that is, mineral fertilisation increased SOC by 9.4% (CI [+6.3, +12.6]) on average. In contrast, crop residue retention and organic amendments showed greater positive effects on SOC in the same synthesis (Beillouin et al. 2023).

Management practices, such as the application of manure and mineral fertilisers, crop rotations, and tillage, are commonly implemented in farming systems, but the analysis dealing with combined and complex agricultural practices remains limited (Beillouin et al. 2023). The combination of manure and mineral fertiliser has shown greater positive effects on SOC accrual and soil microbial biomass than applying manure or mineral fertiliser alone (Gross and Glaser 2021; Zhong et al. 2010). Integrated crop-livestock systems, including leys or cover crops in combination with animal manure, generally have a positive effect on SOC (Henryson et al. 2022; Shi et al. 2022). However, this is not always the case, as illustrated by an eight-year experiment in Germany that showed the tendency of negative or stable annual SOC change (De Los Rios et al. 2022). The effects of farming practices on SOC are mainly driven by C input quantity and quality but are also modulated by highly variable soil pedoclimatic conditions, which may result in contradictory results. Yet, few studies identified the main mechanisms for C gains or loss (Basile-Doelsch et al. 2020). To provide evidence-based management recommendations for SOC accrual that consider local pedoclimatic conditions, we need a better understanding of the mechanism of SOC changes concerning C inputs, soil microbial communities shift and interactions with minerals and other site characteristics.

Although subsoil stores a substantial amount of carbon, the assessments of management practices are mostly limited to the topsoil or tillage layer (Siddique et al. 2023), and studies of subsoil often find highly variable responses to management practices in deeper soils (Minasny et al. 2017; Skadell et al. 2023). Due to large variation and the fact that subsoil responses are reported for less than 10% of data from LTE studies according to a synthesis of reviews (Bolinder et al. 2020), more LTE studies reporting management effects on subsoil C decomposition and stabilisation are needed. Quantifying carbon inputs from different sources and studying microbial communities can help our understanding of the mechanisms of how management practices influence soil carbon storage (Jastrow et al. 2007; Lange et al. 2015). Furthermore, denser sampling of depth intervals than usually practised, particularly in the transition zone between topsoil and subsoil, would provide more insights into the distribution of SOC changes in soil profiles.

Several meta-replicated LTEs (i.e., experiments with an identical layout) are running at sites with different pedoclimatic conditions throughout Sweden. In this study, we used data from three of the Swedish soil fertility experiments that compare two farming systems: one representing a livestock-based system with perennial leys and manure application, and another system growing only annual cash crops, referring to ley rotation and arable rotation, respectively. Both systems are subject to different mineral fertilisation regimes. The objective was to evaluate the long-term rotation and fertilisation effect on SOC in both topsoil and subsoil, considering C inputs to soils, microbial community composition and site characteristics. The hypotheses are: (1) ley rotation has a positive effect on SOC in the topsoil and, to a lesser extent, even in subsoil because of more root C input from perennial ley compared with annual crops; (2) fertilisation increases biomass production and provides more C input to soils, which results in SOC accrual; (3) microbial community composition differs between the two farming systems because of differences in the quality of C inputs.

TABLE 1 | Site characteristics for the three long-term soil fertility trials in central Sweden.

Site	Location	Altitude (m)	pH[a]	Bulk density[a]	C/N ratio[a]	Soil texture	Clay%[b]	Sand%[b]	Annual mean temperature (°C)	Annual precipitation (mm)
Bjertorp	58°14′ N, 13°08′ E	90	6.0–6.8	1.31–1.35	11.5–13.0	Silty clay	30.0	16.0	6.2	571
Högåsa[b]	58°30′ N, 15°27′ E	80	5.5–6.4	1.27–1.43	12.4–13.9	Topsoil silty sand, subsoil loamy sand	7.4	77.4	6.8	569
Vreta Kloster	58°29′ N, 13°08′ E	47	6.2–6.8	1.28–1.36	10.3–10.9	Topsoil silty clay, subsoil clay	47.6	8.5	6.8	569

[a]Ranges of measured pH, estimated bulk density and C/N ratio for 0–20 cm soil are from profile sampling in recent years.
[b]Clay and sand content of 0–20 cm soil at Högåsa and Vreta Kloster are from Kirchmann et al. (2005).

2 | Methodology

The ‘Swedish soil fertility trials’ comprise a series of field experiments at 12 sites across Sweden, which were started in the 1950s and 1960s and designed to evaluate the impact of agricultural specialisation and intensification on soil fertility (Carlgren and Mattsson 2001). The number of mixed farms decreased and grain production became separated from animal husbandry. As a result, manure disappeared and straw was left in the fields on the farms specialised in grain production, while leys were more frequently included in the farming systems specialised in livestock production (Persson 2007). Because of different climatic conditions, the crops selected in the rotations and rotation length were different in southern, central and northern Sweden. The aim of the Swedish fertility trials is to investigate how farming systems with and without livestock affect soil fertility and crop production in the long term. The rotations in these trials represented common crop sequences at that time on farms with livestock and those focusing only on annual cash crops.

2.1 | Study Site and Experimental Design

In this study, we selected three sites with contrasting soil properties but similar climatic conditions in central Sweden from the series of soil fertility trials (R3-9001), that is, Bjertorp, Högåsa and Vreta Kloster that started in 1966. There are two other sites in this series of trials in central Sweden, Fors and Kungsängen, but they were excluded from this study because of lacking compatible soil profile sampling. Soil texture at Bjertorp is silty clay, and silty sand at Högåsa in the 0–20 cm soil and loamy sand in the soil below 20 cm. At Vreta Kloster, the texture is silty clay in the 0–20 cm soil and clay in the soil below 20 cm (Carlgren and Mattsson 2001). Site characteristics are summarised in Table 1.

The experimental design follows a split-split-block design at all sites, and treatments were set at three levels (Addy et al. 2024; Ivarsson and Bjarnason 1988; Poeplau et al. 2016). At level one, two 6-year crop rotations were replicated in two blocks; at level two, four doses of phosphorus and potassium (PK) fertilisation were nested under each rotation, and at level three, four doses of N fertilisation were nested under each PK level. The ley rotation includes spring barley (*Hordeum vulgare*) undersown with grass/clover ley, followed by 2 years of ley (which is cut two times during the first and once during the second year), winter wheat (*Triticum aestivum*), oats (*Avena sativa*), and then winter wheat again. The crops in the arable rotation, representing a farming system without livestock, are similar to those in the ley rotation, except that the leys are substituted by oats and oilseed rape (*Brassica napus* ssp. *napus*). The winter wheat after ley in the ley rotation received 30 Mg ha^{-1} animal manure (fresh weight) but the crop residues from all cereals were removed. The animal manure was applied as solid farmyard manure before 2010 and as slurry thereafter. In contrast, the arable rotation received no manure, and all crop residues were left in the field. Both rotations are mouldboard ploughed every year in autumn, except the 2 years with leys in the ley rotation. The leys are established with barley (i.e., under-seeded) using a mixture of two grasses and two legume species, including meadow fescue (*Festuca pratensis*), timothy (*Phleum pratense*), alsike clover (*Trifolium pratense*) and red clover (*T. hybridum*).

The four PK treatments include an unfertilized control (treatment PK0), a replacement treatment PKR, where the mass of nutrients removed in harvested products during the preceding six-year rotational period is replaced by applying the same amount of PK mineral fertiliser split into two doses every third year. In treatments, PK3 and PK4, PK rates are enlarged beyond their replacement, by 20 kg P and 50 kg K ha^{-1} $year^{-1}$, and 30 kg P and 80 kg K ha^{-1} $year^{-1}$, respectively. The N treatments also include an unfertilized control and three doses of N, 41, 82 and 125 kg N ha^{-1} $year^{-1}$, denoted by N0, N41, N82 and N125, respectively. More details about this series of experiments were presented in previous publications (Carlgren and Mattsson 2001; Ivarsson and Bjarnason 1988; Lück et al. 2011).

2.2 | Time Series of Topsoil Sampling and Analysis

The 0–20 cm soils have been sampled routinely once per rotation since the start of the experiments. This depth was chosen to represent topsoil, reducing the risk of mixing of soils below the tillage layer. This 0–20 cm soil layer refers to topsoil in the time series SOC. Ten samples from each of the two replicates were mixed, air-dried and sieved to 2 mm, and the composite sample was analysed for several soil properties, including total C and total N by dry combustion, and pH measured in water. However, samples from 1966 and 2013 were analysed separately for each replicate. The initial soil C was determined by the loss on ignition or Walkley–Black wet oxidation (Poeplau et al. 2016). In this study, total C is considered SOC because carbonate was only observed in the soil horizon below 58 cm at Vreta Kloster (Kirchmann et al. 2005).

2.3 | Recent Soil Profile Sampling, SOC Stocks Calculation and PLFA Analysis

To investigate the effect of rotation and fertilisation on the distribution of SOC and microbial communities within the soil profile, we conducted a detailed sampling in four selected treatments in each rotation at the three sites during recent years, that is, in treatments without PK and N fertilisation (PK0N0), no PK but highest N rate (PK0N125), highest PK but no N (PK4N0) and highest PK and N rate (PK4N125). These treatments were selected because they allowed us to test our hypotheses on the most extreme treatments. All treatments were sampled in nine soil depth layers, including 0–20, 20–22.5, 22.5–25, 25–27.5, 27.5–30, 30–35, 35–40, 40–50 and 50–60 cm. The short depth intervals between 20–30 cm and 30–40 cm were chosen to see whether there were significant SOC changes in the transition zone of top and subsoil and how deep the treatment effects were. Bjertorp, Högåsa and Vreta Kloster were sampled in October 2013, 2017 and 2019, respectively. For SOC and PLFA analyses, auger samples were taken at five locations within each plot. We used an auger with a diameter of 25 mm for sampling the 0–20 cm layer. Another auger with a diameter of 20 mm was inserted in the same drilling hole to take samples at 20–60 cm depth that were sliced in the field according to the depth layers mentioned above. Plot-wise composite samples for element analysis were stored at 2°C and sieved at 2 mm before analysis. Samples for PLFA analysis were freeze–dried.

We calculated SOC stocks in soil profiles for the aforementioned extreme treatments. As fine soil fraction mass and bulk density were not measured for each sampling layer, we estimated bulk density of fine soil using either site-specific regression based on field measurements or pedotransfer functions derived from the Swedish agricultural soil database (Table S1). As the pedotransfer functions used 25 cm as the boundary between top and subsoil when they were developed, we adapted this boundary and referred 0–25 cm to topsoil in the analysis of SOC and PLFAs profiles. Gravel content at Högåsa was measured as detailed in the supplement, and it was negligible at Bjertorp and Vreta Kloster.

Soil carbon stocks were calculated from $BD_{fine\ soil}$ and carbon content of fine soil ($[SOC]_{fine\ soil}$) corrected for the volume fraction of gravel according to Poeplau et al. (2017):

$$SOC_{stock} = [SOC]_{fine\ soil} \times BD_{fine\ soil} \times depth_i \times \left(1 - f_{gravel}\right) \quad (1)$$

where *depth* is the thickness of the sampled soil layer *i*, f_{gravel} is the volume fraction of gravels greater than 2 mm in a whole soil sample calculated as.

$$f_{gravel} = \frac{V_{gravel}}{V_{sample}} \quad (2)$$

and the bulk density of fine soil ($BD_{fine\ soil}$, ≤2 mm) was then calculated from the mass and volume of bulk samples and gravel content.

$$BD_{fine\ soil} = \frac{M_{sample} - M_{gravel}}{V_{sample} - V_{gravel}} \quad (3)$$

where M_{sample} and M_{gravel} are the dry mass of the cylinder sample and the dry mass of sieved gravel and stones in g (> 2 mm), V_{sample} and V_{gravel} are the volume of the cylinder and the volume of gravel and stones in cm^3, V_{gravel} was determined by dividing the mass of gravel and stones by an assumed rock density of 2.6 g cm^{-3}.

We applied the equivalent soil mass method (ESM) (Ellert and Bettany 1995) to overcome the limitation of fixed depth sampling in comparing carbon stocks among treatments. We modified the SimpleESM R script (Ferchaud et al. 2023) by including the gravel correction. The SimpleESM R script calculates soil carbon stock using three methods, including the fixed depth (FD) method, the classic ESM method (Ellert and Bettany 1995) and an alternative ESM method that additionally incorporates a cubic spline model (McBratney and Minasny 2010; Rovira et al. 2015). We chose the mean of the two plots with the lowest BD, that is, treatment with the highest rates of PK and N fertilisation (PK4N125) in the ley rotation at each site as the reference soil mass, to avoid extrapolation of soil depths beyond the sampled range.

Soil samples from treatments PK0N0 and PK4N125 were freeze–dried for phospholipid fatty acids (PLFAs) analysis, covering soil depths of 0–20, 22.5–25, 25–27.5, 27.5–30 and 35–40 cm at the Högåsa and Vreta Kloster sites, and 0–20, 30–35 and 50–60 cm at Bjertorp. About 1 g of the freeze–dried samples was fractioned with 5 mL chloroform, 20 mL acetic acid and 5 mL methanol. Methylated PLFAs were then quantified by gas chromatography (Börjesson et al. 2014, 2012). We grouped individual PLFA into Gram-negative, Gram-positive, actinobacteria and fungi for analysing the composition of microbial communities. Unsaturated PLFAs with one double bond or cyclic structure were grouped as Gram-negative bacteria, and all other branched PLFAs except for methyl structures were grouped as Gram-positive bacteria. Actinobacteria were the sum of methyl-branched fatty acids 10Me16:0, 10Me17:0 and 10Me18:0, and fungi were represented by the sum of 18:2 and 18:3 (Willers et al. 2015). The sum of cyclopropyl PLFAs to the sum of their monoenoic precursors (abbreviated as *Cy*/*pre* ratio), (cy17:0 + cy19:0)/(16:1ω7 + 18:1ω7) was calculated as a stress indicator (Moore-Kucera and Dick 2008). Bacterial PLFAs were summed from straight-chain saturated PLFAs, monosaturated PLFAs, cyclopropyl saturated PLFAs and terminally branched PLFAs. The bacteria: fungi ratio was calculated as the bacterial PLFAs divided by fungal PLFAs (18:2 + 18:3). All identified PLFAs in each sample were summed as total PLFAs (nmol g^{-1} soil). The ratio of total PLFAs and SOC was also calculated.

2.4 | Carbon Inputs

Carbon inputs from above- and below-ground crop residues were calculated from the dry mass of the harvested product (grain or ley forage) recorded for each treatment at each site except for a few crop failures using allometric functions (Bolinder et al. 2007). The allocation coefficients of small-grain cereals were used for winter wheat, spring wheat, oats and barley not under-seeded with leys, coefficients of under-seeded grain were used for under-seeded barley in ley rotation, coefficients of perennial forages were used for leys and coefficients of oilseed crops were calculated from data on harvest index and shoot-to-root ratios from Palosuo et al. (2015). We assumed that all biomass contained 45% C (Kätterer et al. 2011). The belowground C input is the sum of C from roots and extra-root C (rhizodeposits), and the aboveground C input is the stubble in the ley rotation and all straw in the arable rotation. Carbon input from organic amendments was calculated from their dry matter and ash content. Carbon input from manure before 2010 was 0.462, 0.407 and 0.488 Mg C ha^{-1} for Högåsa, Vreta Kloster and Bjertorp, respectively. Carbon input from slurry applied from 2011 onwards was about 0.113 Mg C ha^{-1} for all sites. For the years with crop failure due to drought or fallow, we assigned 0.2 and 0.3 Mg C ha^{-1} to belowground C input (roots + extra-root) and aboveground C input for all treatments, respectively. Allocation coefficients are presented in Table S2, and the steps of calculations are detailed in Supporting Information.

2.5 | Statistical Analysis

To understand the general trend of the treatments on topsoil SOC, we analysed the effect of crop rotation, PK fertilisation and N fertilisation on the time series of topsoil SOC content using MIXED MODEL with SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Bjertorp site had 11 points in time while the other two sites had nine. Because the two replicate samples from the same treatment were analysed as a composite sample, sites became replicates in the data analysis of time series SOC. First, crop rotation, PK fertilisation, N fertilisation, sampling year and their interactions were set as categorical fixed effects in the mixed models. Nested experimental units were treated as random effects in multiple levels and crossed with the sampling year, and the temporal correlation of repeated measurements was accounted for by selecting the covariance structure of compound symmetry (CS), spatial power (SP(POW)) and variance components (VC). The significance level (alpha) of 0.05 was used throughout the statistical analysis. The insignificant interactions of higher orders were removed one by one to reduce the full model. The Kenward–Roger method was chosen for calculating the degrees of freedom because of unbalanced data, and a random effect level was removed when the estimated variance was zero. Least square means (LS-means) were compared with test the significance of main fixed effects at $p = 0.05$ level. Second, the sampling year was treated as a continuous variable in a mixed model regression to compare the trajectory curve of main effects on topsoil SOC. Third, the plot-based sampling in 1966 and 2013 had two replicates for each treatment, which allowed us to analyse the treatment effects on SOC changes at each site. In the last comparison, site was treated as a fixed effect in addition to the nested rotation and fertilisation effects, and means of SOC changes at each site were compared by slicing the interaction of site, rotation and fertilisation effects.

We compared SOC contents of the main effects at nine sampling depths along the soil profiles sampled in the extreme treatments between 2013 and 2019. In the mixed model, sampling depth was treated as a fixed effect in addition to crop rotation, PK fertilisation and N fertilisation. Experimental units were treated as random effects, and the covariance structure was selected to account for the spatial correlation between sampled depths. We removed insignificant higher-order interactions from the full model and compared means of SOC content for the main effects at the same depth using the lsmestimate statement. We assumed that sampling a few years apart would not change the pattern created by decades of treatments. Thus, sampling year differences between sites were not considered when analysing the profile sampling between 2013 and 2019. Regarding the SOC stocks calculated using ESM methods (Ferchaud et al. 2023), 0–25 cm carbon stock was considered as topsoil according to the pedotransfer functions used for estimating bulk density. Cumulative SOC stocks in the topsoil and subsoil (below 25 cm), calculated using the ESM method, were compared similarly for each sampling layer.

We compared belowground C inputs and C exported in straw between two rotations using a mixed effects model. Time series of aboveground and belowground and total C inputs were square root transformed to meet the requirements of normal distribution of residuals. Crop rotation, PK fertilisation, N fertilisation and their interactions were set as fixed effects and experimental units were crossed with year in the random effects. Levels of random effects were dropped one by one until the model converged without errors and warnings. Least-square means, standard error, confidence intervals and differences in C input and export between the two rotations were back-transformed for interpreting the effect of C input on SOC changes.

We compared grouped and total PLFAs to test treatment effects on viable soil microbial biomass and the composition of microbial communities. Because only four extreme treatments (PK0N0 and PK4N125 under each rotation) were sampled across all three sites and commonly sampled at 0–20 and 35–40 cm depths (Bjertorp at 30–35 cm), we excluded other treatments that were sampled only at Bjertorp for a compatible comparison. We recoded PK and N fertilisation as one fertilisation factor PKN with two levels (PKN0, PKN4) because of no combined interactions in the sampling. Depth was first treated as a fixed effect to test the depth effect, and later, the main effects were further tested depth by depth. Site, rotation and PKN fertilisation were all set as fixed effects for testing rotation and PKN effects on grouped PLFAs variables, including total PLFAs, bacterial PLFAs, fungal PLFAs, ratio of total PLFAs to SOC, bacteria to fungi ratio, Gram-negative, Gram-positive, actinobacteria and *Cy*/*pre* ratio. Finally, we applied linear regression by site using all available PLFAs at different depths of the topsoil and subsoil to analyse the relationship between SOC and total PLFAs.

3 | Results

3.1 | Rotation and Fertilisation Effect in Time Series Topsoil SOC

The time series of three LTEs in central Sweden showed decreasing SOC content in 0–20 cm topsoil in all treatments (Figure 1). In the mixed model with year as a categorical variable, rotation, N fertilisation level and year were all significant fixed effects, but the interaction of rotation and N level was not. Overall, the ley rotation had significantly higher SOC content in the topsoil compared with the arable rotation. Least-square means of time series SOC were 2.02% ± 0.05% and 1.90% ± 0.05% for the ley rotation and arable rotation, respectively (Table 2). The SOC content was higher in treatments with higher N application rates, but only N82 and N125 had significantly higher SOC than the control without N fertilisation (Table 2). Comparing rotation and N fertilisation levels by year showed that significant differences in SOC between the two rotations were established after 10 years and maintained thereafter, while the effect of N fertilisation became significant only from 1995 onward. From 1966 to 2019, SOC decreased from 2.22% ± 0.07% to 2.04% ± 0.07% in the ley rotation and to 1.89% ± 0.07% in the arable rotation, and absolute changes in SOC were −0.31, −0.27, −0.24 and −0.19 percentage units in N fertilisation level from N0 to N125, respectively (Figure 1). As PK fertilisation and the interactions with other factors were not statistically significant in the F test of fixed effects, no further comparisons were made for PK fertilisation.

In the mixed model regression with year as a continuous variable, regression slopes in arable rotation were steeper than in ley rotation under the same N fertilisation level, and negative slopes increased with N fertilisation rate in both rotations (Figure 2, Table S3). Although SOC loss rate was lower under higher N fertilisation rates, this positive effect started leveling off at N82 and N125 (Figure 2).

Analysing changes in topsoil SOC between 1966 and 2013 at each site with the mixed model showed similar results as in the SOC time series. Rotation and N fertilisation were significant fixed effects, but PK fertilisation and the interactions with rotation and N levels remained not statistically significant and dropped out from the reduced model. After 47 years of the experiment, SOC content had decreased by 0.14% ± 0.03% and 0.30% ± 0.03% (absolute change) in ley rotation and arable rotation, respectively. The SOC loss decreased with N fertilisation rates, from 0.26% ± 0.03% in N0 to 0.22% ± 0.03%, 0.20% ± 0.03% and 0.16% ± 0.03% for N41, N82 and N125, respectively. Site had a significant effect on SOC changes when it was treated as a fixed effect. Despite the overall positive effect

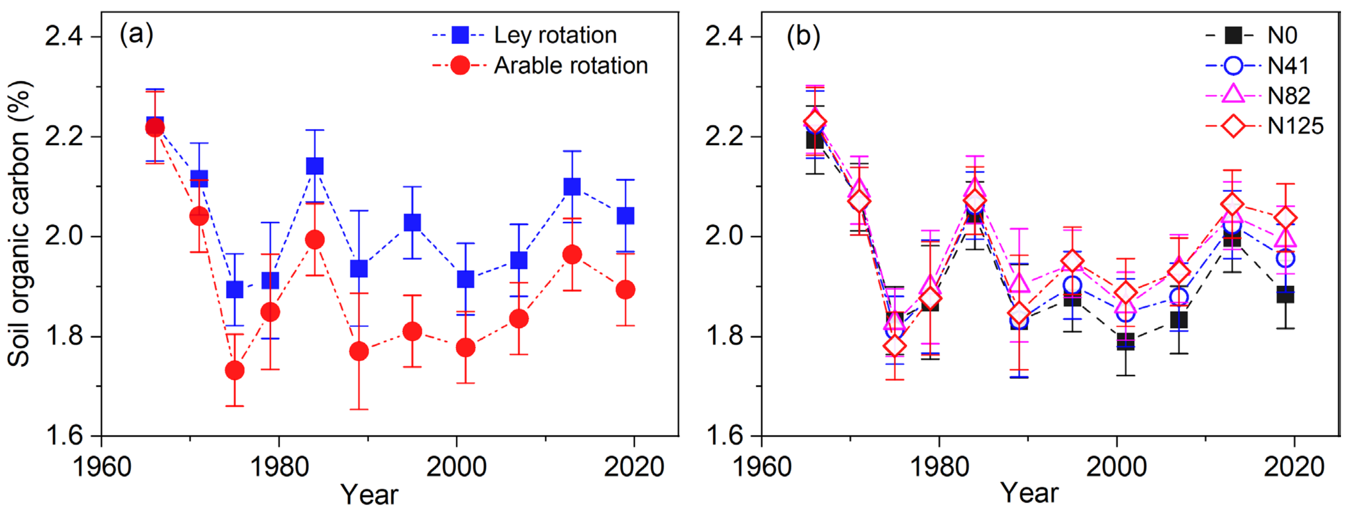

FIGURE 1 | Least-square means of soil organic carbon content in 0–20 cm for rotations (a) and nitrogen fertilisation levels (b) from 1966 to 2019. Error bars stand for the standard error of LS-means. LS-means from the mixed model are pooled from three sites with a total of 926 observations.

TABLE 2 | Least-square means (LS-means) for the time series of soil organic carbon content (%) in 0–20 cm by rotation and nitrogen fertilisation levels ($\alpha = 0.05$).

Factor	Level	LS-means	Standard error	Differences of LS-means[a]	Standard error of differences	DF	t	Pr > |t|	95% confidence interval	
									Lower	Upper
Rotation	Ley	2.02A	0.05							
Rotation	Arable	1.90B	0.05	−0.12	0.05	20.2	−2.31	0.03	−0.24	−0.01
N	0	1.93a	0.04							
N	41	1.95ab	0.04	0.02	0.02	69.8	1.35	0.18	−0.01	0.06
N	82	1.98b	0.04	0.05	0.02	69.8	3.06	0.00	0.02	0.09
N	125	1.98b	0.04	0.05	0.02	70.9	2.66	0.01	0.01	0.08

[a]LS-means of soil organic carbon in arable rotation was compared to ley rotation, and statistical significance is indicated by capital letters. Soil organic carbon in N fertilisation was compared to N0 (no N fertiliser) and statistical significance is indicated by lowercase letters.

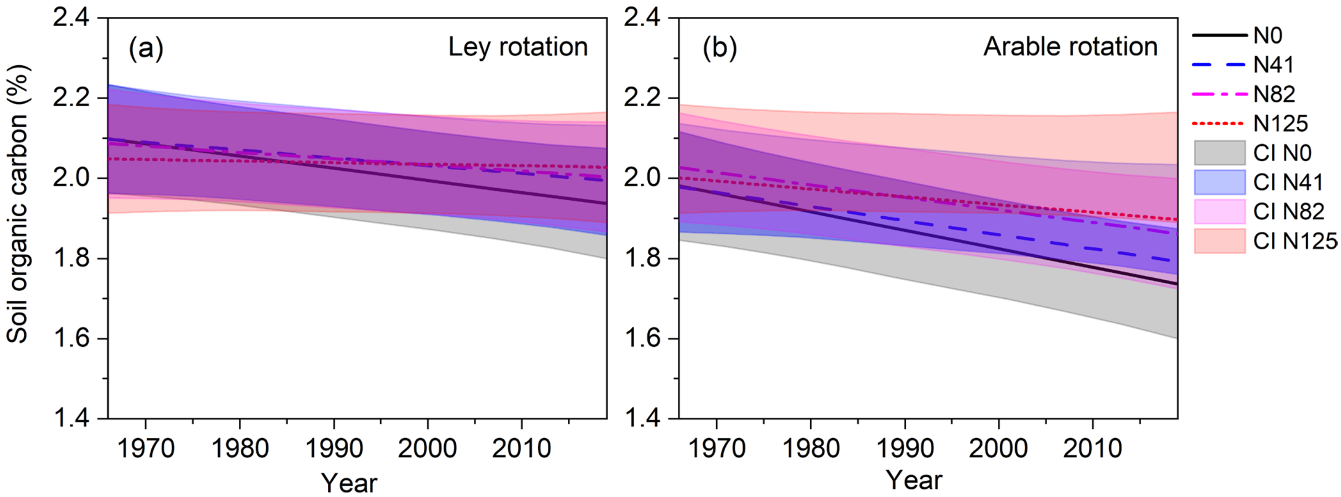

FIGURE 2 | Mixed model regression of soil organic carbon content in 0–20 cm with time in ley rotation (a) and arable rotation (b). Soil organic carbon contents from 1966 to 2019 were used in regressions. Bands are 95% confidence intervals (CI) of the predictions, and regression coefficients are reported in Table S3.

of ley rotation and N fertilisation on SOC, the magnitude of SOC changes differed between sites (Figure 3). For example, SOC loss was the largest in the sandy soil at the Högåsa site, while the heavy clay soil at Vreta Kloster lost the least amount of SOC or even gained SOC under high fertilisation rates during the same period. Moreover, the loss of SOC in the arable rotation was significantly higher than in the ley rotation under the same N fertilisation levels at Högåsa (Figure 3b), and a significant difference between rotations was also observed at Vreta Kloster but only at the N0 level. In contrast, a significant difference in SOC loss was only observed between N0 and N125 at Vreta Kloster under both ley and arable rotations (Figure 3c).

3.2 | Rotation and Fertilisation Effect in Profile SOC and Stocks

After about five decades of the experiment, SOC content in soil profiles under extreme treatments (PK0N0, PK0N125, PK4N0 and PK4N125) showed consistent positive effects of rotation with leys and N fertilisation in the 0–20 cm soil layer (Figure 4). Positive effects were also observed for soils between 20 and 27.5 cm depth. For instance, SOC content was significantly higher in the ley rotation than in the arable rotation for 0–25 cm depths (Figure 4), with differences ranging from 0.12% ± 0.06% to 0.14% ± 0.06% in three sampled depths (Table S4). Compared with N0, N125 had a positive effect on SOC content in the soil up to 27.5 cm and was significantly higher at 22.5–25 cm depth. However, negative effects were observed at some depths in the subsoil. For example, SOC content was higher in N0 below 27.5 cm depth compared with N125, and was statistically significant at 30–35 and 35–40 cm depths (Figure 4, Table S4).

The top 25 cm of soil stored more than 60% of the carbon stocks in 0–60 cm reference soil mass. Cumulative SOC stocks were only significantly different at 22.5–25 cm depth between the ley and arable rotations (Table 3). After about five decades of the experiment, cumulative SOC stocks in 0–25 cm reference soils under ley rotation were 3.3 ± 1.6 Mg C ha^{-1} significantly higher than stocks under arable rotation. The N125 treatment had 2.9 ± 1.6 Mg C ha^{-1} more C in the top 25 cm soil compared to N0, but the difference was not significant. These positive effects of rotation with leys and N fertilisation started diminishing below

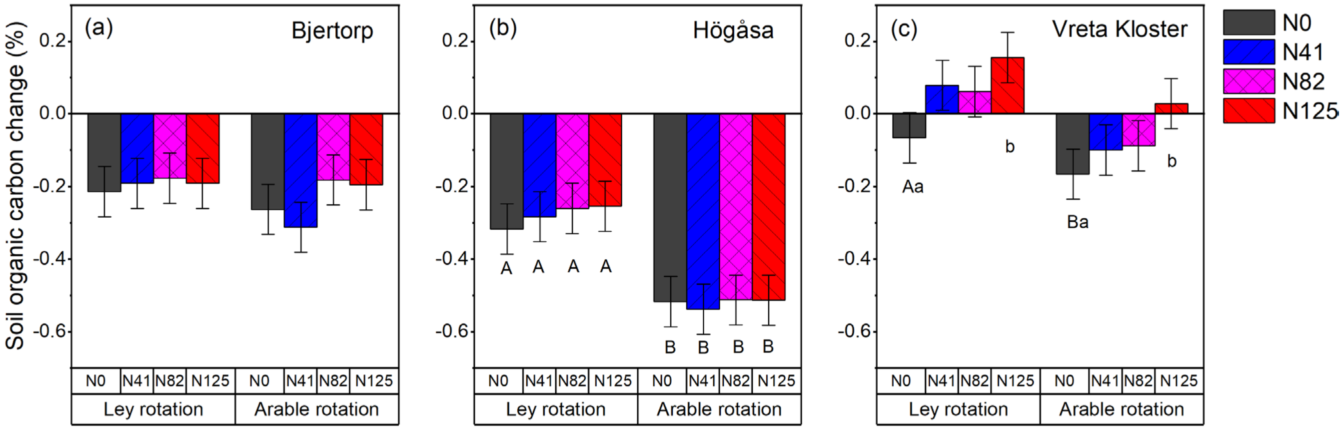

FIGURE 3 | Least-square means of absolute change of soil organic carbon (SOC) in 0–20 cm soil from 1966 to 2013 at Bjertorp (a), Högåsa (b) and Vreta Kloster (c). Changes were calculated from plot-based sampling in 1966 and 2013 (64 observations per site), negative values represent loss of SOC. Error bars are standard error. Significant differences in LS-means of SOC change between ley and arable rotation under the same N fertilisation level were highlighted by different uppercase letters, while lowercase letters represent significant differences between N fertilisation levels under the same rotation.

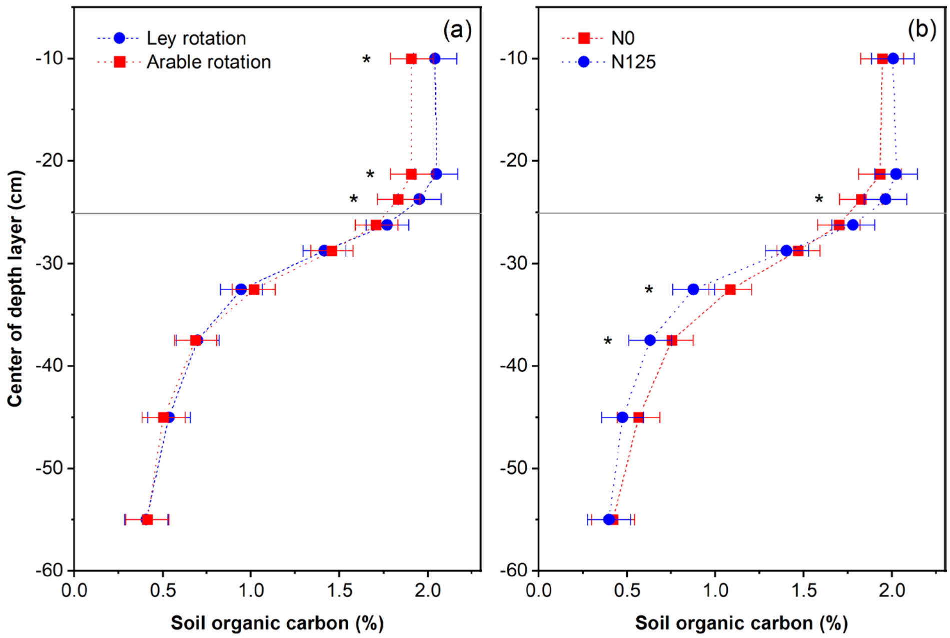

FIGURE 4 | Effect of rotation (a) and N fertilisation rate (b) on least-square means of soil organic carbon content at different soil depths based on profile sampling in the most extreme treatments (PK0N0, PK0N125, PK4N0 and PK4N125 in each rotation). LS-means are derived from three sites with a total of 432 observations. Bjertorp, Högåsa and Vreta Kloster were sampled in 2013, 2017 and 2019, respectively. The numerical differences of LS-means between two rotations and N fertilisation levels are presented in Table S4.

30 cm. As a result, cumulative SOC stocks in 60 cm reference soil mass were 99.3 ± 2.9 and 98.7 ± 2.9 Mg C ha^{-1} for the ley and arable rotations, respectively (difference 0.6 ± 2.4 Mg C ha^{-1}), and 98.5 ± 2.9 and 99.6 ± 2.9 Mg C ha^{-1} for N0 and N125, respectively (difference − 1.0 ± 2.4 Mg C ha^{-1}).

3.3 | Carbon Inputs

Based on the annually recorded crop grain and ley biomass yield, C input from roots, extra-root (or rhizodeposition), straw and manure and C in exported straw were calculated and compared for the two crop rotations. The C inputs from belowground (roots and extra-root) in the ley rotation were significantly higher than those in the arable rotation, that is, 1114 ± 31 versus 524 ± 21 kg C ha^{-1}, respectively (Table 4). Because of exporting straw, the ley rotation had only 594 ± 29 kg C ha^{-1} input from aboveground residues (including that from leys, see the Supporting Information for details), compared with 1518 ± 46 kg C ha^{-1} from retained straw in the arable rotation (Table 4).

The ley rotation, representing farming systems with livestock, received an average of 396 ± 3 kg C ha^{-1} year^{-1} manure C input in addition to high belowground C inputs; yet C inputs were not sufficient to maintain SOC levels across sites. The arable

TABLE 3 | Least-square means of cumulative soil organic carbon stocks ($Mg C ha^{-1}$) in the topsoil (0–25 cm) and subsoil (25–60 cm) for the two rotations and the lowest and highest N fertilisation rates.

Depth of reference soil mass (cm)[a]	Ley rotation	Arable rotation	Difference (ley–arable)	DF	*t*	Pr > |*t*|	N0	N125	Difference (N0–N125)	DF	*t*	Pr > |*t*|
0–20	51.5 ± 2.2	48.7 ± 2.2	2.8 ± 1.6	372.3	1.73	0.0851	49.0 ± 2.2	51.2 ± 2.2	−2.2 ± 1.6	372.3	−1.39	0.1669
20–22.5	57.8 ± 2.2	54.7 ± 2.2	3.0 ± 1.6	372.3	1.90	0.0585	55.0 ± 2.2	57.5 ± 2.2	−2.5 ± 1.6	372.3	−1.57	0.1181
22.5–25	**63.8 ± 2.2A**	**60.5 ± 2.2B**	**3.3 ± 1.6**	**372.3**	**2.08**	**0.0386**	60.7 ± 2.2	63.6 ± 2.2	−2.9 ± 1.6	372.3	−1.84	0.0667
25–27.5	6.3 ± 2.2	5.9 ± 2.2	0.4 ± 1.6	372.3	0.25	0.8048	6.0 ± 2.2	6.1 ± 2.2	−0.1 ± 1.6	372.3	−0.08	0.9374
27.5–30	11.5 ± 2.2	11.5 ± 2.2	0.0 ± 1.6	372.3	0.02	0.9876	11.5 ± 2.2	11.5 ± 2.2	0.0 ± 1.6	372.3	−0.02	0.9820
30–35	18.6 ± 2.2	19.7 ± 2.2	−1.1 ± 1.6	372.3	−0.70	0.4847	19.7 ± 2.2	18.7 ± 2.2	1.0 ± 1.6	372.3	0.63	0.5262
35–40	23.4 ± 2.2	25.0 ± 2.2	−1.6 ± 1.6	372.3	−0.99	0.3228	25.0 ± 2.2	23.4 ± 2.2	1.6 ± 1.6	372.3	1.00	0.3197
40–50	30.2 ± 2.2	32.3 ± 2.2	−2.1 ± 1.6	372.3	−1.29	0.1971	32.3 ± 2.2	30.2 ± 2.2	2.0 ± 1.6	372.3	1.28	0.2004
50–60	35.6 ± 2.2	38.3 ± 2.2	−2.7 ± 1.6	372.3	−1.69	0.0915	37.9 ± 2.2	36.0 ± 2.2	1.9 ± 1.6	372.3	1.19	0.2330
0–60	99.3 ± 2.9	98.7 ± 2.9	0.6 ± 2.4	375.6	0.25	0.8017	98.5 ± 2.9	99.6 ± 2.9	−1.0 ± 2.4	375.6	−0.44	0.6608

Note: Significant differences are indicated by bold style.
[a]Depth layers refer to the reference treatment, that is, highest PK and N fertilisation in arable rotation at each site. Depths in other treatments were adjusted according to the equivalent soil mass method and no extrapolation beyond the sampled depths.

TABLE 4 | C input and export from two crop rotations.

C input component (kg C ha^{-1})	Rotation	LS-means ± standard error	95% confidence interval upper	95% confidence interval lower	Difference of LS-means	DF	t	Pr > |t|	95% confidence interval upper	95% confidence interval lower	Ley/arable
Roots	Ley	913 ± 33	850	979	137	1119	30	< 0.0001	120	155	2.7
	Arable	343 ± 20	304	384							
Extra-root	Ley	598 ± 21	556	640	88	1119	30	< 0.0001	77	100	2.6
	Arable	227 ± 13	202	254							
Belowground (0-25 cm)	Ley	1114 ± 31	1054	1175	110	1115	30.37	< 0.0001	96	125	2.1
	Arable	524 ± 21	483	567							
Exported straw	Ley	1593 ± 39	1517	1671	1592	1120	112.21	< 0.0001	1537	1649	
	Arable	0	0								
Aboveground (stubble for I and all straw for II)	Ley	594 ± 29	539	652	213	1119	−42.08	< 0.0001	233	193	0.4
	Arable	1518 ± 46	1428	1610							
Manure[a]	Ley	396 ± 3									
	Arable	0	0								

[a]Values for manure are arithmetic means and standard errors. The rest of the variables were compared in the mixed model with square root transformation; presented LS-means, standard errors of LS-means and the differences were back transformed values.

rotation, representing farming systems without livestock, retained all straw in the field, but this was not enough to maintain SOC levels either. Although PK and N fertilisation had significant effects on C input, that is, higher C inputs at higher fertilisation rates, the differences within PK or N fertilisation levels were less prominent compared with the rotation effect (data not shown).

3.4 | PLFAs and Microbial Communities

Analysing grouped PLFAs showed more distinct differences between rotations than between fertilisation treatments in the topsoil, despite that site having a significant effect. Total and bacterial PLFAs (nmol g^{-1} soil) in 0–20 cm soil under ley rotation were about 1.5 times those under arable rotation (Figure 5), while no significant difference was found in fungal PLFAs. Compared with arable rotation, significantly higher PLFAs were found in the ley rotation for Gram-positive bacteria, Gram-negative bacteria and actinobacteria (Table S5). Among the compared ratios, the ratio of total PLFAs and SOC, and the ratio of bacterial and fungal PLFAs were also significantly higher in the ley than in the arable rotations (Figure 5). Compared with unfertilized treatments, fertilisation with the highest rate of PKN showed significantly higher PLFA values in total PLFAs, bacterial PLFAs, Gram-positive bacteria, Gram-negative bacteria, and actinobacteria. However, there was no significant fertilisation effect on the compared ratios (Table S5). The *Cy*/*pre* ratio did not significantly differ between rotations or fertilisation rates. The comparison of grouped PLFAs and ratios in 35–40 cm subsoil showed no significant differences between rotations and between PKN fertilisation levels (Table S6).

Site had a significant effect on most of the compared PLFA variables. In general, Bjertorp had higher total and grouped bacterial PLFAs. For example, total PLFAs in 0–20 cm soil were 1.8 times those at Vreta Kloster and Högåsa sites (89.2 ± 3.8 vs. 48.3 ± 3.8 and 48.3 ± 4.4 nmol g^{-1} soil), though their SOC values were not significantly different (1.9% ± 0.1% vs. 2.0% and 2.1% ± 0.1%). Soil depth exerted a great impact on grouped PLFAs. Total PLFAs and grouped bacterial and fungal PLFAs in the topsoil were substantially higher than those in the subsoil (Tables S5 and S6). Nevertheless, despite the wide range of SOC in sampled soil depths, the regression between total PLFAs and SOC at each site showed a strong linear relationship regardless of the soil depths and treatment within a site ($R^2 = 0.86$, $n = 108$, Figure 5). Notably, the regression slope at Bjertorp was about two times the slope values at Vreta Kloster and Högåsa (Figure 5).

4 | Discussion

4.1 | Rotation and Fertilisation Effect on C Input and Topsoil SOC Change

We analysed time series of topsoil SOC and derived consistent treatment effects from mixed models and linear regression analysis using all available data points. Despite the general decreasing trend of SOC over time and large fluctuation in time series SOC (Figures 1 and S1), the crop rotation with perennial leys had higher SOC than the arable rotation with only annual crops, and higher N fertilisation rates resulted in higher SOC content in the topsoil of the three Swedish LTEs (Figure 1, Table 2). The ley rotation, incorporating leys in 1/3 of the rotation length, reduced SOC absolute loss by 0.14% ± 0.04% from 1966 to 2013 compared with the arable rotation, and the highest N fertilisation rate (125 kg N ha^{-1} $year^{-1}$) reduced SOC loss by 0.10% ± 0.03% compared with the no N fertilisation control during the same period. Poeplau et al. (2016) reported a similar magnitude of absolute SOC change (0.15%) for all paired contrasting treatments after about 50 years across 10 sites of the Swedish fertility trials. Given that the initial SOC average at our three sites was similar, ranging from 2.10% to 2.39%, the isolated treatment effect using simple subtraction of initial SOC from final SOC between treatment pairs is unlikely to be affected by the correlation between larger SOC loss at higher initial SOC in our case.

An increase in the proportion of perennials in crop rotations will in most cases lead to SOC accrual (Kätterer and Bolinder 2022). However, perennial leys in 1/3 of the rotation

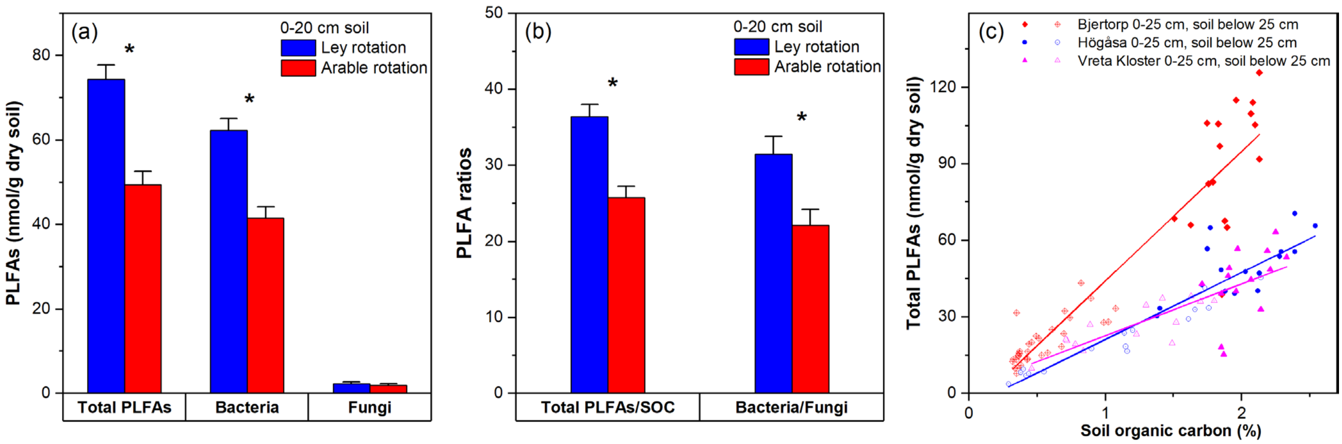

FIGURE 5 | LS-means of total, bacterial and fungal PLFAs (a) and their ratios (b) per rotation across sites in the 0–20 cm soil layer and linear regression of total PLFAs on soil organic carbon (SOC) per site across treatments (c). LS-means in the 0–20 cm soil are derived from 23 observations. Regressions are $y = -6.649 + 50.780*SOC$, $y = -5.102 + 26.291*SOC$ and $y = 2.342 + 20.325*SOC$ for Bjertorp, Högåsa and Vreta Kloster, respectively, with $R^2 = 0.86$, $n = 108$.

length with low to moderate N fertilisation rates (highest 125 kg N ha^{-1}) were not sufficient to maintain initial SOC levels in our study. Similarly, De Los Rios et al. (2022) found that grain crop rotations with leys in one out of three years lost less SOC stocks than continuous silage maize, but it was not sufficient to increase SOC stocks even with a high N fertilisation rate. In contrast, increasing the proportion of ley to 3/4 of rotation length resulted in larger positive effects on SOC and SOC sequestration compared with cereal monoculture in Sweden (Börjesson et al. 2018). Moreover, data from 15 sites under Nordic conditions showed that systems with leys retained on average 0.5 Mg C ha^{-1} $year^{-1}$ more carbon, compared with systems with only annual crops (Kätterer et al. 2013). Another review by Land et al. (2021) reported similar results to a depth of 25 cm (for details see also Kätterer and Bolinder (2022)), but the effect depends also on the initial SOC levels governed by land use and management history as shown for other LTEs in northern Sweden (Kätterer et al. 2012). Meta-analyses confirmed that perennialisation of annual systems with herbaceous perennials significantly increased SOC stocks by 24.2% in comparison to rotations with only annual crops (Siddique et al. 2023), and changing annuals to perennial crops led to an average 20% increase in SOC at 0–30 cm (6.0 ± 4.6 Mg ha^{-1} gain) and a total 10% increase over the 0–100 cm soil profile (5.7 ± 10.9 Mg ha^{-1}) in 20 years period (Ledo et al. 2020). Guillaume et al. (2022) found that an increase of temporary grassland by 10% in crop rotation would induce a SOC gain of 0.40 ± 0.13 kg m^{-2}, regardless of clay content and pH.

Belowground C input is strongly associated with changes in SOC. In our study, SOC change in the topsoil from 1966 to 2019 was linearly correlated with belowground C inputs (Figure 6, Pearson correlation coefficient $r = 0.45$, $n = 96$, $p < 0.0001$). Higher N fertilisation rates corresponded to higher belowground C input in each rotation, but the average belowground C input in the ley rotation was more than two times the input in the arable rotation (Table 4, Figure 6). Moreover, the high belowground C input in the ley rotation was mainly attributed to leys. For example, when we differentiate leys from other crops in ley rotation, the average belowground C input was 1954 ± 27 kg C ha^{-1} for leys and 848 ± 9 kg C ha^{-1} for the other crops. Although aboveground straw C input in the arable rotation quantitatively compensated for its lower belowground C input compared with the ley rotation, retaining all straws in the field did not result in SOC accrual or lower SOC loss (Table 4, Figure 6). This suggests that C input from straw contributed less to SOC balances than belowground C input. Poeplau et al. (2015) also observed limited positive effects of straw incorporation on SOC in other Swedish LTEs, which were attributed to lower retention of aboveground crop residue C in sandy soils. Perennial forage crops are known to produce higher root biomass than cereals (Bolinder et al. 2012; Börjesson et al. 2018). Furthermore, many studies have shown that the fraction of C deriving from belowground residues that is retained in soil is roughly twice that from aboveground plant residues despite different estimation methods (Berti et al. 2016; Jacobs et al. 2020; Kätterer et al. 2011). The estimated retention coefficient in LTEs for solid farmyard manure was similar to that for roots, while estimations for slurry were lower and closer to that for aboveground residues (Berti et al. 2016; Kätterer et al. 2011; Liang et al. 2021). Moreover, the partial or total substitution of mineral fertilisers with organic amendments in croplands led to a greater increase in SOC of +34% (CI +20, +49), compared with SOC increment of +9.4% (CI +6.3, +12.6) by mineral fertiliser alone (Beillouin et al. 2023). Therefore, the higher belowground C input and higher retention coefficients of belowground C input and manure in the rotation with perennial leys favoured topsoil SOC balances in our study, that is, less SOC loss compared with the rotation with only annuals.

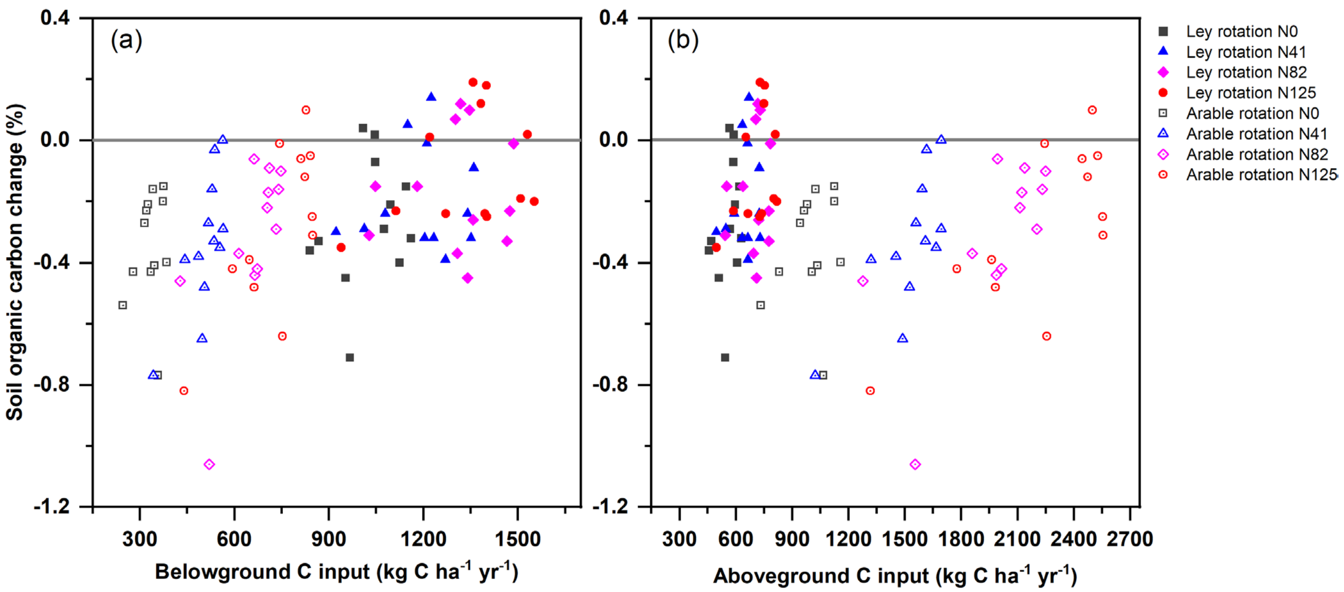

FIGURE 6 | Changes in soil organic carbon (SOC) in 0–20 cm soil between 1966 and 2019 versus averaged annual C input from belowground (a) and aboveground straw (b) by rotation and N fertilisation levels. SOC changes by site and treatment are absolute differences of SOC values between 1966 and 2019 ($n = 96$), and C inputs are arithmetic means by site and treatment averaged from 5136 observations. Pearson correlation coefficient between SOC and belowground C input $r = 0.45$, $p < 0.0001$. Filled symbols represent ley rotation and unfilled are arable rotation. N0-N125 represents N fertilisation rates.

4.2 | Rotation and Fertilisation Effect on SOC Stocks in Soil Profiles

The significant positive effects of leys and N fertilisation on SOC stocks in our study were limited to the top 25 cm of soil (Figure 4, Table 3); while the effect remained positive, it became smaller towards the 30 cm depth. Maximum differences of cumulative SOC stocks, $3.7 \pm 2.4\,\mathrm{Mg\,C\,ha^{-1}}$ for the rotation effect and $3.1 \pm 2.4\,\mathrm{Mg\,C\,ha^{-1}}$ for the N fertilisation effect, appeared at 25–27.5 cm reference soil depth. However, the differences in cumulative SOC stocks changed sign further down the profile, suggesting that the positive effects of rotation with leys and N fertilisation on SOC stocks were diminishing with increasing soil depths in subsoils. Consequently, corresponding treatment differences in cumulative SOC stocks decreased with depth to 0.6 ± 2.4 and $1.0 \pm 2.4\,\mathrm{Mg\,C\,ha^{-1}}$ at 0–60 cm reference soil depth for the rotation and N fertilisation effect, respectively. In this study, the observed differences in SOC stocks were $3.3 \pm 1.6\,\mathrm{Mg\,C\,ha^{-1}}$ in 0–25 cm topsoil for the rotation effect, that is, the ley rotation retained about $66 \pm 32\,\mathrm{kg\,C\,ha^{-1}\,year^{-1}}$ more C than arable rotation during the 50-year experiment period. This observation is much lower than the reported differences of $520\,\mathrm{kg\,C\,ha^{-1}\,year^{-1}}$ from 15 LTE sites under Nordic conditions (Kätterer et al. 2013), which is likely related to a lower proportion of leys in our study.

Overall, N fertilisation slightly increased SOC stocks in topsoil but depleted subsoil SOC stocks (Table 3, Figure 4). Conflicting results of mineral fertilisation's impact on subsoil SOC stocks have been reported in LTEs with various interpretations. Whether fertilisation promotes SOC accumulation in subsoils or stimulates the mineralisation of SOC in subsurface horizons, and by which mechanisms, are still elusive. For example, Skadell et al. (2023) analysed 10 LTEs in Germany and found that mineral fertilisation increased SOC stocks by 9% in topsoil and 6% in subsoil, attributed to increased net primary production and subsequently increased above- and below-ground C inputs. In contrast, the LTE in Puch showed soil organic matter (SOM) increment in 0–25 cm topsoil with N fertilisation because of C in the free light fraction, while 25–60 cm subsoil had SOM decrement likely due to roots' relocation in the Ap horizon with N fertilisation at the surface (Shahbaz et al. 2017). Although belowground C input increased with N fertilisation rates under each rotation (Figure 6) in our study, adding N fertiliser together with more belowground plant-derived residues likely favoured SOC decomposition in the subsoil.

The negative effects on subsoil SOC stocks due to N fertilisation might be attributed to the following reasons: (1) positive priming effect in subsoil. Continuous or pulse input of nutrients or fresh organic matter may activate slow-growing microbial species and cause SOM decomposition, thus generating a real priming effect (Blagodatskaya and Kuzyakov 2008; Wang and Kuzyakov 2024). N leached to subsoil from mineral fertilisation or bioturbation by earthworms under leys provided N to microbes, which may have stimulated the decomposition of SOC in subsoil in our case. (2) Subsoil pH may have affected SOC decomposition. Increasing soil pH in the acidified rhizosphere from pH 6 to 10 was found to accelerate the mineralisation of 'native' SOC in sandy soils (Li et al. 2007; Wang and Tang 2018) through increased availability of essential nutrients and root exudates to microbes, and subsequently increased microbial biomass and activity. In our study, topsoil pH was higher in the order of Högåsa, Bjertorp and Vreta Kloster during the experimental period (Figure S1). In the latest profile sampling, Högåsa had pH ranging around 6.1 across sampled soil depths, while pH at the other two sites increased with increasing soil depths, and pH became above 7.0 in the deeper layers, which may have accelerated SOC decomposition, leading to a negative N fertilisation effect on subsoil SOC.

Our hypotheses on the positive effect of perennial forage crops and N fertilisation on SOC were confirmed only for the topsoil, but these practices did not result in SOC sequestration. In our study, the rotation with ley not only had much higher belowground C input but also received C from manure, contributing to the positive effect on topsoil SOC stocks in farming systems with livestock compared to the system without livestock. A worldwide meta-analysis (Gross and Glaser 2021) and another for Chinese cropland (Sun et al. 2024) showed trends toward positive effects of manure applications on subsoil SOC even below 30 cm depth. However, we did not observe similar trends in the subsoil in our study, which might be due to the low application rate of manure in the ley rotation. Nevertheless, in addition to adding C input to soils, applying manure has shown other benefits on SOC storage, for example, by promoting the incorporation of manure C into microbial biomass and increasing microbial C use efficiency (Berti et al. 2016; Liu et al. 2018). The analysis of the joint effect of ley rotation and manure application in northern LTEs showed that manure had a greater positive effect on SOC content at a lower proportion of ley in crop rotations and the effect was larger in clay soils than in sandy soils (Joona et al. 2024). We could not separate the effect of ley and manure in our study, but under the same proportion of leys and manure application rate, SOC concentration responses were more positive in the heavy clay soil at Vreta Kloster than at the other two sites (Figure 3).

We sampled soils below 20 cm in narrow depth intervals, which allowed us to observe more precisely where the rotation and N fertilisation effects changed from positive to negative at a few depths in the soil profile. According to SOC changes at different sampling depths and approximate ploughing depths, the boundary of topsoil and subsoil was likely to lie between 25 and 30 cm. Although changing the boundary depth for top and subsoil in this study did not result in different conclusions, sampling of subsoil in thicker layers may miss the opportunity to identify how deep management practices can affect SOC, which makes it more difficult to understand the mechanisms and processes involved for induced SOC changes. We, therefore, recommend narrow sampling intervals in the upper parts of the subsoil in future studies for a better quantification of management effects and understanding of underlying processes.

4.3 | Microbial Communities and SOC

We found that viable microbial biomass and bacterial communities were more affected by rotation than mineral N fertilisation (Figure 5, Table S5). This may be attributed to different substrate quality and quantity and site factors. Manure and slurry in general contain more readily available nutrients such as N and P, which may promote rapid microbial growth and carbon use efficiency, especially in bacterial communities, while plant residues with high C/N ratios and complex compounds favour fungal-microbial communities (Guo et al. 2022; Zhong et al. 2010). In our study, manure application was included in the ley rotation, and this system corresponded to higher viable microbial biomass (total PLFAs), bacterial PLFAs and bacteria/fungi ratio than the system without leys and manure application. Similar positive effects have been reported for manure. For example, Zhong et al. (2010) found that amounts of total PLFAs, bacterial, Gram-negative, and actinobacterial PLFAs were highest in the combined manure and mineral fertilisation, followed by the manure-only treatment and least in the mineral N treatment. A global meta-analysis has shown that manure application increased soil microbial biomass and bacterial diversity but decreased fungal diversity in cropland (Guo et al. 2022). Although plant residues generally had more positive effects on fungal biomass and the fungi/bacteria ratio than manure (Liu et al. 2023), the aboveground and belowground plant residues have different effects on fungal communities. Ley rotation with manure application had higher belowground C input in our study. Considering the decomposability of roots being lower than that of aboveground residues (Heikkinen et al. 2021), the high amount of root-derived C from the ley contributed substantially to higher fungal PLFAs despite the lower aboveground residue compared with the arable rotation. As a result, the ratio of bacterial/fungal PLFAs remained higher in ley rotation with manure application.

Mineral fertilisation resulted in a positive effect on total microbial PLFAs and bacterial PLFAs in our study. Compared with the unfertilised treatment, both total PLFAs and bacterial PLFAs were significantly higher in PKN-fertilised treatments at the highest application rate (Table S5), suggesting that mineral PKN fertilisation promoted soil microbial biomass. Our observation is consistent with the comparison of total PLFAs under the mineral fertilised and unfertilised plots in Zhong et al. (2010) and Verdenelli et al. (2019), showing that balanced mineral fertilisation possibly increased the availability of essential nutrients, plant residues and root exudates that microorganisms need. A meta-analysis of LTEs regarding fertilisation response revealed that mineral fertiliser application led to a 15.1% increase in microbial biomass compared to unfertilised controls and that the magnitude of the effect was pH-dependent (Geisseler and Scow 2014). We did not observe any pH-related adverse effect of long-term mineral fertilisation on total and bacterial PLFAs, probably because liming maintained soil pH within the optimal range for crops and microbes.

Strong positive correlations between total PLFAs and SOC content were observed at all three sites regardless of treatment and sampling depth within each experimental site (Figure 5). Notably, the regression slope at Bjertorp was much steeper than at the other two sites, indicating a larger increment of microbial biomass per unit of SOC. This may be attributed to soil structure and clay content. Börjesson et al. (2014) reported a similar positive correlation in four LTEs, but the slope in their study was independent of the experimental site, and the microbial communities analysis showed larger differences between sites than between treatments. Clay particles can bind microbial products through adsorption, increase the formation of microaggregates and decrease SOC mineralization, but the higher microbial biomass in soil with high clay content was associated with high specific surface area and water content irrespective of mineralogy (Rakhsh et al. 2020). The Vreta Kloster site had the highest clay content; despite its larger water-holding capacity, the poor soil structure may limit water availability to microbes and plants under dry conditions or create more anaerobic conditions in the event of heavy rains, which may explain less total PLFAs but smaller SOC loss (mineralization) at Vreta Kloster. In contrast, the less clayey soil at Bjertorp, with observed good soil structure, not only made water more available to microbes and plants but also favoured the formation of macroaggregates and occluded particulate SOC.

This study showed that manure application and ley cultivation in the farming system with livestock were beneficial for total viable microbial biomass and bacterial communities compared with the farming system without livestock. This supports our hypothesis that microbial community composition is affected by farming systems because of differences in the quality of C inputs. The strong linear correlation between total PLFAs and SOC, irrespective of treatment and topsoil and subsoil within each site, suggests that microbial communities have an important role in SOC dynamics. Mineral PKN fertilisation in general resulted in higher total PLFAs and grouped communities, but the ley rotation with manure application had larger beneficial effects.

The findings of this study are also subject to limitations. For example, we calculated root and extra root C input using carbon allocation coefficients proportional to crop yields in all treatments. A field study showed that belowground C inputs to soils from maize and wheat were independent of yield and absolute amounts of belowground inputs responded little to fertilisation (Hirte et al. 2018). More measurements of root biomass and extra root C in situ under different treatments for other major crops in the future would improve the estimation of belowground C inputs. Our detailed soil profile sampling was carried out once but in different years for each site, and the SOC changes during these years were not accounted for in this study. Sampling all sites in the same year would reduce the temporal variation of analysed soil properties. Furthermore, the exact ploughing depth was not recorded in our LTEs. The typical ploughing depth in Scandinavian regions varies from 18 to 30 cm (Rasmussen 1999), but it was not measured in the Swedish LTEs and may have increased over time from 20 to 25 cm (Poeplau et al. 2015). Recording actual ploughing depth and measuring bulk density more frequently in the future would improve the delimitation of subsoil and reduce the uncertainties in assessing management effects in subsoils.

5 | Conclusion

Realising the potential of SOC sequestration or reducing C losses in cropland requires combining multiple management strategies. In this study, we examined two contrasting farming systems from three Swedish LTEs. Incorporating perennial leys, adding manure and exporting straw in farming systems with livestock resulted in higher SOC stocks in topsoils, compared with the system without livestock and only annual crops where crop residues were retained. The leys contributed high amounts of belowground C inputs. Considering their higher C retention coefficient compared to aboveground crop residues, belowground C inputs played an important role in promoting soil carbon stabilisation in the ley rotation. The rotation with a ley period and applying manure favoured soil microbes, especially bacterial communities, promoting the incorporation of manure and belowground C inputs into microbial biomass and benefiting the SOC balance in the long term. Nitrogen fertilisation increased plant C inputs and reduced SOC loss in the topsoil, but had a negative effect on SOC at a few depths in the subsoil. The variable responses of topsoil SOC in the three long-term experiments indicate that site characteristics should be taken into account when developing multiple local management strategies for SOC accrual in cropland.

Author Contributions

Rong Lang: data curation, methodology, investigation, formal analysis, visualization, writing – review and editing, writing – original draft, conceptualization. **Martin A. Bolinder:** data curation, funding acquisition, writing – review and editing, conceptualization, methodology, investigation, project administration. **Gunnar Börjesson:** data curation, writing – review and editing, conceptualization, methodology, investigation. **Thomas Kätterer:** data curation, funding acquisition, writing – review and editing, conceptualization, methodology, investigation, project administration, resources.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The description of the Swedish soil fertility experiments (R3–9001 series) and contact for retrieving archived data are available at https://www.slu.se/en/departments/soil-environment/research/soil-nutrient-cycling/slu-field-research-plant-nutrition/.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.