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Nitrous oxide emissions from grass–clover swards as influenced by sward age and biological nitrogen fixation

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Abstract

Grassland renovation by cultivation and reseeding has been shown to increase short-term emissions of N_2O, but there is uncertainty about long-term effects, despite the potential impacts of reseeding on sward composition and soil functions. A field experiment was therefore carried out to determine how N_2O emissions from previously renovated grasslands varied in the intermediate to long-term, compared with an undisturbed permanent grassland (PG). Plots on the PG site were renovated, either two (G2) or five (G5) years prior to the two experimental years. In each sward age and experimental year, annual N_2O-measurements were conducted on a weekly basis and compared with the undisturbed PG. Plots were either unfertilized or were fertilized with slurry (240 kg N ha^{-1} $year^{-1}$). On average, annual N_2O emissions were 0.39 kg N/ha for the unfertilized swards, and 0.91 kg N/ha for slurry-fertilized swards. Sward age had no effect on N_2O emissions. With increasing sward age the proportion of legumes in the sward was reduced, but a minimum biological nitrogen fixation (BNF) of 88 kg N/ha was maintained even in the fertilized PG. Both sward age and BNF were of limited importance for the annual N_2O emissions compared with the effects of soil carbon content and nitrogen surplus levels. However, measured N_2O emissions were low in all sward age treatments, with a low risk of additional N_2O emissions when BNF is taken into account in fertilizer planning.

KEYWORDS

eco-efficiency, GHG emissions, grassland renovation, nitrogen surplus, permanent grassland, soil organic carbon

1 | INTRODUCTION

Agricultural production systems affect the emissions of all major greenhouse gases (GHG), namely carbon dioxide (CO_2) (Freibauer, Rounsevell, Smith, & Verhagen, 2004), nitrous oxide (N_2O) (Reay et al., 2012) and methane (CH_4) (Haque, 2018). The livestock sector alone has been estimated to contribute about 18% of the global anthropogenic GHG emissions (Haque, 2018). Projected increments in consumption of animal products will increase the challenge of balancing environmental concerns and food provision (Rojas-Downing, Nejadhashemi, Harrigan, & Woznicki, 2017). Hence, one of the main challenges for both policy makers and agricultural scientists is to improve the data relating to GHG emissions from livestock agriculture, and to identify opportunities to reduce emissions associated with ruminant production (Carswell, Gongadze, Misselbrook, & Wu, 2019).

Grasslands play a substantial role in ruminant production globally and are of particular importance for European agriculture as they support much of the continent's livestock and comprise more than 20% of the total land area (EU-28) (Eurostat, 2015). In addition to providing grazed and conserved forage for ruminant meat and dairy production, grasslands also contribute to a range of ecosystem services, including climate regulation through sequestration of carbon (C), prevention of soil erosion, as well as landscape and biodiversity protection (Taube, Gierus, Hermann, Loges, & Schönbach, 2014). Permanent grasslands in particular act as a net C sink, through the accumulation of soil organic matter due to the absence (or only occasional use) of soil tillage, and a permanent vegetation cover with high belowground productivity (Loges et al., 2018), thereby contributing to C storage and climate change mitigation. With regard to N_2O, however, grasslands are generally regarded as a net source, although the amount of N_2O emitted varies depending on inputs of nitrogen (N)-containing fertilizers and environmental conditions (Dobbie & Smith, 2003a; Flechard et al., 2007; Skiba et al., 2009). Nitrous oxide is a potent and long-lived GHG, with a global warming potential, on a per-molecule basis relative to CO_2, of 265 over a 100-year horizon. Anthropogenic N_2O emissions are predominantly of agricultural origin, linked to fertilizers and cultivation. This has led to a considerable focus on identifying opportunities to adapt agricultural management to reduce this emission (Misselbrook, Prado, & Chadwick, 2013).

In recent years, there has been increased interest and adoption of ley systems that use grass–clover mixtures. Their superior yields (Nyfeler et al., 2009) and large N fertilizer replacement potential have been shown to be effective across a range of mixture compositions and environmental conditions (Suter et al., 2015). The N-replacement benefits of legume-based ley mixtures are mainly a result of their increased resource use efficiency and biological nitrogen fixation (BNF) (Nyfeler, Huguenin-Elie, Suter, Frossard, & Lüscher, 2011). Clover-based ley systems are generally considered to provide a sustainable N source, and they require fewer inputs, and have a lower environmental impact than ley systems based mainly on mineral N fertilizers (Flessa et al., 2002; Li et al., 2013). However, the turnover of N-rich clover residues can increase the availability of soil mineral N and hence affect N losses via N leaching (Rasmussen, Eriksen, Jensen, & Høgh-Jensen, 2008) or via N_2O-emissions (Flessa et al., 2002). In addition, root turnover and root senescence rates in the clover fraction increase over time (Hammelehle, Oberson, Lüscher, Mäder, & Mayer, 2018). This can result in increased N concentrations in the soil, thereby raising the potential for N_2O emissions to increase over the duration of the ley. A further problem that frequently arises is deterioration of grass–clover swards with age, as ingress of weed species and decreasing proportions of clover reduce the agricultural value of the ley mixture. This may be addressed by oversowing, but ultimately, if that fails, the deterioration might require a complete sward renewal (i.e. by cultivation and complete reseeding) to maintain productivity and forage quality (Conijn, 2004). However, these methods should be limited to severely degraded swards, as grassland renovation results in substantial CO_2 emissions: recent studies have shown soil organic C losses of 32.2 t/ha within two years following grassland renovation on poorly drained gleys (Necpálová et al., 2013b) and high N_2O emissions (Mori & Hojito, 2007; Reinsch, Loges, Kluß, & Taube, 2018; Velthof et al., 2010). Accordingly, in the year of renovation, the grassland acts as a source of greenhouse gases, rather than a sink. However, although these effects have been observed as a short-term response, only a few field studies have considered the impact of grassland renovation on N_2O-emissions in the longer term. These can be affected by reduced soil C stocks and by changes in the botanical composition (Kayser, Seidel, Muller, & Isselstein, 2008). Hence, this study had the following objectives:

- to determine how long the benefits of grassland renovation on sward composition and forage quality persist,
- to analyse the effect of grassland renovation on N_2O fluxes in the long-term and
- to compare N_2O-emissions over time from fertilized treatments, receiving organic fertilizer, and from unfertilized treatments with only BNF as N input.

2 | MATERIALS AND METHODS

2.1 | Experimental Site

The experiment was conducted on a permanent grassland at the experimental farm “Lindhof” (54°27’N, 9°57’E; elevation 10 m a.s.l.) of Kiel University in northern Germany. This investigation was carried out within a long-term observation area established in 2005, described in detail by Reinsch et al. (2018). The soil type was classified as sandy loam (pH 5.7) with 11% clay, 29% silt and 60% sand, and 1.7% C_{org} in the topsoil (0–30 cm soil depth) (Reinsch et al., 2018). The site has a long-term (1981–2010) mean annual temperature of 8.9°C and long-term average annual rainfall of 778 mm.

The experiment comprised a 2 × 3 × 2 factorial design with three replicates of each treatment. Factors were N fertilization, sward age after grassland renovation and experimental year. The N fertilization treatments were cattle slurry supplying 240 kg N ha^{-1} $year^{-1}$ (N240), and a non-N-fertilized control (N0). The slurry was applied with trailing hoses on four occasions each year, one application for each grass growth phase (supplying 80, 60, 60, 40 kg N/ha). Slurry applications in the first experimental year were on 4 April, 31 May, 7 July and 6 September, and in the second year they were 2 April, 30 May, 9 July and 6 September. The sward was managed by taking four cuts per year, each harvested at a "silage" growth stage. All plots received 45 kg P/ha, 100 kg K_2O/ha, 24 kg Mg/ha and 68 kg S/ha every two years, applied as mineral fertilizer. Sward age treatments were arranged as follows: permanent grassland plots that had been seeded previously in 1994 were ploughed to a soil depth of 25 cm and reseeded with a grass–white clover mixture at 30 kg/ha; in proportions by seed weight: *L. perenne* (0.70), *Poa pratensis* (0.12), *Phleum* *pratense* (0.12) and *Trifolium repens* (0.06). Ploughing and seeding of individual plots were carried out in September 2005, 2006, 2008 and 2009. This enabled the creation of reseeded grassland swards with equal sward ages in each of the two experimental years (year 1 was March 2010 – April 2011, and year 2 was March 2011 – April 2012). Consequently, the treatment plots in each of the two experimental years were either 2-year-old or 5-year-old swards (G2 swards and G5 swards), and the undisturbed 16-year-old permanent grassland (PG) served as a control (see Table 1). The plot size of each replicate was 6 m × 12 m ($n = 3$).

The weather conditions during the two experimental years were generally similar to the long-term average (Figure 1). The exceptions were July 2010, when rainfall was substantially below average (18 mm vs. 84 mm long-term), with possible implications for grass growth, and December 2010 when temperatures were unusually low for several weeks (average −3.5°C vs. +2.2°C long-term). Comparing the two experimental years, growing conditions were more favourable in the second experimental year, with a higher mean temperature (9.9°C vs. 8.3°C) and more precipitation (937 mm vs. 808).

2.2 | Herbage yield, forage quality and sward composition

Each plot was harvested with a Haldrup forage harvester (Haldrup GmbH, Ilshofen, Germany) at a cutting height of 5 cm. The total fresh matter (FM) weight of each plot was recorded immediately after harvesting. Sub-samples (~150 g of FM) were taken, oven-dried to constant weight at 58°C to determine their dry-matter content and then milled to pass a 1-mm sieve (Cyclotech mill, Foss analytical, Hilleroed, Denmark) for determination of N concentration and energy content in terms of net energy for lactation (NEL) by near-infrared reflectance spectroscopy (NIRS) using a NIR-System 5,000 monochromator (Foss analytical).

To determine the species composition of the harvested herbage, prior to each harvest subsamples were taken from each plot, using lawn shears, from an area of 0.5 m^2. These hand-harvested samples were subdivided into grass, legumes and unsown dicot species.

2.3 | Nitrogen balance

For calculation of the N balance at field level, BNF was considered using the total N-difference method (Hardarson & Danso, 1993; Laidlaw, Christie, & Lee, 1996; Ledgard, 1991; Peoples, Faizah, Rerkasem, & Herridge, 1989). For this method, additional clover-free subplots (1 m^2) were introduced into each plot at the beginning of each experimental year by manual removal of clover plants. This procedure was repeated several times per year to prevent clover re-establishment and ingress of stolons. Subsequently, the BNF was calculated using the following formula:

$$\mathrm{BNF} = \mathrm{N}_{\mathrm{mixed}} - \mathrm{N}_{\mathrm{grass}} \tag{1}$$

where N_{mixed} is the N yield (kg N/ha) in aboveground biomass in the grass–clover sward and N_{grass} is the N yield (kg N/ha) in the pure grass stand of each respective treatment. The difference between the two values represents the BNF. For N derived from N_2 fixation and translocated to stubble, stolon and root tissues, an equivalent of 0.7 was used, based on the estimates of Jorgensen and Ledgard (1997). Nitrogen balance ($N_{balance}$), expressed in kg N/ha for all treatments, was calculated by subtracting the N output from the N input, as shown in Equation 2. The N input was derived from the slurry application in the fertilized treatment, which provided 240 kg N/ha (N_{manure}), and from the calculated amount of BNF. Nitrogen yield (N_{yield}) in the harvested biomass was considered to be the N output.

$$\mathrm{N}_{\mathrm{balance}} = (\mathrm{N}_{\mathrm{manure}} + \mathrm{BNF}) - \mathrm{N}_{\mathrm{yield}} \tag{2}$$

The N deposition from rainfall was not considered in the $N_{balance}$ calculation as all treatments were exposed to similar levels of deposition. The long-term annual average N deposition from rainfall (1989–2005) in this area is 12.5 kg N ha^{-1} year^{-1} (LANU, 2005).

2.4 | Soil characteristics

Soil sampling was conducted every two weeks to a soil depth of 20 cm from each plot during the two years of experimentation (April 2010–April 2012). Samples were stored at −17°C in a freezer until further processing. For the determination of the mineral N status (N_{min}), soil samples were extracted with 0.01 mol $CaCl_2$. Soil nitrate (NO_3^-) and ammonium (NH_4^+) concentrations were determined photometrically using a dual channel continuous flow analyser (Skalar Analytical Instrument, Breda, the Netherlands). Dry-matter weight of soil samples was obtained by oven-drying the fresh samples at 105°C to constant weight.

TABLE 1 Year of grassland renovation (R) and respective year of measurement (M) for each of the treatments in the factor "sward age." Measurements were conducted during the two experimental years (1st year: 2010, 2nd year: 2011). Permanent grassland was established in 1994

	2005	2006	2007	2008	2009	2010	2011
Permanent grass (PG) (1st and 2nd year)						M	M
2-year old (G2) (1st year)				R		M	
2-year old (G2) (2nd year)					R		M
5-year old (G5) (1st year)	R					M	
5-year old (G5) (2nd year)		R					M

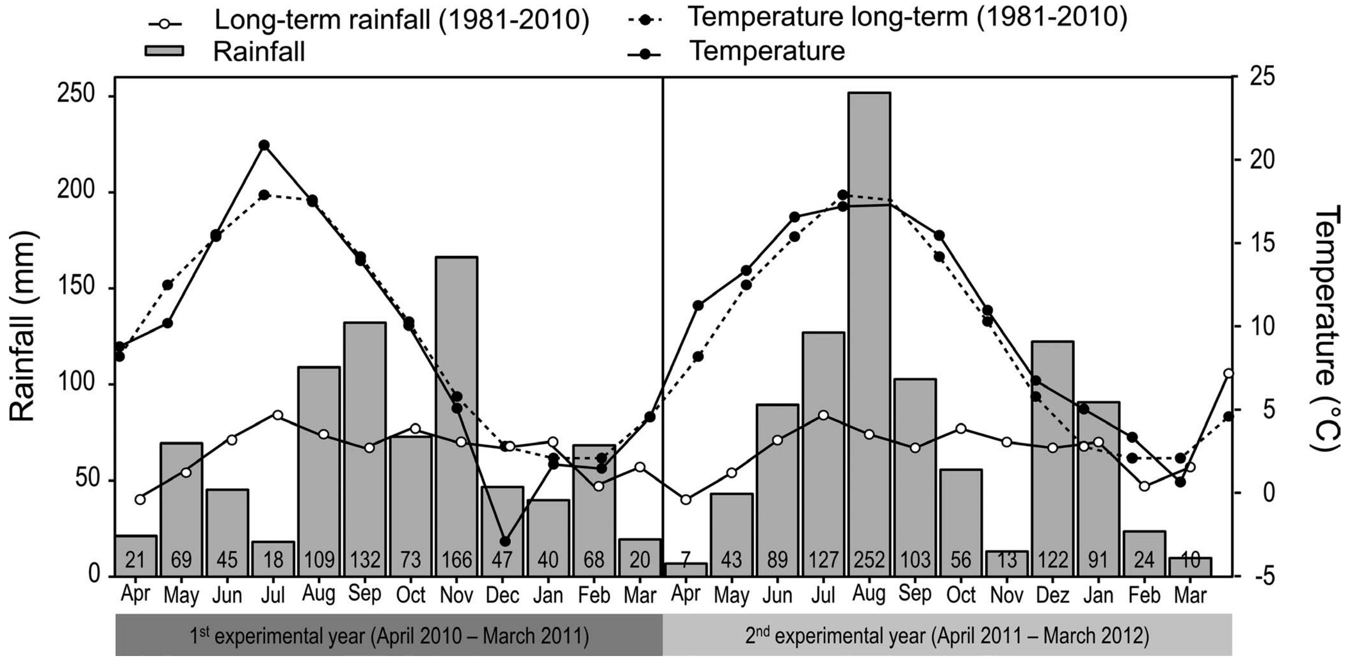

FIGURE 1 Daily averages of air temperature and precipitation per month (bars) during the two experimental years in comparison with the long-term average

Additional soil samples were taken to a soil depth of 30 cm in autumn 2010 and 2011. Each sample was bulked from three replicates per plot. Soil samples were dried in an oven at a temperature of 30°C to constant weight. In the pre-treatment for laboratory analysis, dried soil was sieved to pass a 2-mm sieve, and subsequently ball-milled to improve homogeneity of samples. Total concentration of soil C and N in each sample was measured by dry combustion using a C/N-Analyser (Vario Max CN, Elementar Analysensysteme, Hanau, Germany). For calculation of soil carbon (SOC) and nitrogen stocks (N_{tot}) as well as N_{min}, the soil bulk density of each plot was measured before ploughing and after each experimental period.

2.5 | N_2O measurements

Emissions of N_2O were measured at least once a week for a period of two years (April 2010–March 2012) using the closed chamber method (Hutchinson & Mosier, 1981). For a better evaluation of background and $N_{balance}$-related emissions, the unfertilized clover-free subplots (see section 2.3) were also used for N_2O-flux measurements. The measurement procedure is described as follows. At the beginning of each experimental year, a PVC-collar with a diameter of 60 cm and a height of 15 cm was dug into the soil to a depth of 10 cm at the centre of each plot and sub-plot to provide a base for the measuring chambers. During N_2O measurements, gas-tight chambers with a height of 35 cm were placed on top of these collars for 40 min. The area between the basal ring and chamber was sealed with a taut butyl rubber band. To allow gas sampling during measurements, the chambers were equipped with a rubber septum on the top. Gas samples were taken at 20-min intervals (0, 20 and 40 min) and stored in 12 ml pre-evacuated septum-capped vials (Labco, High Wycombe, UK) using a 30 ml syringe. Gas samples were analysed for N_2O concentrations using a gas chromatograph (model 7890a, Agilent technology Inc., Santa Clara, CA, USA) equipped with a ^{63}Ni electron-capture detector using helium as the carrier gas and argon-methane as make-up gas. The analytical precision was regularly determined by three certified gas standards (300, 620, 1,510 ppb). The standard derivation (*SD*) of 10 repeated measurements was < 3 ppb (1.8 (300), 2.8 (620), 2.9 ppb (1,510 ppb)). The gas chromatographic procedure involved a detector temperature of 320°C, a column temperature of 40°C and an injector temperature of 200°C. All samples were measured within two weeks of field sampling. Data were processed using the software *Chem Station* (Version B.01.04, Agilent technology Inc., Santa Clara, CA, USA). Fluxes were calculated for each treatment and replicate by linear regression between the measured N_2O concentrations and time. According to the chamber specification, measurement procedure and the described analytical performance, the minim detectable flux rate was $\pm$ 0.8 µg N_2O-N m^{-2} hr^{-1} The accumulated N_2O emissions were calculated by plotwise linear interpolation between the measured daily fluxes. The emission factors (EF) were calculated by dividing the cumulative N_2O emissions by the sum of all N inputs (BNF and N_{manure}). This procedure is contrary to that of IPCC guidelines (IPCC, 2006), where the annual N_2O background emissions from clover-free and unfertilized plots have to be deducted from emissions. However, as we hypothesized that clover proportion may have an effect on annual N_2O emissions, and clover content is affected by the absence of fertilization, the comparison between fertilized and unfertilized plots would not have been possible. Hence, the emission factors are merely guidance to illustrate the maximal amount of incorporated N that is emitted in the form of N_2O.

TABLE 2 Soil characteristics and annual N_2O emissions for the different sward ages (G2 = two years after grassland renovation, G5 = 5 years after grassland renovation; PG = undisturbed permanent grassland) and for the two tested fertilizer levels (N0 = without fertilizer addition; N240 = 240 kg N ha^{-1} $year^{-1}$ applied as cattle slurry), averaged across the two experimental years. Abbreviations are as follows: SOC = soil organic carbon stock in the top soil (0–30 cm), N_{tot} = total Nitrogen stock in the top soil (0–30 cm), BNF = biological nitrogen fixation, $N_{balance}$ = the annual nitrogen excess or deficit in the swards, EF = emission factors (fraction of nitrogen (from slurry and BNF) emitted as nitrous oxide emissions). Values in brackets show the standard error (SE). Uppercase letters indicate significant differences between the different sward ages. Lowercase letters indicate differences for the tested slurry application ($p < .05$). ANOVA results are presented below for the factors: sward age (A), fertilization (F) and their interaction (A*F). Numerator and denominator degrees of freedom are presented as lowercase numbers for each factor. *F*-values are presented as well as level of significance as asterisk (* $p < .05$, ** $p < .01$, * $p < .001$)

Age	Fertil.	DM yield (t DM/ha)	Energy yield (GJ NEL/ha)	Legume share (%)	Unsown share (%)	SOC (t C/ha)	N_{tot} (t N/ha)	BNF (kg N/ha)	$N_{balance}$ (kg N ha^{-1} a^{-1})	N_2O (kg N ha^{-1} a^{-1})	EF (%)
G2	N0	8.7 (1.0)^{a}	57.2 (6.9)^{a}	44 (5)^{Aa}	11 (4)	62.6 (3.9)^{Aa}	6.0 (0.4)	319 (46)^{Ba}	56 (20)^{Aa}	0.25 (0.05)^{a}	0.12 (0.06)^{A}
G2	N240	10.7 (1.0)^{b}	70.2 (7.0)^{b}	17 (3)^{Ab}	12 (4)	68.4 (1.5)^{Ab}	6.5 (0.1)^{A}	169 (22)^{Bb}	172 (37)^{Ab}	0.55 (0.06)^{b}	0.13 (0.01)^{A}
G5	N0	9.7 (0.4)^{a}	64.3 (3.0)^{a}	30 (4)^{Ba}	13 (5)	71.0 (2.1)^{ABa}	6.8 (0.2)	224 (18)^{A}	−19 (19)^{Ba}	0.47 (0.16)	0.22 (0.09)^{AB}
G5	N240	11.5 (0.3)^{b}	75.4 (2.4)^{b}	13 (4)^{ABb}	15 (5)	74.7 (1.5)^{Bb}	7.0 (0.1)^{A}	133 (45)^{AB}	95 (41)^{ABb}	0.70 (0.22)	0.21 (0.07)^{AB}
PG	N0	9.9 (0.6)^{a}	66.7 (3.4)^{a}	24 (2)^{Ba}	22 (5)	73.3 (2.5)^{Ba}	6.9 (0.2)^{a}	193 (27)^{Aa}	−34 (18)^{Ca}	0.45 (0.10)	0.25 (0.06)^{B}
PG	N240	11.6 (0.6)^{b}	77.7 (4.3)^{b}	13 (5)^{Bb}	20 (6)	81.0 (2.7)^{Cb}	7.6 (0.2)^{Bb}	88 (31)^{Ab}	65 (47)^{Bb}	1.48 (0.57)	0.41 (0.11)^{B}
ANOVA											
$A_{2,29}$		5.9**	10.1***	47.6***	5.7**	97.2***	47.3***	22.3***	52.2***	1.1	8.0**
$F_{1,29}$		81.0***	69.3***	133.1***	0.0	37.5***	18.1***	59.3***	43.7***	22.2***	5.9*
$A*F_{2,29}$		0.6	1.0	1.9	0.1	2.1	2.2	2.4	0.2	0.9	0.4

2.6 | Statistical analysis

The data evaluation started with the definition of an appropriate statistical mixed model. A linear mixed model was defined, utilizing sward age (A) and fertilizer rate (F), as well as their interaction as fixed factors, while the experimental year (Y) was used as random factor. Based on a residual analysis, the data were assumed to be normally distributed and heteroscedastic and the model was adjusted accordingly to account for the heteroscedasticity. Based on this model, an analysis of variance (ANOVA) was conducted. Significance of factors was declared at $p < .05$ (Table 2). Additionally, multiple contrast tests were conducted in order to assess differences across factor levels (Bretz, Horthorn, & Westfall, 2011). Additionally, a multiple regression analysis was used to test if variables (soil C stocks, N surplus) could explain the annual N_2O fluxes. For statistical analyses, the R programming language (R Core Team, 2019) was used.

3 | RESULTS

3.1 | Herbage yield, nitrogen balance and soil functions

Biomass and energy yields were similar across sward ages, and despite a tendency for both values to be higher in PG than in G2 swards, by an average of more than 10%, the differences were not significant (Table 2). Within swards, however, legume proportions decreased continuously with sward age and were almost twice as high ($p < .001$) in G2 swards as in PG. There was a concomitant increase in unsown species from, on average, 12% in the two-year-old swards to 21% in the permanent grassland, but again these differences were not significant. The renovated swards had an increased amount of *L. perenne* ($p < .05$) in the G2 swards compared with PG, and PG also showed a non-significant tendency for higher proportions of *Poa trivialis*, *Dactylis glomerata* and *Taraxacum ssp.* (results not shown).

Slurry application increased ($p < .001$) biomass yields on average by 20% and energy yields by 19% across all sward ages (Table 2). The benefits of fertilization for increased biomass and energy yield were uniform across sward ages. However, fertilization decreased ($p < .001$) the proportion of legumes in the mixture by 51% on average, with the largest reduction occurring in the G2 swards, from 44% to 17%. As a result, the concentration of crude protein in the G2 swards was reduced ($p < .01$) from 169 g CP kg DM^{-1} in the unfertilized swards to 148 g CP kg DM^{-1} in the fertilized G2 swards, despite the fertilizer addition (results not shown). Sward age did not significantly affect crude protein concentrations, with crude protein concentrations of 161 g CP kg DM^{-1} and 166 g CP kg/DM in the unfertilized G5 and PG respectively. The energy content was not affected by the CP reduction from fertilization and was generally between 6.5 and 6.7 MJ NEL per kg dry matter across all sward ages and fertilization levels (Table 2). The proportions of unsown species were not significantly affected by slurry application.

Both sward age and fertilizer application had a significant ($p < .001$) impact on the $N_{balance}$. Accordingly, with increasing sward age the unfertilized swards showed a decline ($p < .001$) in their $N_{balance}$ from a surplus of 56 kg N ha^{-1} a^{-1} in the G2 swards to a deficit of 34 kg N ha^{-1} a^{-1} in PG (Table 2). In the fertilized plots, there was initially a large N surplus of 172 kg N ha^{-1} a^{-1} in the G2 swards, but the surplus in PG was smaller, at 65 kg N ha^{-1} a^{-1} ($p < .05$). In association with the decrease in legume proportion, the BNF also decreased by 43% with increasing sward age ($p < .001$) and was also generally lower in the fertilized plots ($p < .001$). On average, the BNF in fertilized plots was only 53% of the BNF in the unfertilized plots. In unfertilized plots, the BNF ranged from 193 kg N ha^{-1} a^{-1} in PG to 319 kg N ha^{-1} a^{-1} in the G2 swards, while in the fertilized plots the BNF range was from 88 to 169 kg N ha^{-1} a^{-1} for the PG and G2 swards respectively.

Both soil N_{tot} and SOC continued to accumulate as sward age increased (Table 2). The tendency of the N_{tot} increase was similar in the unfertilized and fertilized treatments with 0.3 t N ha^{-1} a^{-1} and 0.1 t N ha^{-1} a^{-1} increments from the G2 swards to G5 respectively. The SOC increased from the G2 swards to PG for both the unfertilized (+9.5 t C ha^{-1}, $p < .01$) and the fertilized (+12.6 t C ha^{-1}, $p < .001$) swards. The C/N ratio showed a range from 10.4 to 10.7 across all sward ages and fertilization levels.

The highest concentrations of N_{min} per 100 g dry soil were observed in autumn, from September to December, with the highest daily N_{min} status being 115 kg N/ha, which was recorded in the unfertilized G5 swards (Figure 2). However, during the entire period, soil N_{min} values were low in all treatments and never exceeded 46 kg N/ha (Figures 2 and 3). Within N_{min}, NH_4^+ concentrations were higher than NO_3^- concentrations and accounted for 0.67 of the total, on average (Figures 2 and 3).

3.2 | N_2O fluxes

Over the entire experimental period, N_2O fluxes ranged between −34 and 569 µg N_2O-N m^{-2} hr^{-1}, for the fertilized treatments and between −41 and 182 µg N_2O-N m^2 hr^{-1} for the unfertilized treatments. From April to October, emissions were low in all treatments, with the highest fluxes of 123 µg N_2O-N m^{-2} hr^{-1}. Highest fluxes were observed during winter, in both unfertilized and fertilized treatments (Figure 3), with more than 0.60 of the accumulated annual N losses occurring from November to March. As a result, when looking only at soil temperatures above 5°C, soil temperature was not correlated with N_2O fluxes ($r = .0$, $p = .56$). Annual N_2O emissions were lowest in the 2-year-old sward, amounting to 0.25 kg N_2O-N ha^{-1} $year^{-1}$. In comparison, PG emitted 0.45 kg N_2O-N ha^{-1} $year^{-1}$. Fertilized treatments showed slightly higher N_2O fluxes within 14 days following slurry application, but these were not significantly different to fluxes from the unfertilized treatments. The application of N fertilizer resulted, on average across all sward ages, in additional emissions of 0.51 kg N_2O-N ha^{-1} $year^{-1}$.

Throughout the entire year, calculated EFs were generally very low, ranging from 0.12% to 0.41% of the available N from N inputs (Table 2). Emission factors were higher ($p < .01$) in PG than in the G2 swards. While fertilization generally increased ($p < .05$) EFs, this effect was not significant in any individual sward age. When looking at the background emissions from clover-free subplots, the background emissions increased over time. Hence, the annual accumulated measured emissions from the clover-free subplots accounted for 0.17 (SE 0.13), 0.25 (SE 0.06) and 0.46 (SE 0.18) kg N_2O-N/ha in G2, G5 and PG respectively. Accordingly, the emissions directly allocated to the BNF in PG would be zero after deducting background emissions.

Concentrations of NH_4^+ in the soil were not significantly correlated with N_2O emissions ($r = .04$, $p = .12$). In contrast, NO_3^- exhibited a slight positive correlation with N_2O emissions ($r = .12$, $p < .001$). Also, SOC ($r = .36$) and $N_{balance}$ ($r = .36$) showed a positive correlation with annual N_2O-emissions ($p < .001$). A linear regression analysis was sufficient to predict annual N_2O emissions [N_2O (kg N ha^{-1} $year^{-1}$) = −1.297 + SOC * 0.0256 + $N_{balance}$ * 0.0018] (Figure 4).

4 | DISCUSSION

4.1 | Herbage yield, nitrogen balance and soil functions

Despite the 2-year-old sward having a higher proportion of *L. perenne*, grassland renovation did not result in improved yields and forage quality. This is in agreement with several other reported studies of investigated swards where there were only minor signs of deterioration prior to restoration (Iepema et al., 2020; Velthof et al., 2010; Vliegher & Carlier, 2007). Furthermore, yield and quality improvements following grassland renovation are likely to be limited to the more intensively managed systems. Accordingly, Hopkins, Gilbey, Dibb, Bowling, and Murray (1990) conducted a study at a range of sites across the UK and found that increased yields obtained from renovated grasslands, compared with permanent grasslands with identical management, occurred particularly in the first year after grassland renovation. In subsequent years, the yield improvements from the newly renovated grassland swards were restricted mainly to treatments that received high rates of N fertilizer. As the benefits of N on highly fertilized plots are generally lower in grass–legume mixtures than in pure grass stands, due to the BNF of the legumes, the absence of yield or quality improvements reported in the present work is in accordance with these findings.

The benefits of grass–clover mixtures for herbage productivity and ruminant production are well established and have been shown in numerous previous experiments and across several sites and species; for instance, yields from grass–clover mixtures receiving 150 kg N ha^{-1} $year^{-1}$ have been shown to be comparable to grass monocultures receiving 450 kg N ha^{-1} $year^{-1}$ across a wide range of environments and legume proportions (Nyfeler et al., 2009; Suter et al., 2015). However, in several of these sites, legume persistence was a problem, with legume proportions and biological N fixation dropping substantially in the third experimental year (Suter et al., 2015). The decrease in legume proportion has been found to

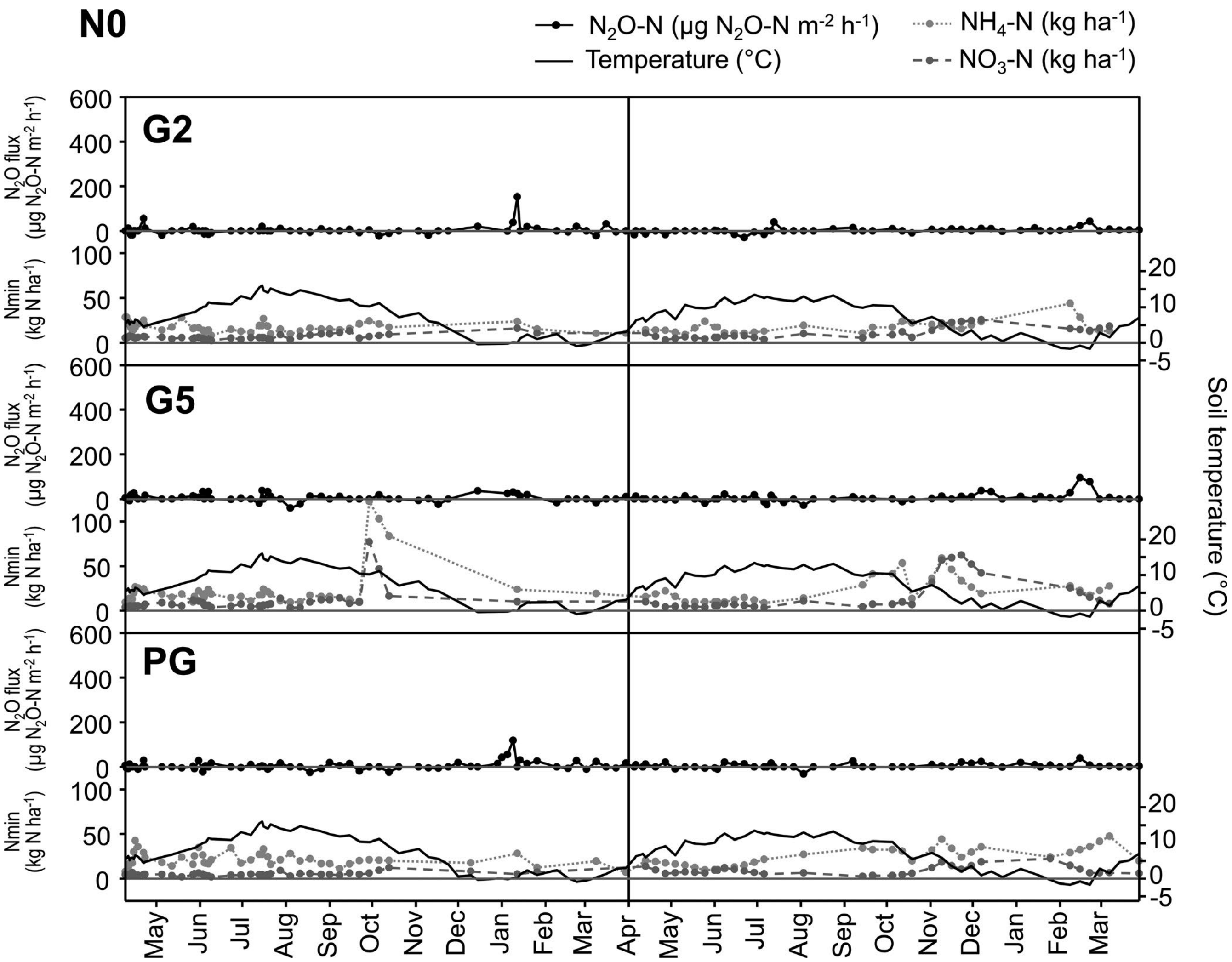

FIGURE 2 Daily average values for N_2O fluxes (μg N_2O-N m^{-2} hr^{-1}) and soil temperature (°C), as well as the N_{min} fractions (NO_3 and NH_4 in kg N/ha) in the non-fertilized (N0) plots across all sward ages (G2 = two-year-old swards, G5 = five-year-old swards, PG = permanent grassland) from April 2010 to March 2012

be a consequence of both the N amount that was applied, as well as the minimum temperature in winter, with cold winters resulting in limited legume survival (Elgersma & Schlepers, 1997). Lack of persistence is often one of the major obstacles for the widespread adoption of forage production based on grass–legume mixtures. An important finding in the present study was that the unfertilized plots in the 16-year-old permanent grassland still had a legume content of 24% and a BNF rate of almost 200 kg of N ha^{-1} $year^{-1}$. This was higher than reported in many previous studies and shows the potential for greater adoption of grass–legume mixtures in practice. Even the slurry-fertilized permanent grassland, which represents a typical semi-intensive management system in Europe, still had a legume proportion of 13% and a BNF of 88 kg/ha of N. The high N fixation potential of swards, even when the legume content is relatively low, is consistent with the findings of Suter et al. (2015) that showed across sites and in most years, that a legume content of around 25% of the sward was sufficient to fix 95% of the maximum N fixation potential of legumes. Consequently, higher contents of legumes, or even legume monocultures, do not necessarily result in any additional N benefit. However, until now this had been shown only in relatively short-term experiments, yet legume persistence is generally considered to be an issue for many farmers who struggle to maintain sufficient amounts of white clover in productive grasslands (Guckert & Hay, 2001). Hence, a measurable benefit of legumes to the N fixation potential of the sward, with no yield losses even after almost 20 years, without any additional N inputs have, to our knowledge, seldom been documented (Tyson, Roberts, Clement, & Garwood, 1990).

Grass–clover swards managed with low N fertilization have a higher efficiency in N transfer and N use efficiency (Nyfeler et al., 2011). This was also apparent in the present study, where each kg of slurry-N substituted on average 0.5 kg N from BNF, indicating a higher nitrogen use efficiency in the unfertilized grass–clover swards, compared with the fertilized plots. This resulted in an improved N balance, as particularly the fertilized plots received total N inputs (BNF plus slurry) of 300 kg N ha^{-1} a^{-1} across all sward

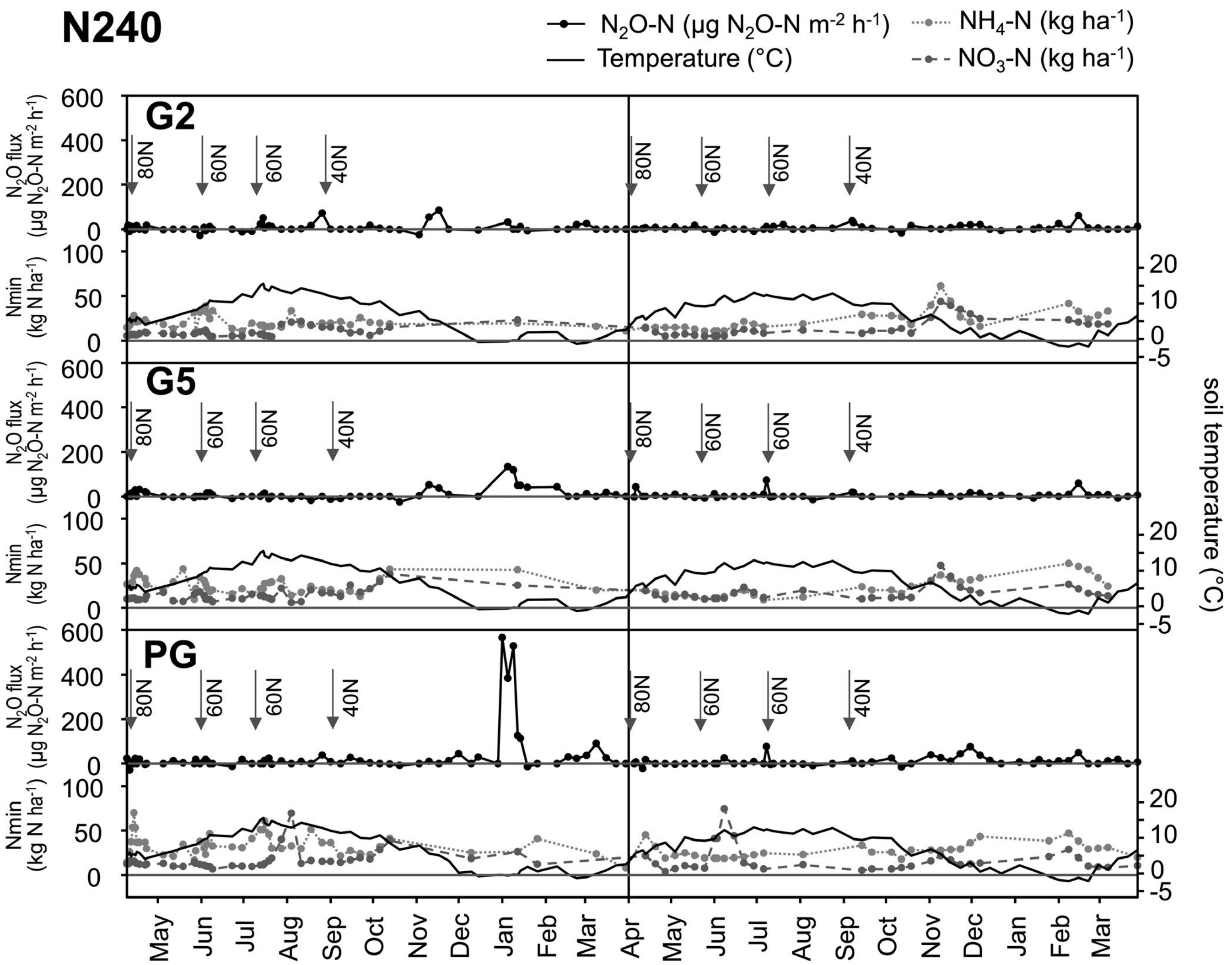

FIGURE 3 Daily average values for N_2O fluxes (μg N_2O-N m^{-2} hr^{-1}) and soil temperature (°C), as well as the N_{min} fractions (NO_3 and NH_4 in kg N/ha) in the plots fertilized with 240 kg of N in the form of slurry (N240) across all sward ages (G2 = two-year-old swards, G5 = five-year-old swards, PG = permanent grassland) from April 2010 to March 2012. Arrows indicate date and total amount of N fertilization

ages. This exceeded the N demand of clover-grass mixtures, resulting in substantial N surpluses of up to 172 kg N ha^{-1} a^{-1} in the two-year-old swards. However, this N surplus was not linked to high gaseous emission peaks. This was likely due to the low SOC stocks as a result of high soil C losses after ploughing prior to grassland renovation (Reinsch et al., 2018). Hence, C sequestration rates were at their maximum on recently renovated swards. As N immobilization is linked to C sequestration, it can be assumed that most of the N surplus in the young swards became immobilized in the soil (Loges et al., 2018), avoiding substantial N mining even at high biomass yields. However, after 5 years of grassland renovation, when SOC is almost at the same level as that of PG, our study showed elevated mineral N concentrations in the topsoil during winter were observed in the unfertilized swards. This indicates a significant decay of clover residues late in the year after several years of excessive BNF. In contrast, the fertilized 5-year-old grassland swards revealed a more balanced soil mineral N status, showing that moderate use of slurry-N can limit the development of an excessive clover proportion during the year after grass–clover establishment.

4.2 | N_2O fluxes from grassland at different sward ages

Renovation of permanent grasslands is known to increase N_2O emissions in the short term (Mori & Hojito, 2007; Necpalova, Casey, & Humphreys, 2013a; Reinsch et al., 2018; Velthof et al., 2010). Accordingly, in an additional experiment conducted at the same site as this study, the estimated annual N_2O emissions ranged from 1.9 to 21.3 kg N_2O-N in the year of reseeding, depending on the time of the year when renovation was conducted (Reinsch et al., 2018). However, very little is known about the long-term effects of grassland renovation on N_2O fluxes. In the study reported here, in year two after the grassland renovation, the N_2O fluxes had already decreased substantially and annual emissions were generally low for all

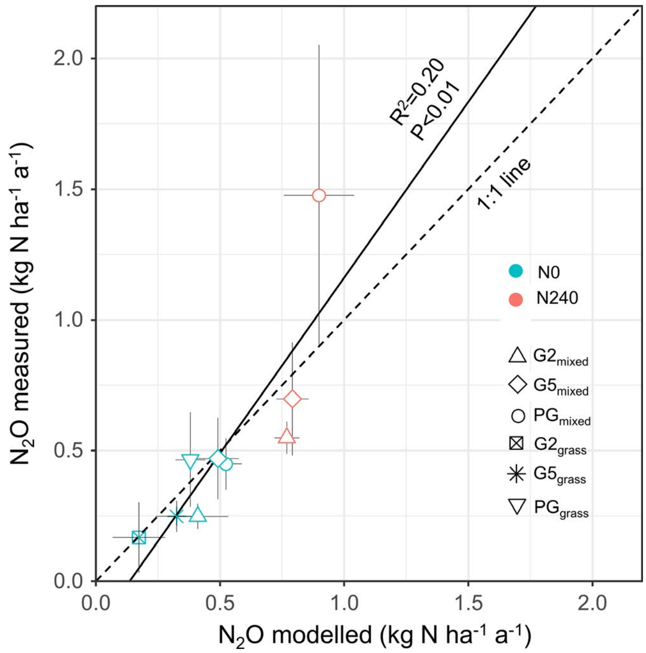

FIGURE 4 Measured versus modelled annual nitrous oxide emissions (kg N_2O-N/ha) of grass-clover swards. Each symbol represents the average accumulated N_2O emissions of one sward age (G2, G5, PG) and fertilization level (0N and 240N) of grass clover (mixed) as well as clover-free subplots (grass). N_2O measurements in clover free-subplots were only performed in the 0N treatment. Error bars represents the standard error. The solid line represents the regression equation [N_2O (kg N ha^{-1} year^{-1})= −1.297 + *C-stock* * 0.0256 + *Nsurplus* * 0.0018], where *C-stock* is the soil carbon stock in the upper soil layer (t C/ha (0-30 cm soil depth)) and *Nsurplus* is the field-level nitrogen balance per year (kg N ha^{-1} year^{-1}). For $N_{surplus}$, the annual N yield, fertilizer rate and biological nitrogen fixation ares taken into account

sward ages, despite the short-term high peaks that occurred in winter. Although the PG swards had emissions that were two to three times higher than those in the G2 swards, these differences were not significant. This was surprising, as we had anticipated an effect of both sward age and fertilization on N_2O emissions. Sward age was anticipated to have an indirect effect, based on the soil water retention capacity and the botanical composition. Additional analyses at the same experimental site have shown that the PG swards had a much higher water retention capacity compared with the newly renovated swards, as soil structures require time to stabilize after disturbance (Ajayi, Horn, Rostek, Uteau, & Peth, 2019). Accordingly, the PG swards had significant higher (PG: 80%) water-filled pore spaces (WFPS) than the young swards (G2: 65% and G5:70% WFPS) (data not shown) and hence, they were anticipated to have higher N_2O emissions (Bateman & Baggs, 2005; Dobbie & Smith, 2003b). At the same time, the legume proportion decreased significantly with time. Considering this, the difference in N_2O emissions based on sward age was relatively small, and it appears that by far the highest emissions occured in the year after reseeding (Reinsch et al., 2018). Similarly, fertilization with slurry had only a limited impact on the emissions, when the total N input was assumed from BNF and fertilization. There are, however, indications that emissions from the N derived from BNF are much lower than emissions from fertilizer applications (Schmeer et al., 2014). Similarly, Li, Lanigan, and Humphreys (2011) did not find any significant differences in clover-based pasture systems for different fertilizer levels. Consequently, several authors have recommended the exclusion or recalculation of emission factors for BNF for the IPCC inventories (Carter & Ambus, 2006; Rochette & Janzen, 2005).

Reasons for the generally observed low emissions from legume-based swards might include a combination of high N uptake by plants due to the high root length and root density commonly observed in grass–legume swards (Chen et al., 2016), together with the low levels of nitrate in the root zone. For instance, soil N concentrations rarely exceeded 20 mg NO_3-N/l, which has been indicated as the critical value for increased N_2O fluxes at high water-filled pore space values during laboratory experiments (Senbayram, Chen, Budai, Bakken, & Dittert, 2012). This is a common phenomenon in grass–clover mixtures, as rhizobial turnover rates result in much lower N fluxes compared with those for mineral fertilizer application. Consequently, grass–clover mixtures experience a continuous addition of small N amounts, in contrast to the short-term "hot spots" of nitrate in the soil from mineral N fertilizer application (Ball & Ryden, 1984). The slow, yet long-term addition results in more plant uptake and less substrate for soil nitrification and denitrification processes. In addition to the generally low emissions, there were also periods with negative fluxes distributed evenly across summer and winter. Negative fluxes from grassland have also been reported elsewhere (Glatzel & Stahr, 2001; Necpálová et al., 2013b). There is, however, some dispute about whether negative fluxes indicate N_2O uptake from the atmosphere during low availability of soil N (Davidson, Savage, Verchot, & Navarro, 2002; Parsons, Mitre, Keller, & Reiners, 1993; Verchot et al., 1999), or if they represent technical and calculation errors, particularly if these fluxes are close to the detection limit (Parsons et al., 1993). However, despite the existing uncertainty regarding the validity of negative fluxes and the factors that contribute to their occurrence (Freney, Denmead, & Simpson, 1978; Parsons et al., 1993; Verchot et al., 1999), they should nevertheless be considered in annual GHG budgets in order to avoid flux overestimations (Chapuis-Lardy, Wrage, Metay, Chotte, & Bernoux, 2007).

Although in the present study we found very little or no fluxes for most of the observation period, it is important to identify the times when fluxes were highest in order to identify the risks and vulnerability to emissions in the production system. Therefore, we identified two main sources of emissions. First, using multiple regression, we identified positive effects of the N surplus, as well as of the soil C stocks on N_2O emissions in semi-intensive grass–clover swards but not for swards with N inputs only. Increased risks for N_2O emissions on soils with high SOC stocks have also been described elsewhere (Baggs et al., 2003; Jäger, Stange, Ludwig, & Flessa, 2011; Ruser et al., 2006). In a model simulation, Li, Frolking, and Butterbach-Bahl (2005) estimated that the additionally produced N_2O due to enhanced C stocks in reduced-tillage farming systems could offset the amount of sequestered C, when N_2O is expressed in CO_2-equivalents. This was apparent in the increasing SOC stocks in PG compared with G2, and the concomitantly increased emission factors. While this effect was found to be small in our experiment, it could become more important with increasing amounts of applied N or when using mineral fertilizer. Hence, the idea of paying attention to balanced N levels to reduce N_2O emissions from grassland systems (Pfab et al., 2011; Van Groenigen, Velthof, Oenema, Van Groenigen, & Van Kessel, 2010) is more important in the context of older swards with high C stocks than in younger swards. Second, the measured fluxes occurred predominantly during winter when the soil was frozen and there was no N uptake by plants, and these fluxes contributed more than 60% of the annual N_2O emissions from grasslands. Almost the entire major fluxes took place during January 2011. The soil had had become frozen in December 2010 and temperatures in January 2011 fluctuated around 0°C, and frost–thaw cycles were considered responsible for these substantial emission peaks. This is a common phenomenon, and frost–thaw cycles have been identified as one of the processes that can increase the N_2O flux by up to 39-fold on temperate grasslands (Burchill, Li, Lanigan, Williams, & Humphreys, 2014). The reasons why frost–thaw cycles increase emissions are not entirely understood but the effect is unlikely to be monocausal. Relevant factors include the physical destruction of organic compounds, as well as microbes. As a consequence, large amounts of additional nutrients and C compounds are made available, thus resulting in N_2O peaks when the soil thaws and intact soil microbes become active again (Koponen et al., 2006; Koponen & Martikainen, 2004). Additionally, anaerobic conditions might be created by snow cover, thus increasing N_2O emissions from denitrification, which are then released during thawing (de Bruijn, Butterbach-Bahl, Blagodatsky, & Grote, 2009). According to these findings and measured N_2O peaks during winter in our PG treatment, it may be concluded that high emission peaks during freeze–thaw cycles are more likely in old permanent grassland swards as they show higher SOC values and higher microbial activity. This would result in higher rates of released C and faster response of frozen soil on oxygen limiting conditions, thereby accelerating N_2O production to a higher level compared with younger swards. However, while frost–thaw cycles evidently were involved in emission peaks, soil temperatures generally did not have an effect on N_2O emissions. This was unexpected, as soil temperature has repeatedly been shown to affect N_2O fluxes (Breuer & Butterbach-Bahl, 2005; Grant & Pattey, 2008). This was likely a result of the generally high N uptake during summer and the low losses, both in gaseous form as N_2O and as nitrate leaching, to the groundwater. The dominant effect of frost-thaw cycles on annual N_2O emissions has been reported previously in other grassland renovation experiments (Reinsch et al., 2018). While this appears to contradict our statement regarding the impact of high SOC stocks on elevated N_2O emissions, as frost–thaw cycles are limited to a brief period of the year, we consider the statement to hold true irrespective of this. In previous studies, it was also shown that the risk for N_2O emissions increases when there are large amounts of decomposable organic matter available during a freeze–thaw cycle. Thus, independent of when the emissions occur, SOC stocks are a relevant factor for their magnitude and are important for an accurate estimation of annual N_2O emissions. However, as a result of climate change and warmer winters, the likelihood and frequency of periods with soil frost occurrence is decreasing in many temperate regions. Hence, re-evaluation of these processes and interactions might be required in the future.

5 | CONCLUSIONS

According to our findings, grassland renovation does not necessarily lead to improved grass–clover yields in the medium term if the existing sward is already in good condition. Sward renovation can also be expected to have no long-term effects on N_2O emissions. Thus, sward age of grassland does not have a direct effect on elevated N_2O emissions. However, high N_2O emissions can be expected if high N surpluses coincide with high C stocks, and the latter are more likely in older grassland swards. Thus, a low N surplus should be sought in permanent grassland. In order to quantify N surplus-related N_2O emissions from grass–clover swards, the amount of symbiotically fixed N should be considered in N-budget calculations. Finally, even without fertilization, high productivity and high forage quality can be achieved and maintained for long periods of time using grass–legume mixtures. At the same time, these systems display improved N balances compared with intensively slurry-fertilized swards. This confirms the potential for greater use of grass–legume mixtures to support climate-smart agriculture based on forage production from grasslands.

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