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Microbial controls of nitrogen retention and N_2O production in cropping systems supporting soil carbon accrual

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ABSTRACT

The fate of nitrate is central for increasing nitrogen use efficiency in cropping systems. It is influenced by ammonifiers and denitrifiers, two microbial guilds that compete for nitrate and contribute to nitrogen retention and loss, respectively, with the latter in the form of dinitrogen gas and the greenhouse gas nitrous oxide (N_2O). We hypothesized that cropping systems causing higher soil carbon:nitrate favor ammonifiers and thereby lower N_2O emissions and improve crop yield. We sampled long-term field experiments comparing annual cereal, w/wo straw return, and ley rotations under four fertilization regimes replicated in three pedoclimatic zones in Sweden. Soil carbon content has decreased in the cereal rotations since the establishment of the experiments, whereas positive effects of leys on soil carbon varied depending on clay content. Nevertheless, the ley rotations displayed consistently lower nitrate levels irrespective of fertilization regime, lower N_2O production rates, and similar or higher cereal yields compared to cereal cropping. Sequencing of 16S rRNA genes showed major differences in the soil microbiome between ley and cereal rotations, with some effects of fertilization. To tease apart effects on the functional guilds, we quantified the genetic potential of ammonifying (*nrfA*), denitrifying (*nirK*, *nirS*) and N_2O reducing (*nosZ*I and *nosZ*II) microbial communities. Nitrate availability rather than carbon content explained the apparent control of carbon:nitrate on ammonifiers *vs* denitrifiers, with lower levels favoring the former. Altogether, our findings highlight the importance of integrating carbon and nitrogen management strategies to improve soil carbon content while also reducing N_2O emissions from cropping systems.

1. Introduction

Nitrogen (N) loss resulting from the low N-use efficiency of fertilized soils, where only half of the N inputs are recovered in the harvested yield (Lassaletta et al., 2014), have made agriculture the main source of N pollution (Kanter et al., 2020). This surplus N is partly lost through emissions of gaseous N-compounds, including the ozone-depleting and greenhouse gas nitrous oxide (N_2O) (Ravishankara et al., 2009). Nitrous oxide has a long half-life (ca. 120 years) and a warming potential 300 times greater than CO_2 (Prather et al., 2015). The N_2O concentration in the atmosphere has been increasing at an accelerating rate (Thompson et al., 2019) and emissions from fertilized soils account for ca. 50 % of the anthropogenic emissions at the global scale (Tian et al., 2020). Improving the management of N is thus critical to increase the sustainability of agriculture and mitigate climate change (Battye et al., 2017).

In fertilized soils, N_2O emissions are tightly linked to the fate of nitrate (NO_3^-). When not assimilated by crops and other organisms or lost by leaching, NO_3^- can be respired by phylogenetically diverse soil bacteria and archaea via denitrification or ammonification (Pold et al., 2024; Saghaï and Hallin, 2024). Both processes couple the oxidation of carbon (C) compounds to the reduction of NO_3^- under anoxic conditions, with contrasting consequences for the fate of N. Denitrification is a modular pathway where NO_3^- is sequentially reduced to N_2, with N_2O as an intermediate, and represents the main source of N_2O in terrestrial ecosystems (Scheer et al., 2020; Liang and Robertson, 2021). The last step of denitrification, the reduction of N_2O to N_2, can also be performed by non-denitrifying microorganisms utilizing N_2O produced by other community members (Hallin et al., 2018). By contrast, NO_3^- is reduced to ammonium during ammonification, which can support N retention in the system through ammonium assimilation or adsorption to clay particles and soil organic matter. Only small amounts of N_2O are produced during ammonification, suggesting that the contribution of ammonification to N_2O emissions is marginal (Stremińska et al., 2012). The genetic capacity for specific processes ultimately affect N loss and retention in agricultural soils and their contribution to climate change

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(Yoon et al., 2019).

Previous work has shown the influence of the C:NO_3^- ratio on ammonifier *vs*. denitrifier abundance and activity, with high ratios generally increasing the importance of ammonification in relation to denitrification (Kraft et al., 2014; van den Berg et al., 2015; Pandey et al., 2019; Luo et al., 2020). Agricultural management practices like the application of organic amendments, reduced tillage, and retention of crop residues can contribute to maintaining or increasing soil C content, but can also lead to higher greenhouse gas emissions (Xia et al., 2018; Tamburini et al., 2020). Nevertheless, site and region specific studies indicate that cropping systems including perennials emit less N_2O compared to annual crops while also increasing soil C content (Gelfand et al., 2016; Thompson et al., 2016; Putz et al., 2018). Moreover, a recent metagenomic analysis spanning all major terrestrial biomes has shown that the abundance of ammonifiers relative to denitrifiers increases with increasing soil C content when C levels are low, suggesting that C management can help retain N in arable soils (Saghaï et al., 2023). Regarding the N_2O-reducing communities, there is evidence for niche partitioning between clade I and clade II organisms in relation to soil C:N ratio and cropping system (Jones et al., 2014; Domeignoz-Horta et al., 2015; Juhanson et al., 2017), with the abundance of clade II N_2O-reducers being associated with a higher N_2O sink capacity in arable soils (Domeignoz-Horta et al., 2016; Xu et al., 2020; Jones et al., 2022). Deciphering the long-term effects of soil C:N development in a cropping system and management context on communities performing NO_3^- and N_2O reduction, and how this feeds back on N_2O emissions, may therefore be the key to soil management strategies that improve soil C content while decreasing N_2O emissions.

The aim of this study was to assess the effects of long-term cropping and management practices that increase soil C in relation to soil NO_3^- content or decrease NO_3^- availability on the abundance and activity of N cycling guilds that determine N retention or loss and tease apart the effects of soil C and NO_3^-. We hypothesized that such cropping systems and management practices alter niche partitioning between ammonifying, denitrifying and N_2O-reducing communities and favor ammonifiers over denitrifiers and clade II over clade I N_2O-reducing communities. This should decrease N_2O production, increase N retention and improve crop yield. To test this, we selected two long-term field experiments, initially set up to evaluate the effects of cropping systems (annual cereal and short-term ley rotations) and their management (fertilization regimes, retaining the straw in the annual cereal rotations) on soil organic matter in different climate zones across Sweden (Fig. S1). These treatments have created C:N gradients both within and across the cropping systems. We assessed the genetic potential of the microbial communities for NO_3^- reduction via denitrification and ammonification and N_2O reduction by quantifying the abundance of the genes encoding the enzymes involved in the different processes. Crop yield and potential N_2O production rates were used as measures of system responses and analyzed in relation to fertilization regime to assess the N use efficiency of the different cropping systems. We also sequenced the total prokaryotic communities to gain insights into the effects of long-term cropping and management on their diversity and composition.

2. Materials and methods

2.1. Field experiments and soil sampling

Soil samples were collected in long-term field experiments run by the Swedish University of Agricultural Sciences comparing annual cereal rotation with and without retention of crop residues (experiment R3-0020) and short-term ley rotation (experiment R3-0021). The experiments were set up 1970–1980 adjacent to each other and are replicated at three locations across Sweden (Röbäcksdalen, Umeå, 63.8°N, 20.2°E; Säby, Uppsala, 59.5°N, 17.4°E and Lönnstorp, Lomma, 55.7°N, 13.1°E; Fig. S1a). Each experiment is fully factorial with 90 m^2 plots and includes three mineral-N fertilization rates and an unfertilized control, with cereal rotations receiving 0 (Unfert.), 40 (Low), 80 (Medium), and 120 (High) kg N ha^{-1} $year^{-1}$ and ley rotations 0 (Unfert.), 50 (Low), 100 (Medium), and 150 (High) kg N ha^{-1} $year^{-1}$. Phosphorus and potassium are added every second year according to the maintenance principle, i.e. replacing losses from the field with harvested products. The annual cereal rotation has sub-plots where crop residues (i.e. straw) is either removed or retained and incorporated into the soil after harvest. All treatments have four field replicates. The annual cereal rotation includes barley, oats and winter wheat (Table S1) and is tilled (moldboard ploughing) annually. The ley rotation has a four-year cycle starting with barley and an undersown grass and a red-clover mixture followed by three years of ley and then tillage by moldboard ploughing. We sampled all experiments in late summer/early autumn 2020 after harvest and before ploughing, at the end of a four-year ley rotation. Five soil samples were taken at 0–20 cm depth in each plot using a spade, homogenized into a composite sample to represent the plot and sieved through a 4-mm mesh. Samples were stored at −20 °C until further use.

2.2. Yield and soil data

Cereal kernel yields for the period 2000–2012 were used to compare yields across the cropping systems. Yield data for the ley cropping systems was only available for 2008 in Röbäcksdalen, and this site was thus excluded from the yield comparison. Soil pH and NH_4^+ and NO_3^- content were determined at the Soil and Plant Laboratory (Swedish University of Agricultural Sciences, Uppsala, Sweden). Data on total C (hereafter C) content in the soil over the last 25 years prior to sampling and at the start of the experiments at each site, as well as other soil properties (pH, total N) before establishing the experiments were provided by The Department of Soil and Environment, Swedish University of Agricultural Sciences. Soil properties at each site did not differ initially between the two experiments (Table S2).

Clay content was estimated by near infrared spectroscopy (SLU, Skara, Sweden) and differed between sites (Röbäcksdalen: 10 %, Säby: 33 %, Lönnstorp: 20 %). The diffuse reflectance spectra of the samples were recorded with a FieldSpec Pro FR scanning instrument (Analytical Spectral Devices, Boulder, Colorado). Spectra were collected at 1.4–2 nm intervals with a spectral resolution of 3–10 nm. A wavelength interval of 1 nm was interpolated to the instrument output file. The spectral range covered both the visible and near infrared regions, 350–2500 nm. Measurements were made using the High Intensity Contact Probe (ASD Inc., Boulder, CO, USA) equipped with a sapphire window. It has a 100 mm^2 spot size, a built-in DC current stabiliser circuitry and is equipped with a tungsten quartz halogen lamp (4 W−3.8 V) with built-in DC current stabiliser circuitry. The colour temperature of the halogen bulb is 2901 K ± 10 K.

2.3. Potential N_2O production assays

Thawed soil samples (10 g fresh weight) were placed in 125 mL Duran flasks and kept at room temperature overnight. The following day, potential N_2O production rates were determined in in soil slurries after addition of 20 mL of water. The bottles were tightly capped, and the headspace exchanged by flushing with N_2 and evacuating four times to obtain anoxic conditions. The bottles were incubated at 25 °C on a shaker (175 rpm) and after 30 min, 1 mL of substrate was added to reach a final concentration of 3 mM potassium nitrate, 1.5 mM succinate, 1 mM glucose, and 3 mM acetate (Philippot et al., 2011). Gas samples (0.5 mL) were taken from the headspace 30, 60, 90, 120, 150 and 180 min after substrate addition. Nitrous oxide concentrations were determined using a gas chromatograph (Clarus-500, Elite-Q PLOT phase capillary column, PerkinElmer, Hägersten, Sweden) equipped with a ^{63}Ni electron-capture detector and the rate of N_2O accumulation was determined in each bottle by non-linear regression as described by (Pell et al., 1996).

2.4. DNA extraction and quantitative PCR

DNA was extracted from 350 mg of each soil sample using the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. DNA quality was checked using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), before quantification on a Qubit fluorimeter using the Broad Range double stranded DNA kit (Thermo Fisher Scientific). Two independent extractions were done for each sample and pooled prior to further molecular analyses.

The abundance of total prokaryotic and several N transforming communities were determined by quantitative real-time PCR using primers targeting the 16S rRNA gene, the dissimilatory nitrite reductase genes *nirK* and *nirS* in denitrifiers and *nrfA* in ammonifiers and the N_2O reductase gene *nosZ*I and *nosZ*II in N_2O reducers. The qPCR reactions were performed in two independent runs in a reaction volume of 15 µL containing iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.1 % bovine serum albumin (New England Biolabs, Ipswich, MA, USA), primers and 5 ng of template DNA on a CFX Connect Real-Time System (Bio-Rad). Primers, qPCR conditions, and amplification efficiencies are presented in Table S3. Standard curves were generated by serial dilutions of linearized plasmids containing a fragment of the specific gene. The amplifications were validated by melting curve analyses and agarose gel electrophoreses. Potential inhibition of PCR reactions was initially checked by amplifying a known amount of the pGEM-T plasmid (Promega, Madison, WI, USA) with the plasmid specific SP6/T7 primer set (Table S3) and 5 ng of DNA template or non-template controls for each sample. No inhibition was detected with the amount of DNA used.

2.5. Sequencing of 16S rRNA genes

Sequencing libraries of 16S rRNA gene amplicons were prepared using a two-step procedure. In the first step, duplicate PCR reactions were run with the same primers as those used for qPCR. For 16S rRNA gene fragments, the PCR reactions consisted of 5 ng extracted DNA, 1 × Phusion PCR Mastermix (Thermo Fisher Scientific), 0.1 % bovine serum albumin and 0.25 µM of each primer in 15 µL reactions; with cycling conditions as follows: 3 min at 98 °C, 25 cycles of 98 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension step of 10 min at 72 °C. The PCR products were then pooled, and the amplicon size validated by agarose gel electrophoresis. After purification using Sera-Mag beads (Merck KGaA, Darmstadt, Germany), a single 30 µL reaction was performed for the second PCR to add barcodes unique to each sample, using 0.2 µM of barcoded primer and 4 µL of the pooled PCR product from the first PCR. The thermal cycling conditions were as follows: 3 min at 98 °C, 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and an extension step for 5 min at 72 °C. The amplicon size was validated by gel electrophoresis and the final PCR products were purified using Sera-Mag beads. After quantification on a Qubit fluorometer using the High Sensitivity double stranded DNA kit, the sequencing library was created by pooling equal amounts of purified 16S rRNA gene amplicons from each sample. Sequencing was performed on an Illumina MiSeq instrument using the 2 × 250 bp chemistry.

2.6. Sequence analysis

Processing of 16S rRNA gene amplicons was performed in R v. 4.4.0 (R Core Team, 2024) using the 'dada2' package v. 1.21.0 with default parameters to infer amplicon sequence variants (ASVs; Callahan et al., 2016). Primer sequences were removed, and the reads truncated to 240 and 230 bp for forward and reverse reads. Chimeras were discarded using a *denovo* approach with the removeBimeraDenovo function ('consensus' method). Each ASV was then aligned to the SILVA reference database (SSU138.1 Ref NR) using the SINA aligner v. 1.7.2 (Pruesse et al., 2012) and classified using SINA's least common ancestor algorithm. ASVs classified as chloroplast or mitochondria were discarded.

2.7. Community analyses

All analyses were done using the R software. A rarefied table was obtained by averaging the ASV counts over 1000 computations using the *rrarefy* function in 'vegan' v. 2.6–4 (Oksanen et al., 2018). The alpha-diversity indices Shannon's diversity and Pielou's evenness were calculated using 'vegan'.

Beta-diversity analyses focused on frequently occurring ASVs, as defined by species abundance distributions across all sites, to reduce the influence of site-specific differences in community composition. An index of dispersion corresponding to the ratio of the variance to the mean abundance multiplied by the occurrence was calculated (Hubbell, 2001) to split the dataset according to the frequency of occurrence of each ASV (Magurran, 2007). This index was then used to model whether ASVs followed a stochastic (Poisson) distribution and those falling below the 2.5 % confidence limit of the χ^2 distribution were discarded (Krebs, 1999). The resulting prokaryotic communities represented 75 % of the reads in the rarefied datasets. Zero counts were replaced using a Bayesian-multiplicative replacement ('zCompositions' package v. 1.5.0–3 (Martín-Fernández et al., 2015); and the zero-replaced ASV tables were centered log-ratio transformed using the 'compositions' package v. 2.0–8 (van den Boogaart et al., 2022) to account for the compositional nature of sequencing data (Gloor et al., 2017). Differences in community composition and structure were visualized with principal component analysis (PCA) using the *rda* function in 'vegan'. Vector and surface fitting of the soil properties on the ordinations were done using the *envfit* ($p < 0.001$, permutations = 9999) and *ordisurf* functions in 'vegan'.

2.8. Statistical analyses

Permutational multivariate analyses of variance (PERMANOVA) were conducted on the table containing the frequently occurring ASVs to assess the effects of site, crop rotation and fertilization regime using the adonis2 function in 'vegan'. Pair-wise DESeq2 analyses (v. 1.46.0 (Love et al., 2014); were conducted to detect differential ASV abundances between the ley and the cereal rotations at each site ($p < 0.01$).

Effects of site, rotation, and fertilization regime on soil properties and functional gene abundances and gene abundance ratios were assessed using analyses of variance (ANOVA). Tukey's HSD test was used as post-hoc test ('stats' package v. 4.2.2). The abundance of individual denitrification and N_2O reduction genes were combined before calculating gene abundance ratios; *nirK* + *nirS* for denitrification ('*nir*') and *nosZ*I + *nosZ*II for N_2O reduction ('*nosZ*'). Differences in C:NO_3^- ratio between treatments were assessed using Kruskal-Wallis tests with multiple comparisons computed according to Fisher's least significant difference and the false discovery rate correction available in the 'agricolae' package v. 1.3–7 (de Mendiburu, 2019). Uncertainty analyses were performed to assess (i) the evolution of soil C content over time and (ii) differences in N_2O production rates between fertilized and unfertilized soils. First, the best estimate for a given treatment was calculated as the difference between the treatment mean and that of the corresponding control. Uncertainty was then calculated by propagating the errors given by the standard deviation of the measurements. Finally, 95 % confidence intervals were calculated using the t-distribution to account for small sample size ($n < 30$).

Linear mixed effects models were used to compare grain yield in annual cereal (with and without straw return) and ley rotations at the end of each four-year cycle over the period 2000–2012 ($n = 4$ years and $n = 192$ data points), with year and fertilization regime as random factors ('lme4' package v. 1.1–35.3 and 'lmerTest' package v. 3.1–3; (Bates et al., 2015; Kuznetsova et al., 2017). Models were also run to assess the effect of cropping system on kernel yields within each fertilization regime with year as random factor. Estimated marginal means and confidence intervals were calculated using the 'emmeans' package v 1.10.1.

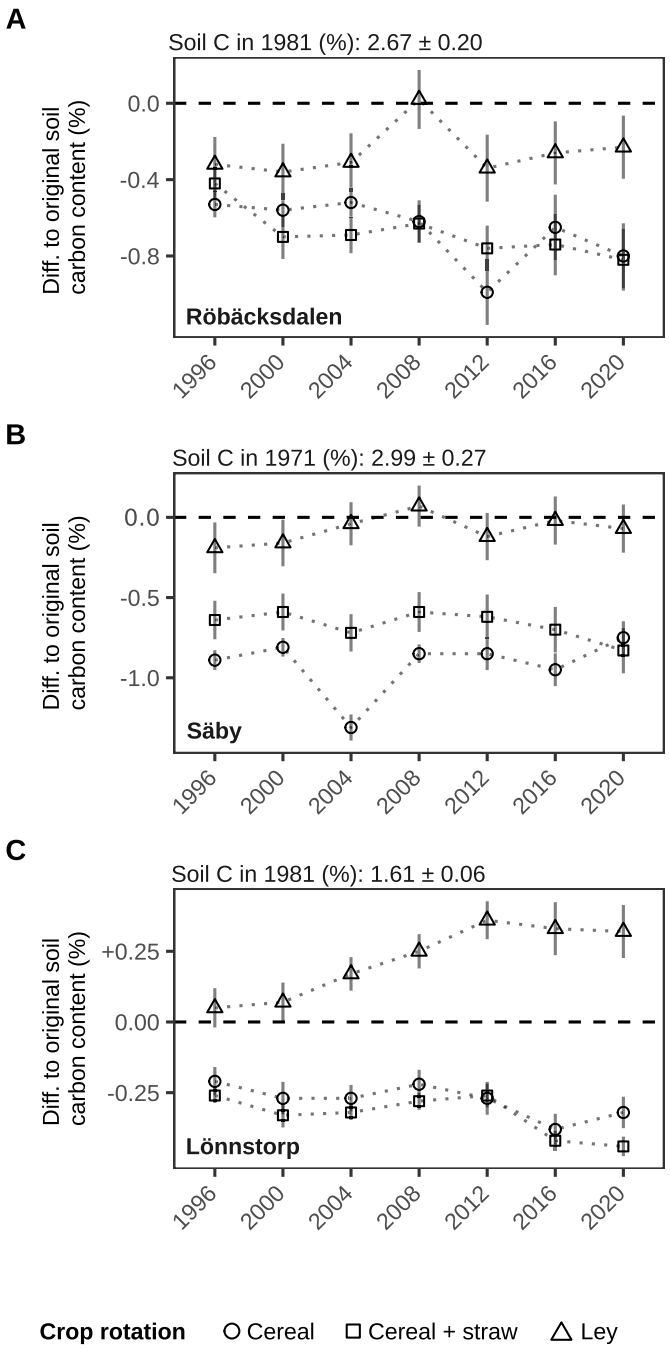


Fig. 1. Development of total carbon (C) content in the topsoil (0–20 cm) year 1996–2020. Changes in in soil C was calculated as the difference in C content before the establishment of the field experiments in cereal and ley rotations at (A) Röbäcksdalen, (B) Säby and (C) Lönnstorp. The initial soil carbon content is indicated for each site above the graphs. Error bars represent the 95 % confidence interval. Confidence intervals that do not include zero are considered significantly different from the control. Symbols indicate crop rotations.

Spearman correlations were used to assess the relationships between functional gene abundance ratios (*nrfA*:*nir*, *nosZ*:*nir* and *nosZ*II:*nosZ*I) and potential N_2O production rates, cereal kernel yield and soil $C:NO_3^-$ ratio. All figures were plotted using the 'ggplot2' package v. 3.3.5 (Wickham, 2016).

Table 1
Effect of site, crop rotation, and fertilization regime on soil properties based on analyses of variance (ANOVA). Three-way ANOVA (n = 4): nitrate, carbon to nitrate ratio ($C:NO_3^-$); Two-way ANOVA (n = 16): ammonium, total C, total N, pH. Only significant effects are shown ($p < 0.05$).

	Ammonium	Nitrate	Total C	Total N	$C:NO_3^-$	pH
	$F_{4,135}$	$F_{12,108}$	$F_{4,135}$	$F_{4,135}$	$F_{12,108}$	$F_{6,132}$
Site (S)	39.82	249.99	374.88	460.76	83.05	98.96
Rotation (R)	22.89	133.97	63.92	114.80	167.99	/
Fertilization (F)	/	182.42	/	/	112.08	3.31
S x R	8.43	26.66	51.53	47.79	13.51	/
S x F	/	16.52	/	/	4.47	2.35
R x F	/	38.70	/	/	17.53	/
S x R x F	/	15.51	/	/	7.36	/

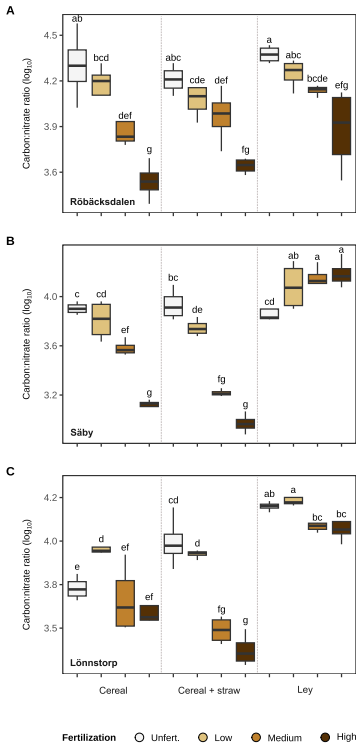


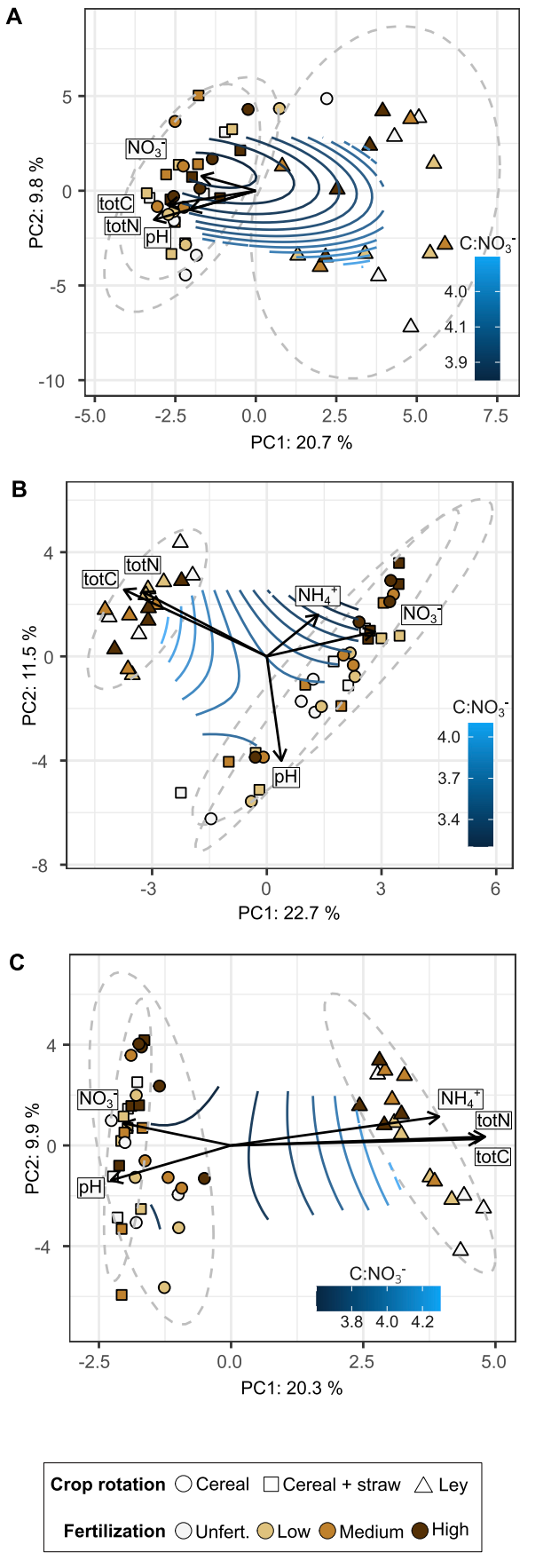
Fig. 2. Soil carbon:nitrate ratio in the different treatments in (A) Röbäcksdalen, (B) Säby and (C) Lönnstorp. Significant differences are indicated with different letters (ANOVA, $p(F) < 0.05$, followed by Tukey's HSD test, n = 4). Boxplots are colored according to fertilization regime.

3. Results

3.1. Soil properties

Long-term data shows that soil C content has decreased since the establishment of the experiments in the annual cereal rotations and has either stabilized or continues following a decreasing trend in the last 25 years, despite some year-to-year and site variation (range: −0.26 to −1.31 %; Fig. 1). Overall, straw retention had no or minor effects on soil C. By contrast, ley cropping resulted in maintained or increased soil C up to +0.36 % over time in the more clayey soils (Säby and Lönnstorp) and reduced C loss compared to the annual cereal rotation in the lighter soil in Röbäcksdalen.

Data from the sampling campaign in 2020 shows that the long-term practices resulted in gradients of soil properties across the sites (Fig. S1B). Soil NH_4^+ and total C and N content were influenced by crop rotation, with small and large interactions with site, respectively



(caption on next column)

Fig. 3. Principal component analysis plots of the prokaryotic communities in all treatments in (A) Röbäcksdalen, (B) Säby, and (C) Lönnstorp. Symbol shape indicates crop rotation and color fertilization regime, with ellipses representing the 95 % confidence level for the distribution of points for each rotation. Soil properties that are significantly correlated ($P < 0.05$) with the community structure are shown as arrows indicating direction and strength of the correlation. Contours represent the carbon to nitrate ratio ($C:NO_3^-$, $\log_{10}$) data fit to sample ordination scores. Variance explained by each principal component is shown in the axes labels. NO_3^-: nitrate, NH_4^+: ammonium; totC: total carbon; totN: total nitrogen.

(Table 1). These were driven by opposite patterns in total C and N content between annual cereal and ley rotations, with higher total C and N in the ley in Säby and Lönnstorp and vice versa in Röbäcksdalen (Fig. S2). There were no differences in total C or N between fertilization regimes. Soils under ley cropping displayed lower NO_3^- content than those under annual cereal cropping when comparing within each fertilization regime (Fig. S3), which could be explained by high N uptake due to the continuous presence of plants. This resulted in higher $C:NO_3^-$ ratios in ley rotations compared to the annual cereal rotations at all sites (Fig. 2), despite only Säby and Lönnstorp having significantly higher C content in the ley plots (Fig. S2A). Finally, fertilization had a small but significant negative effect on pH, mainly driven by the highest fertilization regime in the annual cereal rotations (Table 1; Fig. S4).

3.2. Genetic potential for N transformations

The genetic potential for ammonification (*nrfA*), denitrification (*nirK* and *nirS*) and N_2O reduction (*nosZ*I and *nosZ*II) tended to be higher in the ley rotations, which was driven by the overall higher abundance of prokaryotes in this system (Fig. S5), with little effect of fertilization (Table S4). Functional differences among rotations were further explored by patterns in gene abundance ratios (Fig. S6). The *nrfA*:*nir* ratio, a proxy for the genetic potential for N retention vs loss, varied between sites but did not differ between rotations within sites, and the genetic potential for denitrification was almost systematically dominant (range: 0.04–1.23). The *nosZ*:*nir* ratio, a proxy for gaseous N loss in the form of N_2 vs N_2O during denitrification tended to be higher in the annual cereal than in the ley rotations, with significant effects in Säby and Lönnstorp. The N_2O-reducing communities were dominated by clade II organisms in all samples (range: 4–66; Fig. S5).

Effects of long-term management on the *nrfA*:*nir* ratio were captured by the soil $C:NO_3^-$ ratio, with a positive correlation between *nrfA*:*nir* and $C:NO_3^-$ in the annual cereal rotations (Spearman's $\rho = 0.34$ and 0.49, $p < 0.05$, in Säby and Lönnstorp, respectively). This effect on the genetic potential for N retention was driven by N fertilization rather than soil C (Fig. S2A and S3A). Higher $C:NO_3^-$ ratios were also associated with higher *nosZ*II:*nosZ*I ratios in Säby (Spearman's $\rho = 0.58$, $p < 0.001$). No relationship between $C:NO_3^-$ ratio and any of the gene abundance ratios was detected in the ley rotations.

3.3. Total prokaryotic community composition

The α-diversity indices of the total prokaryotic communities did not differ between sites (ANOVA, $p(F) > 0.05$). However, the communities were slightly less diverse and less evenly distributed in the ley rotations than in the annual cereal rotations at Röbäcksdalen and Säby (ANOVA, $p(F) < 0.05$; Table S5), but with no significant effect of fertilization (ANOVA, $p(F) > 0.05$).

Principal component analyses showed that community composition differed both between sites (unconstrained PERMANOVA $R^2 = 0.37$, $P < 0.001$) and between rotations (site-constrained $R^2 = 0.05$, $P < 0.001$), with a small effect attributed to fertilization (site and rotation-constrained $R^2 = 0.02$; $P < 0.001$). The community composition differed between ley and annual cereal rotations, but there was little difference between the cereal rotations with and without straw residues

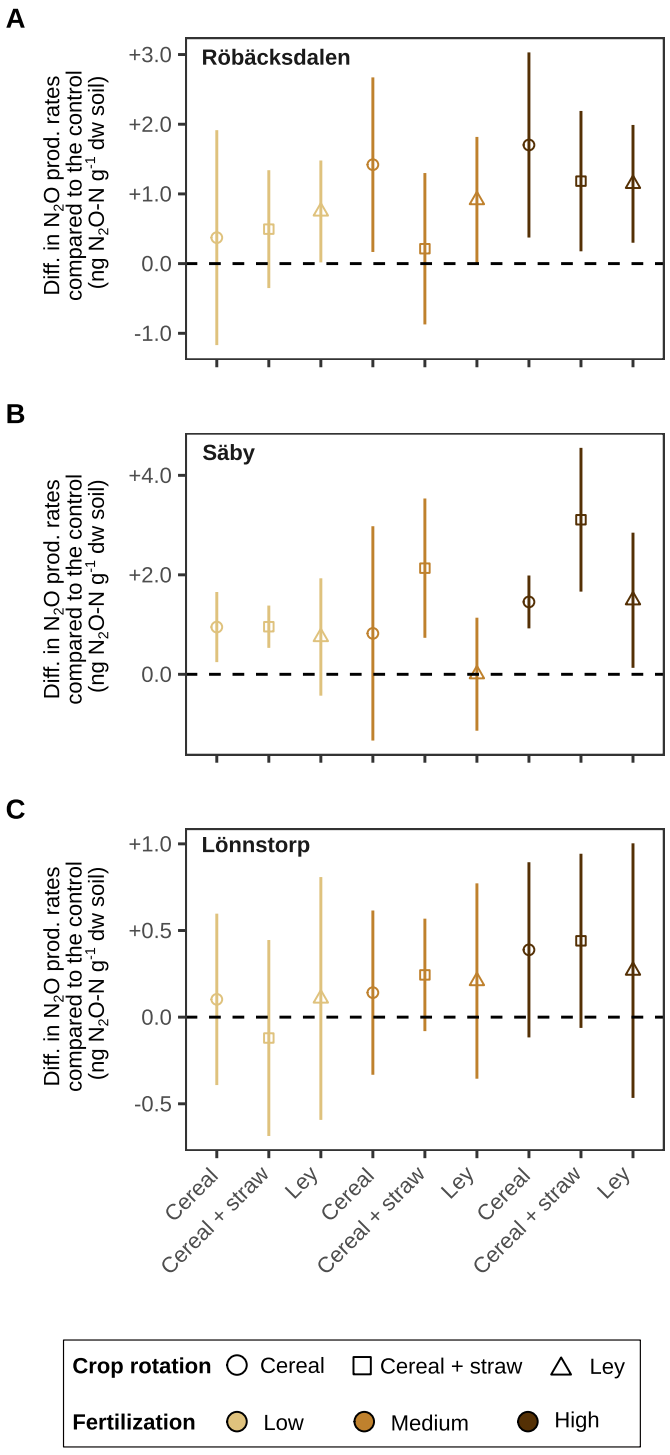


Fig. 4. Difference in soil potential N_2O production rates between fertilized and unfertilized plots in the different crop rotations at (A) Röbäcksdalen, (B) Säby and (C) Lönnstorp. Error bars represent the 95 % confidence interval. Confidence intervals that did not include zero were considered significantly different from the control. Symbols and error bars are colored according to fertilization regime, with symbols indicating crop rotations.

(Fig. 3). Several soil properties were correlated with the two main principal components ($p < 0.001$), with higher pH systematically associated with communities in the annual cereal rotations and C:NO_3^- ratios with those in the ley rotations.

DESeq2 analyses comparing the abundance of ASVs in ley versus annual cereal rotations showed that 31–45 % of the ASVs were significantly affected at each site ($p < 0.01$), with 12–15 % and 17–30 % displaying higher and lower abundances in the ley relative to the annual cereal rotations, respectively (Fig. S7). Most of these ASVs belonged to Acidobacteriota, Actinobacteriota, Gemmatimonadota, Proteobacteria and Verrucomicrobiota. Although the abundance of individual Rhizobiales ASVs could be lower or higher in the ley compared to the annual cereal rotations (Fig. S7), they were collectively more abundant in the ley rotations in all three sites (ANOVA, *F*-ratio = 80.45 and $p(F) < 0.001$, followed by Tuckey's HSD test). The leys also had lower abundance of taxa (the archaeal and bacterial orders Nitrososphaerales and Nitrospirales, respectively) involved in nitrification, the process of ammonia oxidation to nitrate.

3.4. System responses: potential N_2O production rates and cereal yields

The potential N_2O production rates varied between sites (Röbäcksdalen: 1.07 ± 0.36 to 3.71 ± 0.73 ng N_2O–N g^{-1} dw soil; Säby: 2.34 ± 0.27 to 5.45 ± 1.57 ng N_2O–N g^{-1} dw soil; Lönnstorp: 0.91 ± 0.38 to 1.64 ± 0.59 ng N_2O–N g^{-1} dw soil). Higher potential N_2O production rates due to fertilization were observed in Röbäcksdalen and Säby (Fig. 4), but crop rotation had no or little effect when comparing within each fertilization regime (Table S6). The C:NO_3^- ratio and the potential N_2O production rates were negatively correlated in the annual cereal rotations, but no relationship was detected in the ley rotations (Fig. 5). The potential N_2O production rates were negatively correlated with the *nosZ*:*nir* and *nosZ*II:*nosZ*I ratios, but positively correlated to the *nrfA*:*nir* ratio.

Cereal kernel yields in the ley rotations were comparable (in Lönnstorp) to, or higher (in Säby) than those in annual cereal rotations, after accounting for year and fertilization regime (Fig. S8). In the unfertilized and low fertilization treatments, the positive yield effects in the leys were larger compared with the medium and high fertilization rates at both sites (Fig. 6), indicating higher N-use efficiency in the former. Cereal kernel yield was positively correlated with the N_2O production rates and negatively correlated with C:NO_3^- ratio, *nrfA*:*nir*, *nosZ*:*nir*, and *nosZ*II:*nosZ*I abundance ratios in the cereal rotations in Säby and with C:NO_3^- ratio and *nosZ*II:*nosZ*I in the cereal rotations in Röbäcksdalen (Fig. 5).

4. Discussion

Long-term ley cropping had a positive effect on soil C content when compared to the cereal rotations, and in agreement with other studies (Liu et al., 2014; Poeplau et al., 2015; Lang et al., 2025), this effect depends on clay content. By contrast, straw return had a negligible effect on the long-term trajectories of soil C content in the cereal rotations. This agrees with reported SOC stocks in these and other experiments comparing cereal rotations with and without straw return in Sweden (Poeplau et al., 2015) and experiments in Canada (Lemke et al., 2010), but contrast global estimates (Liu et al., 2014; Xia et al., 2018). Northern latitudes are poorly represented in global studies and this discrepancy is potentially due to pedoclimatic conditions (Ledo et al., 2020). Differences between cropping systems in our study are likely linked to the amount and type of the residues, as perennials produce more plant residues than annual crops (Ferchaud et al., 2016), in particular belowground residues that contribute more to C stocks than aboveground residues (Kätterer et al., 2011). The lower frequency of tillage in the ley systems can also have a positive effect on C stocks (Luo et al., 2010).

Despite the differences in the overall prokaryotic community composition between cereal and ley rotations and the higher C:NO_3^- ratios in the leys, differences in the genetic potential for N retention between soils under ley and annual cereal cropping were small, contrasting Putz et al. (2018). Nevertheless, and in agreement with Putz et al. (2018), we observed a positive correlation between C:NO_3^- and *nrfA*:*nir* ratios at two sites, driven by soil NO_3^- rather than C. Previous

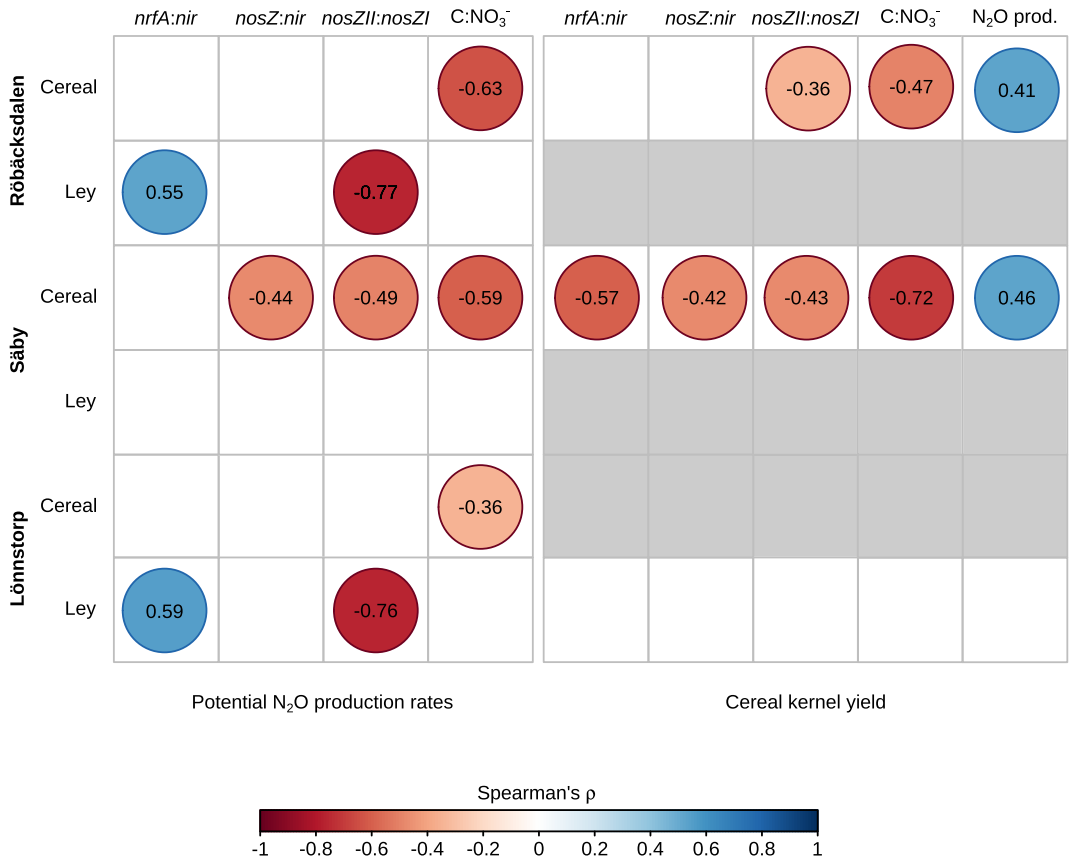


Fig. 5. Biotic and abiotic drivers of N_2O production rates and cereal kernel yields in the different crop rotations at each site. Spearman correlations are colored only if significant ($p < 0.05$; $n = 32$ for cereal rotations and $n = 16$ for ley rotations) according to their strength, with red showing negative correlations and blue positive correlations. Grey boxes correspond to treatments for which yield data was missing.

work conducted across several terrestrial biomes has shown that the relative importance of ammonifiers increases with decreasing soil NO_3^- and that soil C only has a positive effect on the potential for N retention in soils with <1 % C (Saghaï et al., 2023). Our results indicate that NO_3^- availability exerts a stronger control than C on the competition between the two functional groups even in soils with relatively low C content. This was associated with shifts in the prokaryotic community composition due to long-term fertilization, a general effect of N addition that has been well documented in other studies (Dai et al., 2018; Jones et al., 2022). The effect of NO_3^- availability on the *nrfA*:*nir* ratios does not negate that other soil properties also influence the relative importance of ammonifiers and denitrifiers (Saghaï et al., 2023). Altogether, our results align with the observation that ammonifiers have a higher affinity for NO_3^- than denitrifiers (van den Berg et al., 2016). A positive relationship between *nrfA* abundance and ammonification rates has been reported in different ecosystems (Song et al., 2014; Putz et al., 2018; Pandey et al., 2019), suggesting that higher *nrfA*:*nir* along the $C:NO_3^-$ gradient in our experiments may translate into higher N retention in the form of ammonium when soil NO_3^- content is low.

Different factors explained the observed differences among cropping systems in N_2O production rates, being the net effect of production and consumption, which highlights the interplay between system and abiotic and biotic soil properties. In the ley rotations, N_2O production was predominantly associated with the relative abundance of clade I and II N_2O-reducing communities, as shown by the strong negative correlation between N_2O production rates and the *nosZ*II:*nosZ*I ratio. Clade II *nosZ* is mainly found in non-denitrifying N_2O reducers (Hallin et al., 2018) and generally linked to lower net N_2O emissions (Jones et al., 2014, 2022; Domeignoz-Horta et al., 2016; Xu et al., 2020). By contrast, changes in N_2O production rates in the annual cereal rotations were associated with the soil $C:NO_3^-$ ratio at all three sites, indicating that microbial controls of N_2O production have a stronger dependency of abiotic factors in these systems. Our findings support previous work showing that fertilization weakens microbial controls of N_2O emissions by increasing resource availability and altering the relative importance of biotic and abiotic factors as predictors of N_2O emissions (Jones et al., 2022). Regarding the abundance of individual functional genes, we observed an increase of *nirK* and in some cases also *nosZ*I in the ley, where clover covers ca. 25 % of the soil surface (Röing et al., 2005). This is consistent with the fact that denitrifying rhizobia typically have these genes (Graf et al., 2014), and is further supported by the higher abundance of Rhizobiales in the ley rotations at each site. Although *nosZ* clade II systematically dominated the N_2O-reducing communities, the trend towards lower *nosZ*II:*nosZ*I ratio in the ley can be explained by a higher proportion of plant-associated lineages among *nosZ* clade I N_2O-reducers (Juhanson et al., 2017; Ai et al., 2020) and possibly also the role played by C quality for niche differentiation between the two *nosZ* clades (Maheshwari et al., 2023). Surprisingly, we found a positive correlation between the *nrfA*:*nir* ratio and the N_2O production rates at two of the sites. While the primers for *nrfA* adequately capture the most abundant members of the soil ammonifiers (Cannon et al., 2019), existing primers cannot capture all NirK-type denitrifiers (Bonilla-Rosso et al., 2016; Pold et al., 2024). Most of the NirK-type denitrifiers lack *nosZ* and are thus more likely to contribute to N_2O emissions than NirS-type denitrifiers, which typically carry a *nosZ* gene (Graf et al., 2014). Since the denitrifying communities in our samples were largely dominated by NirK organisms (median *nirK*:*nirS* ratio = 5500), differences in primer coverage could explain the counterintuitive relationship between the *nrfA*:*nir* ratio and the N_2O production rates observed here.

Inclusion of three-year leys had a positive effect on cereal yield in the

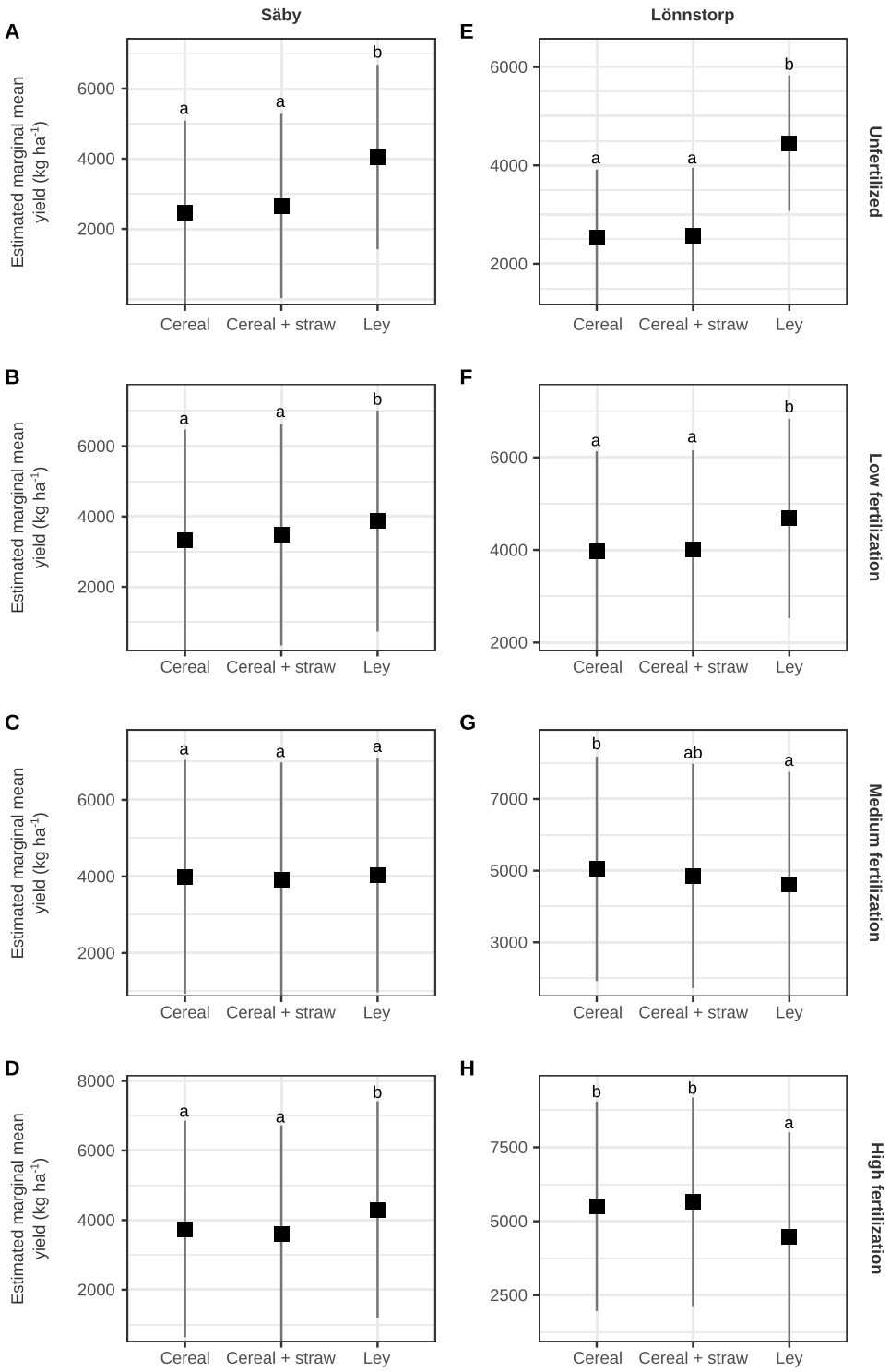


Fig. 6. Grain yield in the different rotations within each fertilization regime in (A–D) Säby and (*E*–H) Lönnstorp. Boxes indicate the estimated marginal (EM) mean computed in a linear mixed model with year as random factor. Error bars indicate the 95 % confidence interval of the EM mean. Means with different letters are significantly different (Šidák-corrected multiple comparisons).

unfertilized and low fertilization treatments, indicating improved N-use efficiency compared to the annual cereal rotations, both with and without straw return. However, this needs to be verified by determining system N balance. The yield increase can be attributed to enhanced ecosystem services in the ley rotations, such as improved soil fertility or nutrient cycling via nutrient conservation by leys and higher N inputs via N_2 fixation (Börjesson et al., 2018; Tamburini et al., 2020; Garland et al., 2021). However, effects of ley cropping on yield were inconsistent at medium and high fertilization regimes, suggesting that fertilization may partly or entirely override the benefits gained with the presence of leys (MacLaren et al., 2022; Nilsson et al., 2023).

5. Conclusions

We found that cropping systems including ley generally increased soil C and displayed similar or higher cereal yields compared to annual

cereal cropping, although effects on yield were dependent on fertilization regime. Nitrous oxide production rates were comparable in both systems, indicating that increases in soil C are not necessarily coupled with higher N_2O emissions in fertilized soils (Lugato et al., 2018). The potential N_2O production rates were primarily associated with microbial factors in the ley rotations, where the continuous presence of plants decreased NO_3^- availability and erased the effect of fertilization, whereas abiotic factors were relatively more important in the annual cereal rotations. Finally, NO_3^- availability, but not C content, explained the apparent control of C:NO_3^- on the competition between ammonifiers and denitrifiers, even if the soils included in this study displayed relatively low C (<2 %). These findings indicate that integrated soil C and N managements are necessary to improve soil C content and maintain crop yields while also mitigating N_2O emissions from arable soils.

CRediT authorship contribution statement

Aurélien Saghaï: Writing – original draft, Visualization, Supervision, Investigation, Formal analysis. **Oliver C. Moore:** Writing – review & editing, Investigation, Formal analysis. **Christopher M. Jones:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Sara Hallin:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Data statement

The raw sequence datasets of the 16S rRNA gene amplicons is available under BioProject accession number PRJNA1187866. The scripts and associated data used to conduct this study are available at Zenodo (10.5281/zenodo.14169768).

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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at https://doi.org/10.1016/j.soilbio.2025.109858.

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