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ORIGINAL ARTICLE

The potential of ryegrass as cover crop to reduce soil N_2O emissions and increase the population size of denitrifying bacteria

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Abstract

Nitrogen (N) fertilization is the major contributor to nitrous oxide (N_2O) emissions from agricultural soil, especially in post-harvest seasons. This study was carried out to investigate whether ryegrass serving as cover crop affects soil N_2O emissions and denitrifier community size. A microcosm experiment was conducted with soil planted with perennial ryegrass (*Lolium perenne* L.) and bare soil, each with four levels of N fertilizer (0, 5, 10 and 20 g N m^{-2}; applied as calcium ammonium nitrate). The closed-chamber approach was used to measure soil N_2O fluxes. Real-time PCR was used to estimate the biomass of bacteria and fungi and the abundance of genes involved in denitrification in soil. The results showed that the presence of ryegrass decreased the nitrate content in soil. Cumulative N_2O emissions of soil with grass were lower than in bare soil at 5 and 10 g N m^{-2}. Fertilization levels did not affect the abundance of soil bacteria and fungi. Soil with grass showed greater abundances of bacteria and fungi, as well as microorganisms carrying *narG*, *napA*, *nirK*, *nirS* and *nosZ* clade I genes. It is concluded that ryegrass serving as a cover crop holds the potential to mitigate soil N_2O emissions in soils with moderate or high NO_3^- concentrations. This highlights the importance of cover crops for the reduction of N_2O emissions from soil, particularly following N fertilization. Future research should explore the full potential of ryegrass to reduce soil N_2O emissions under field conditions as well as in different soils.

Highlights

1. This study was to investigate whether ryegrass serving as cover crop affects soil N_2O emissions and denitrifier community size;
2. Plant reduced soil N substrates on one side, but their root exudates stimulated denitrification on the other side;

[Correction added on 06 January 2021, after first online publication: Georg-August-Universität Göttingen was added for Haitao Wang]

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3. N_2O emissions were lower in soil with grass than bare soil at medium fertilizer levels, and growing grass stimulated the proliferation of almost all the denitrifying bacteria except nosZ clade II;
4. Ryegrass serving as a cover crop holds the potential to mitigate soil N2O emissions.

KEYWORDS
denitrification, perennial ryegrass (*Lolium perenne* L.), soil bacteria, soil CO_2 emissions, soil N_2O emissions

1 | INTRODUCTION

Increasing nitrous oxide (N_2O) concentration in the atmosphere is among the most serious consequences of the anthropogenic alteration of the global nitrogen (N) cycle (Bakken & Frostegard, 2017). In addition to its high global warming potential and long atmospheric lifetime (IPCC, 2013), N_2O has been shown to be the most important emitted compound involved in stratospheric ozone depletion (Ravishankara, Daniel, & Portmann, 2009). The intensive input of mineral N into agricultural soils is one of the crucial factors contributing to soil N_2O emissions (Ju et al., 2009; Song et al., 2018). Denitrification is the predominant N_2O-producing biological process in soils (Bremner, 1997; Hu, Chen, & He, 2015), which is strongly affected by the soil nitrate (NO_3^-) concentration (Köbke, Senbayram, Pfeiffer, Nacke, & Dittert, 2018; Saggar et al., 2013). In the denitrification pathway, denitrifying microorganisms use NO_3^- as an electron acceptor and reduce it to gaseous N_2 in a stepwise manner. Incomplete denitrification results in the emission of gaseous intermediates such as N_2O.

Soil denitrification is regulated by enzymes such as NO_3^-, nitrite (NO_2^-) and N_2O reductases that are produced by microorganisms. In arable soils, plant root architecture and exudation alter soil structure, aeration and biological activity (Bertin, Yang, & Weston, 2003; Kuzyakov & Xu, 2013), as well as soil microbial communities (Berg & Smalla, 2009). The majority of laboratory studies of soil N_2O emissions, however, have not included plants, although it is known that growing plants may increase denitrification activities in soil (Guyonnet et al., 2017; Klemedtsson, Svensson, & Rosswall, 1987). Recent studies investigated how plant and rhizosphere processes affect soil N_2O emissions (Lenhart et al., 2019; Senbayram et al., 2020). On the one hand, plants compete with soil microorganisms for N (Moreau, Bardgett, Finlay, Jones, & Philippot, 2019), on the other hand, plants provide carbon (C) to the soil via root exudates that modulate microbial communities and denitrification activity (Achouak et al., 2019). Apart from effects on the soil N pool, plants consume O_2 and increase soil CO_2 concentrations through root respiration as compared to unplanted soil. It has been estimated that 5% to 21% of all photosynthetically assimilated C is released into the soil in the form of root exudates (Derrien, Marol, & Balesdent, 2004; Nguyen, 2003). Consequently, the C turnover rate in the soil rhizosphere is estimated to be at least one order of magnitude greater than in the bulk soil (Kuzyakov, 2010). It has been suggested that root exudation will increase denitrification (Bijay-singh & Whitchead, 1988), as root-released C can serve as an electron donor (Philippot, Hallin, & Schloter, 2007). Indeed, planted soils are several times greater in density of denitrifiers than unplanted soils (Chèneby et al., 2004; Herman, Johnson, Jaeger, Schwartz, & Firestone, 2006). Growing perennial grasses, such as *Festuca paniculata*, *Bromus erectus* and *Dactylis glomerata* (Guyonnet et al., 2017), barley (*Hordeum vulgare* L.) (Klemedtsson et al., 1987) and maize (*Zea mays* L.) (Mahmood, Ali, Malik, & Shamsi, 1997) has been shown to increase denitrification activities in soil. The stimulation of soil denitrification activity by plants depends on the plant species and soil water content (Bakken, 1988). Furthermore, root exudates have been shown to modulate soil microbial communities (Haichar et al., 2008; Haichar, Santaella, Heulin, & Achouak, 2014).

However, increased denitrification activity does not necessarily mean higher N_2O emissions from soil. Ammonium (NH_4^+) and NO_3^- have different motilities in soil due to the charge-dependent interaction with soil colloids. As a consequence, a depletion zone of NH_4^+ in the rhizosphere can be created by plant root uptake of NH_4^+ as it shows low mobility in most temperate soils (Orcutt, 2000). In contrast, no such depletion zones in the rhizosphere can be expected for NO_3^- due its high mobility in most temperate soils (Kuzyakov & Xu, 2013). The concentration of NO_3^- in soil, however, can rapidly decrease owing to uptake by plant roots (Tinker & Nye, 2000). Therefore, the availability of mineral N in soil is regarded as a major factor limiting denitrification (Philippot et al., 2007; Saggar et al., 2013). The response

of soil N_2O emissions to the application of mineral N fertilizer is exponential rather than linear (Shcherbak, Millar, & Robertson, 2014). Senbayram, Chen, Budai, Bakken, & Dittert (2012) reported that increasing the soil NO_3^- concentration resulted in a higher N_2O/N_2 ratio. The competition for NO_3^- between plants and denitrifiers can result in lower denitrification rates in planted soils (Qian, Doran, & Walters, 1997). Similarly, regulation of denitrifying soil communities by NO_3^- has been reported from different ecosystems (Correa-Galeote et al., 2017; Deiglmayr, Philippot, & Kandeler, 2006; Enwall, Philippot, & Hallin, 2005); however, the effect of the soil NO_3^- concentration on the abundance and diversity of denitrifiers remains to be determined.

Ryegrass is a common cover crop that is used to reduce nitrate leaching (Bergström & Jokela, 2001; Poeplau, Aronsson, Myrbeck, & Kätterer, 2015; Thomsen & Hansen, 2014) and increase soil organic C stocks (Poeplau et al., 2015). The effect of ryegrass on soil N_2O emissions, however, is under-studied. A recent meta-analysis revealed that cover crops have the potential to mitigate N_2O emissions in post-harvest seasons, yet few studies focused on ryegrass (Muhammad et al., 2019). The main aim of this study was therefore to investigate N_2O emissions from soil with ryegrass compared to bare soil under varying fertilizer levels. To achieve this, we used an incubation experiment with two experimental factors: soil planted with grass and unplanted bare soil, each with four levels of N fertilizer addition. Soil N_2O fluxes were determined using the closed-chamber approach. Real-time PCR (qPCR) assays were performed to estimate the abundance of soil bacteria and fungi, as well as microorganisms harbouring genes involved in denitrification. We hypothesized that the presence of grass and the associated belowground modulations would (i) lower soil N_2O emissions at each fertilizer level and (ii) promote the abundance of bacteria, fungi and denitrifiers, as compared to bare soil.

2 | MATERIAL AND METHODS

2.1 | Soil collection

Topsoil (0 to 25 cm) was collected from Reinshof agricultural research station (51°29′50.3"N, 9°55′59.9″E), University of Göttingen, Lower Saxony, Germany. Mean annual precipitation was 651 ± 24 mm and mean annual temperature was 9.2 ± 0.1°C (1981–2010, meteorological station at Göttingen, station ID: 1691, Germany's Meteorological Service). The site had been cropped with winter oilseed rape (*Brassica napus* L.) (2015), winter wheat (*Triticum aestivum* L.) (2016) and winter barley (2017) prior to soil collection on March 23, 2018. The soil was classified as Luvisol (IUSS, 2015) and the texture of the topsoil (0 to 25 cm) was composed of 61% silt, 23% sand and 16% clay. The bulk density was 1.3 g cm^{-3}, the pH was 7.1 ± 0.1, the soil total C concentration was 1.3% and the total N concentration was 0.13%. Following collection, the soil was stored in a polyvinyl chloride (PVC) container for 3 months at room temperature until incubation. Before incubation, the soil was air-dried to 2% gravimetric water content and sieved through a 2-mm mesh to achieve higher homogeneity. PVC cylinders (diameter, 20 cm; height, 20 cm) were used for incubation and sealed with removable lids (height, 5 cm) carrying butyl-rubber septa for headspace gas sampling. Soil moisture was first adjusted to 35% water-filled pore space (WFPS) and soil (equivalent to 4.49 kg dry soil) was filled into the experimental pots in three layers of approximately 3.7 cm each (11 cm in total) for manual compaction to the original bulk density of 1.3 g cm^{-3}, resulting in 4,398 cm^3 of air space (9 cm headspace + 5 cm lid) for gas accumulation when the chambers were closed. The following day, the soil was carefully irrigated in a stepwise procedure to avoid soil compaction and finally adjusted to 60% WFPS.

2.2 | Experimental setup

The experiment was conducted in a fully controlled climate chamber (Fitotron Walk in Plant Growth Room, Type SGR221 LED, Weiss Technik, Leicester, UK). The climate chamber was set to a light intensity of 520 μmol m^{-2} s^{-1} photosynthetically active photon flux density at 25°C air temperature from 6.00 am to 10.00 pm as "day mode" (16 hr), and from 10.30 pm to 6.00 am (8 hr) as "night mode" with no light at 12°C air temperature. The relatively large temperature discrepancy was set in order to mimic conditions close to those in the field.

The experiment consisted of two groups: soil with perennial ryegrass (*Lolium perenne* L.) (DSV AG, Salzkotten, Germany) and bare soil. Each group had four different fertilizer levels (0, 5, 10 and 20 g N m^{-2}, equivalent to 0, 50, 100 and 200 kg N ha^{-1}), resulting in a total of eight treatments. Each treatment was performed in triplicate, yielding a total of 24 pots. Before the first sampling date, grass was sown at a density of approximately 5,000 seeds m^{-2} and pre-incubated for 4 weeks to allow grass establishment in the pots. The treatments with bare soil were treated equally but without plant cultivation. Calcium ammonium nitrate N fertilizer (76% ammonium nitrate (NH_4NO_3) and 24% calcium carbonate ($CaCO_3$)) was applied after dissolution in distilled water. Half of the total N fertilizer was applied after the first collection of soil and gas samples on day 1 (August 3, 2018); the other half

TABLE 1 Total N uptake and C assimilation of grass shoots and roots throughout the experimental period (56 days) at each fertilizer level in soil with grass

	N uptake (g N m^{-2})			C assimilation (g C m^{-2})		
Fertilizer level (g N m^{-2})	**Shoot**	**Root**	**Total**	**Shoot**	**Root**	**Total**
0	2.6 ± 0.1c	1.5 ± 0.2b	4.1 ± 0.1d	58.3 ± 2.6b	58.7 ± 2.8b	117.0 ± 4.4b
5	4.7 ± 0.2c	1.8 ± 0.1a	6.5 ± 0.2c	98.4 ± 4.3a	74.3 ± 0.9a	172.6 ± 4.7a
10	7.4 ± 0.7b	2.0 ± 0.1a	9.3 ± 0.5b	115.8 ± 5.3a	74.8 ± 4.1a	190.5 ± 1.2a
20	11.8 ± 0.7a	2.1 ± 0.1a	13.9 ± 0.5a	123.6 ± 9.2a	68.1 ± 2.6a	191.8 ± 10.8a

Note: Means ± standard errors followed by different lowercase letters indicate significant differences among fertilizer levels within each parameter (one-way ANOVA with Tukey's honestly significant difference (HSD) test or Kruskal-Wallis test with multiple comparison extension) at $p < .05$.

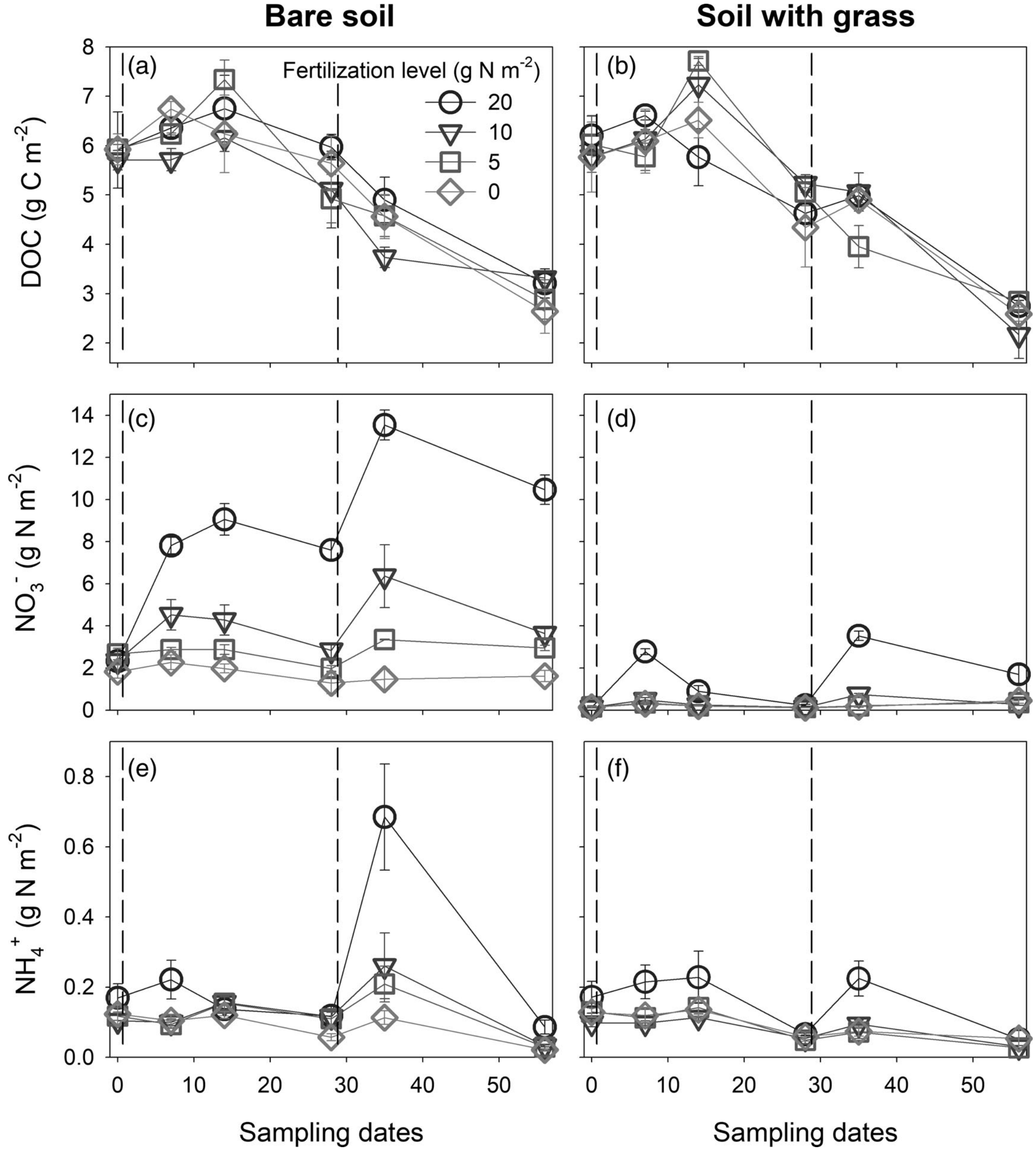

FIGURE 1 Time course of dissolved organic carbon (DOC) concentrations in (a) bare soil and (b) soil with grass, and soil NO_3^--N content in (c) bare soil and (d) soil with grass, and soil NH_4^+-N concentrations in (e) bare soil and (f) soil with grass during the 56 days growing period. Solid lines with points of different grey intensities represent different fertilizer levels (0, 5, 10 and 20 g N m^{-2}); dashed vertical lines indicate fertilization dates (day 1 and 28). Error bars represent the standard error of the mean ($n = 3$)

was applied after 28 days, with a full measuring period of 56 days. After fertilization, soil was irrigated daily, and up to every 2 days in the later period by weighing the pots, to keep the soil moisture at 60 ± 5% WFPS.

Two days before the first fertilization, the grass was cut to a height of 4 cm. Following this, the grass was cut every 2 weeks and the shoot dry matter was determined from air-dried material. At the end of the experiment, the roots were collected as well. Roots were carefully washed, air-dried and weighed. The total C and N of finely ground dry grass shoots and roots were determined on a NA-1500 N elemental analyzer (Carlo Erba, Milano, Italy). Grass N uptake and C assimilation were calculated as:

$$N_{uptake} = DM_{shoot} \times N concentration_{shoot} + DM_{root} \times N\ concentration_{root}; \quad (1)$$

and

$$C_{assimilation} = DM_{shoot} \times C concentration_{shoot} + DM_{root} \times C\ concentration_{root}; \quad (2)$$

where DM refers to the dry matter of the harvested grass. Apparent N recovery (ARN) was calculated as:

$$ARN\ (\%) = \frac{N_{uptake}(fertilized)\text{-}N_{uptake}(unfertilized)}{amount\ of\ N\ applied} \times 100. \quad (3)$$

Gas samples of 25 mL in volume were collected using a syringe inserted in the headspace of sealed lids. Samples were directly transferred to a pre-evacuated 12-mL Exetainer vial (Labco, Lampeter, UK). Gas samples were collected at 0, 20 and 40 min after the pots were sealed. In the first week after each fertilization, gas samples were collected every day to capture the fertilization-induced peaks. In the following 3 weeks, gas samples were taken at larger intervals of 2 to 4 days.

In order to avoid the disturbance of soil structure by soil sampling during the incubation period, we incubated a spare set of pots in parallel to the gas sampling pots for soil sample collection. The setup of these pots was identical to that for pots for gas sampling. Soil samples were taken on day 0 (1 day before the first fertilization), day

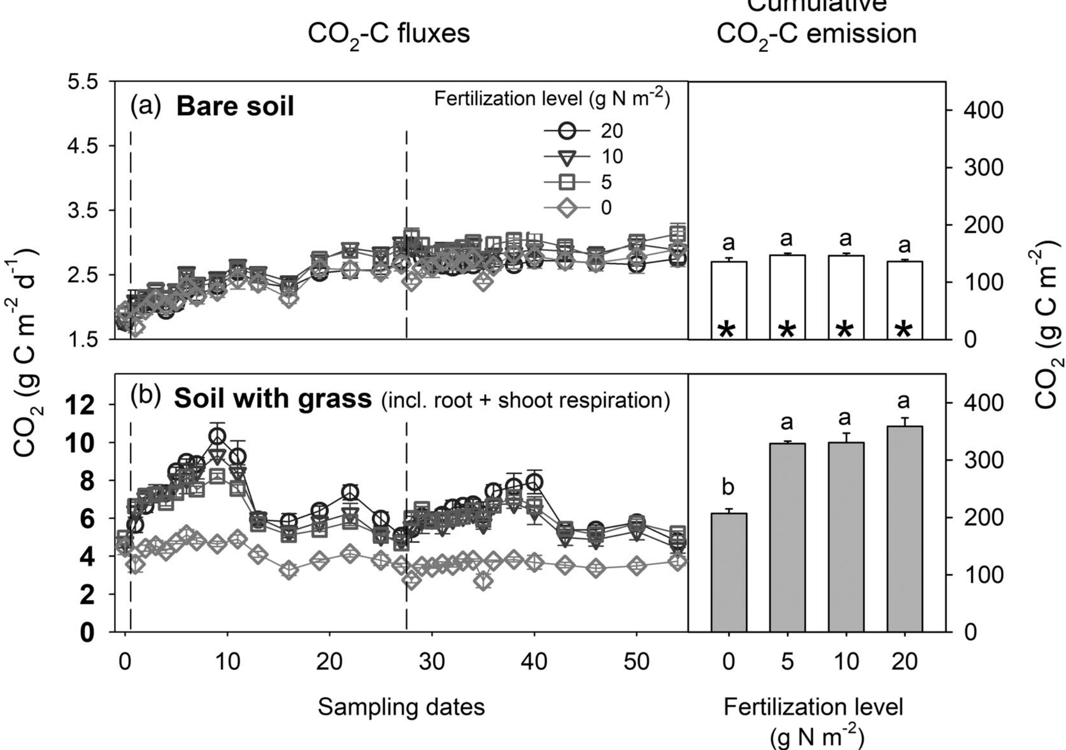

FIGURE 2 CO_2 emission dynamics and cumulative CO_2 emission during the growing period (56 days) from bare soil (a) and soil with grass (b). Error bars represent the standard error of the mean of each treatment ($n = 3$). Solid lines with points of different grey intensities represent different fertilizer levels (0, 5, 10 and 20 g N m^{-2}). Dashed vertical lines indicate fertilization dates (day 1 and day 28). Asterisks indicate significant differences in cumulative CO_2 emission between bare soil and soil with grass at the same fertilizer level (*t*-test or Mann–Whitney *U*-test); lowercase letters indicate significant differences in cumulative CO_2 emission among fertilizer levels within bare soil or within soil with grass (one-way ANOVA with Tukey's honestly significant difference (HSD) test or Kruskal-Wallis test with multiple comparison extension) at $p < .05$

7, day 14, day 28 (before the second fertilization), day 35 and day 56 (final collection of samples). On day 0, day 7 and day 14, soil samples were collected from the first spare pot; on day 35 and day 56, they were collected from the second spare pot. The last soil samples were taken from the pots on which gas measurements were performed. The soil samples (0–11 cm depth) were taken using a 16-mm diameter auger. Remaining holes were filled with reagent glasses (16 mm diameter) to avoid extra water and nutrient losses. Approximately 60 g of fresh soil was taken and sieved through a 2-mm mesh. Soil samples were homogenized and divided for soil NH_4^+ and NO_3^-, and dissolved organic carbon (DOC) analysis. Soil pH, total C and N, and WFPS did not differ among treatments. From the last set of soil samples, aliquots were used for soil DNA extraction and subsequent qPCR analysis.

2.3 | Gas and soil sample analysis

Gas samples were analysed on an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermal conductivity detector for the determination of carbon dioxide (CO_2) concentrations and an electron capture detector for the determination of N_2O concentrations. The flux rates of CO_2 and N_2O were calculated using linear regression of the gas concentration over time (Parkin, Venterea, & Hargreaves, 2012; Wang et al., 2013). Cumulative emissions were calculated by interpolating the values of CO_2 and N_2O emissions.

To determine soil NH_4^+ and NO_3^- concentrations, subsamples (10 g) of sieved fresh soil were extracted by adding 50 mL of 0.0125 M calcium chloride ($CaCl_2$). Mixtures were shaken for 1 hr, filtered (MN615 ¼; pore size, 4–12 μm; Macherey-Nagel, Düren, Germany) and subsequently stored at −20°C until analysis. NH_4^+ and NO_3^- concentrations in the extracts were determined using a San^{++} continuous flow analyzer (Skalar Analytical, Breda, The Netherlands). Soil pH was measured from 10 g of air-dried soil suspended in 50 mL of 0.01 M $CaCl_2$ solution using a pH meter. Total C and N measurements were performed with finely ground air-dried soil using an NA-1500 elemental analyzer (Carlo Erba, Milano, Italy). Prior to the measurement of total C and N, the air-dried soil was fumigated in a hydrogen chloride (HCl) atmosphere

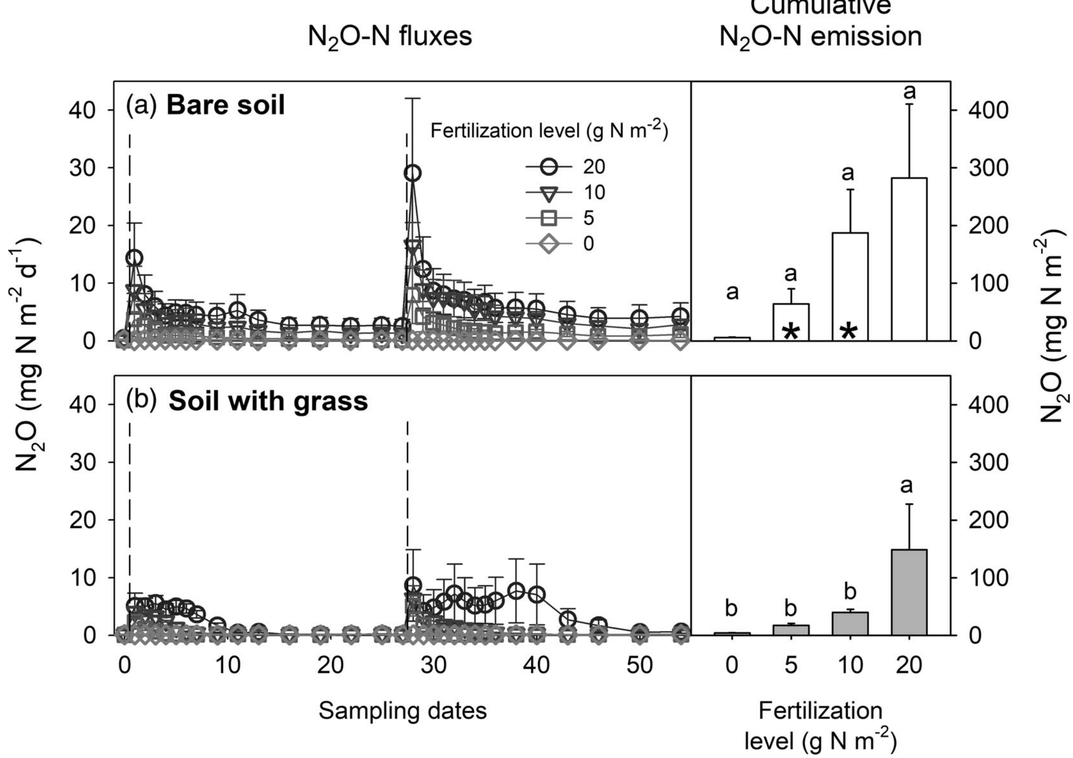

FIGURE 3 N_2O emission dynamics and cumulative N_2O emission during the growing period (56 days) from bare soil (a) and soil with grass (b). Error bars represent the standard error of the mean of each treatment ($n = 3$). Solid lines with points of different grey intensities represent different fertilizer levels (0, 5, 10 and 20 g N m^{-2}). Dashed vertical lines indicate fertilization dates (day 1 and day 28). Asterisks indicate significant differences in cumulative N_2O emission between bare soil and soil with grass at the same fertilizer level (*t*-test or Mann–Whitney *U*-test); lowercase letters indicate significant difference in cumulative N_2O emissions among fertilizer levels within bare soil or within soil with grass (one-way ANOVA with Tukey's honestly significant difference (HSD) test or Kruskal-Wallis test with multiple comparison extension) at $p < .05$

using 3 M HCl for 1 week to remove carbonates (Harris, Horwath, & Kessel, 2001). For DOC measurements, 10 g of fresh soil was extracted using 40 mL of 0.5 M potassium sulphate (K_2SO_4). The solution was shaken for 2 hr and filtered (MN615 ¼; pore size, 4–12 μm; Macherey-Nagel, Düren, Germany). Extracts were stored at −20°C until determination of organic C and total C concentrations using a Total organic carbon/Total inorganic carbon (TOC/TIC) analyser (Multi C/N 2100, Analytik Jena, Jena, Germany).

2.4 | DNA extraction from soil and qPCR

For qPCR analysis, soil was freeze-dried for 72 hr. The freeze-dried material was finely ground using a swing mill (MM400, Retsch, Haan, Germany) for 60 s at 25 Hz. Total DNA was extract from 50 mg ground soil using a modified cetyltrimethylammonium bromide-based protocol (Brandfass & Karlovsky, 2008) as described previously (Beule et al., 2019). Following DNA extraction, the quality and quantity of DNA were examined on 0.8% (w/v) agarose gels stained with ethidium bromide. The extracts were tested for PCR inhibitors as described previously (Guerra, Beule, Lehtsaar, Liao, & Karlovsky, 2020) and diluted 1:50 (v/v) in double-distilled water (ddH_2O) prior to qPCR analysis. We quantified bacterial 16S rRNA and fungal 18S rRNA genes, as well as genes involved in denitrification, namely *narG* and *napA* for NO_3^- reduction, *nirK* and *nirS* for NO_2^- reduction, and *nosZ* clade I and II for N_2O reduction. All reactions were carried out in 4 μL reaction volume (3 μL mastermix +1 μL template DNA or ddH2O for negative controls) on a CFX384 Thermocycler (Biorad, Rüdigheim, Germany). A detailed description of the mastermix composition and thermocycling conditions can be found in Beule et al. (2019).

2.5 | Statistical analysis

All data were tested for homogeneity of variance (Levene's test) and normal distribution (Shapiro–Wilk test). Differences among treatments of cumulative data (N uptake and C assimilation by grass and cumulative CO_2 and N_2O emissions) or data without repeated measurements (soil bacteria, fungi and denitrifiers) were assessed by performing a *t*-test or one-way ANOVA with Tukey's honestly significant difference (HSD) post-hoc test for parametric data, or the Mann–Whitney *U*-test or Kruskal–Wallis test with multiple comparison extension for non-parametric data. Differences among treatments of repeatedly measured data (DOC, NO_3^-, NH_4^+, CO_2 and N_2O fluxes) were analysed using linear mixed effect (LME) models. In the models, either the fertilizer level or the treatment of bare soil versus soil with grass were set as a fixed effect, and sampling date and replicate pot set as random effects. The data were partially $\log_{10}$- or square-root-transformed to meet the criteria for an LME model. Statistical significance was considered as $p < .05$, with marginal statistical significance at $p < .1$. All statistical analyses were performed in R version 3.5.2 (R Core Team, 2018).

3 | RESULTS

3.1 | Grass N uptake and C assimilation

In soil with grass, total plant N uptake, which was calculated by the dry matter of grass shoots and roots, ranged from 6.5 to 13.9 g N m^{-2} in fertilized pots, compared to 4.1 g N m^{-2} in the unfertilized treatment. The ARNs of fertilized treatments were 50% ± 2%. Plant shoot N uptake at the 10 g N m^{-2} fertilizer level was lower than

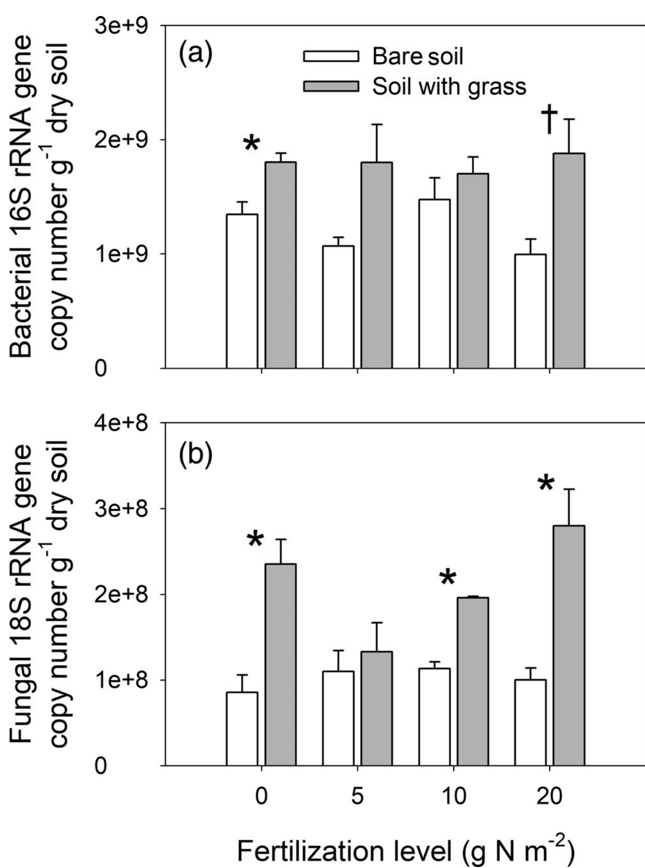

FIGURE 4 Bacterial 16 s rRNA (a) and fungal 18 s rRNA (b) gene copy number per g dry soil in bare soil and soil with grass under different fertilizer levels (0, 5, 10 and 20 g N m^{-2}) at the end of the growing period (day 56). Error bars represent standard error of the mean of each treatment ($n = 3$); asterisks denote differences between bare soil and soil with grass (* $p < .05$); daggers represent marginal differences between bare soil and soil with grass († $p < .1$)

that at 20 g N m^{-2} ($p = 0.006$) and greater than those at 0 and 5 g N m^{-2} ($p < .01$) (Table 1). Plant root N uptake in unfertilized treatments was lower than in the treatments in which 5, 10 and 20 g N m^{-2} were added ($p < .04$) (Table 1). The total N uptake throughout the incubation period (56 days) increased along with increasing fertilizer application ($p < .03$) (Table 1). Shoot C, root C and total C assimilation were greater in fertilized than unfertilized pots ($p < .04$) (Table 1), but did not differ among the 5, 10 and 20 g N m^{-2} fertilization treatments.

3.2 | Soil DOC, NO_3^- and NH_4^+ dynamics during incubation

Dissolved organic carbon was slightly increased in the first 2 weeks, and gradually decreased in the following weeks in both bare soil and soil with grass (Figure 1a, b). DOC concentrations did not differ among bare soil and soil with grass, nor among fertilizer levels (Figure 1a,b). Soil NO_3^--N content in bare soil was always greater than in soil with grass at all fertilizer levels ($p \leq .001$) (Figure 1c,d). Fertilization led to increased NO_3^--N concentrations in bare soil as compared to unfertilized treatments ($p \leq .05$) (Figure 1c). Compared to the background NO_3^- (approximately 2 g of NO_3^--N m^{-2}) in unfertilized bare soil, soil NO_3^--N was close to zero in the unfertilized treatment of soil with grass (Figure 1d). Furthermore, in soil with grass, at the 20 g N m^{-2} fertilizer level, soil NO_3^--N was greater than at all other fertilizer levels ($p \leq .05$) (Figure 1d). When N fertilizer was applied, NH_4^+-N was marginally greater in bare soil than in soil with grass ($p < .1$) (Figure 1e,f).

3.3 | CO_2 and N_2O emissions

The presence of grass strongly enhanced CO_2 emissions compared to bare soil, especially in treatments with fertilizer ($p \leq .003$) (Figure 2). Fertilization had no effect on CO_2 emissions (neither on short-term rates nor on cumulative fluxes) in bare soil (Figure 2a). In soil with grass, however, CO_2 emission rates and cumulative fluxes were increased by fertilizer application ($p \leq .001$) (Figure 2b).

In contrast to CO_2 fluxes, N_2O emissions from bare soil were greater than from soil with grass at each fertilizer level ($p < .05$) (Figure 3). At the 5 and 10 g N m^{-2} fertilizer levels, bare soil showed greater cumulative N_2O emissions than soil with grass ($p < .05$). Cumulative N_2O emissions from soil with grass at the 0, 5 and 10 g N m^{-2} fertilizer levels were lower than at 20 g N m^{-2} ($p < .05$) (Figure 3b).

3.4 | Soil microbial gene abundances in bare soil and soil with grass

At the end of the experiment (day 56), the abundances of bacteria, fungi and denitrification genes in soil were quantified. The fertilization rate did not affect the abundances of bacteria, fungi and denitrification genes (Figure 4, Figure 5). At the 0 and 20 g N m^{-2} fertilizer levels, the soil with grass showed marginally greater bacterial 16S rRNA gene copy numbers than bare soil ($p < .08$) (Figure 4a). Similarly, the number of fungal 18S rRNA gene copies did not differ among fertilizer levels, but were greater in soil with grass than bare soil at the 0, 10 and 20 g N m^{-2} fertilizer levels ($p < .05$) (Figure 4b). The abundance of *narG* was greater in the soil with grass than bare soil at the fertilizer level of 20 g N m^{-2} ($p < .005$) (Figure 5a). At the 0 and 5 g N m^{-2} fertilizer levels, gene copy numbers of *napA* in soil with grass were greater than in bare soil ($p < .06$) (Figure 5b). At each fertilizer level, *nirK* gene copy numbers were greater in soil with grass than in bare soil ($p < .09$) (Figure 5c). The abundance of *nirS* was increased in soil with grass compared to bare soil when 5, 10 or 20 g N m^{-2} of fertilizer was applied ($p < .1$) (Figure 5d). At the fertilization rate of 20 g N m^{-2}, *nosZ* clade I gene copies were marginally greater in soil with grass than in bare soil ($p < .07$) (Figure 5e). No differences between soil with grass and bare soil at any fertilizer level were detected for *nosZ* clade II genes (Figure 5f).

4 | DISCUSSION

4.1 | Soil organic C turnover and CO_2 emissions

The soil microbial community is the main driver of soil respiration and organic C mineralization in bare soils (Li et al., 2018; Liu et al., 2018). The slight increase in DOC in the first 2 weeks may have been due to the rewetting of the dry soil to 60% WFPS (Kalbitz, Solinger, Park, Michalzik, & Matzner, 2000). For example, when Lundquist, Jackson, & Scow (1999) exposed soil to wet–dry cycles, soil aggregates were partly decomposed and their C was found in the DOC fraction. In the first 3 weeks of the experiment, soil CO_2 emissions increased gradually in bare soils, indicating a recovery of the microbial respiration from the rewetted air-dried soil (Figure 2a). As the soil was already pre-incubated for 4 weeks before the application of fertilizer, this may be seen as an indication that the recovery of the soil microbial activity in the bare soil may take approximately 7 weeks under the given conditions. One reason for this

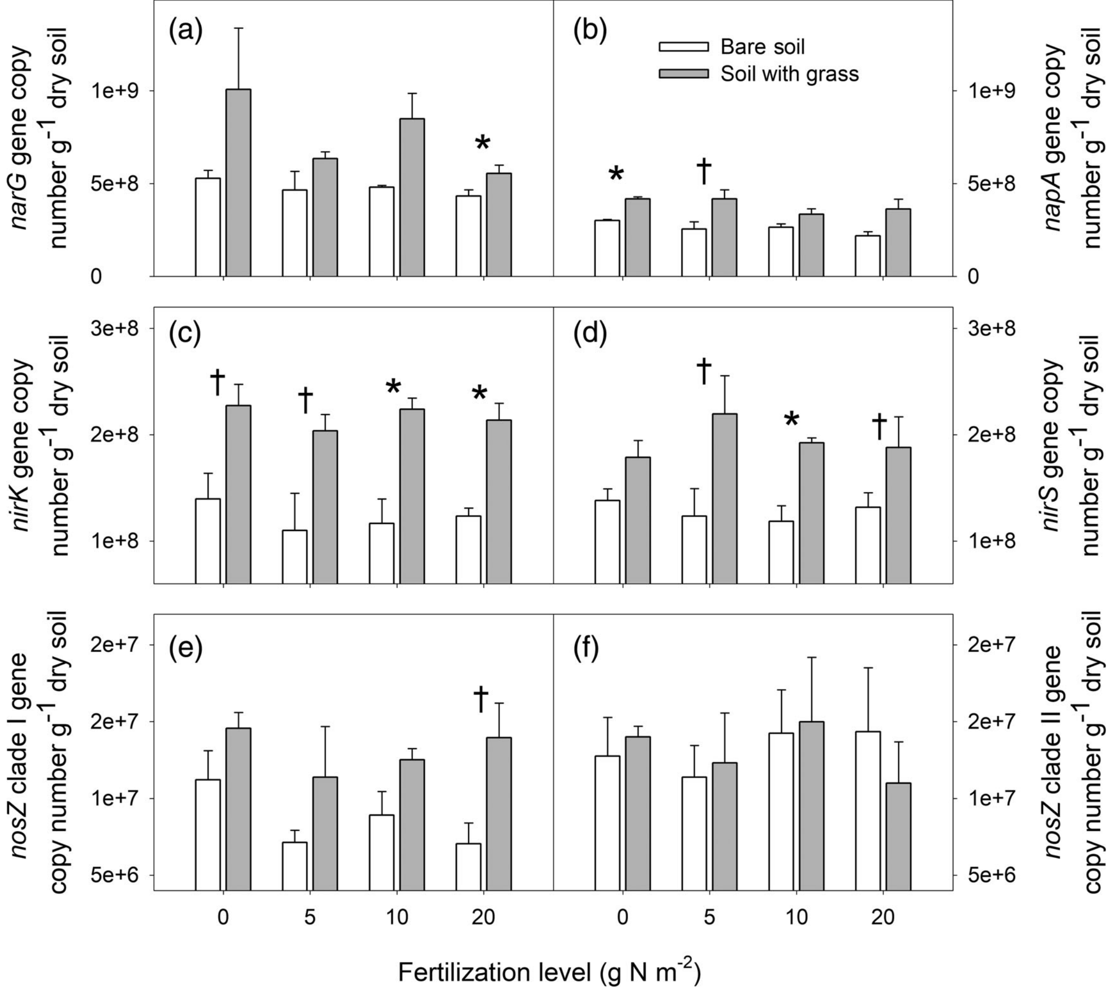

FIGURE 5 *narG* (a), *napA* (b), *nirK* (c), *nirS* (d), *nosZ* clade I (e) and *nosZ* clade II (f) gene copy number per g dry soil in bare soil and soil with grass under different fertilizer levels (0, 5, 10 and 20 g N m^{-2}) at the end of the growing period (day 56). Error bars represent standard error of the mean of each treatment ($n = 3$); asterisks denote differences between bare soil and soil with grass (* $p < .05$). Daggers represent marginal differences between bare soil and soil with grass († $p < .1$)

long recovery period in bare soil may be the limitation of available C. Three weeks after the first fertilization, the stable CO_2 emissions and slow DOC consumption rate may point towards a stabilized soil microbial community.

Pausch & Kuzyakov (2018) reviewed the distribution of C compounds in soil that were released by roots. They concluded that 12% of the assimilated C is emitted from the plant as root-derived CO_2 and 5% is deposited in the rhizosphere. Most plant root exudates have been reported to be readily available to soil microorganisms because they can be metabolized within a few hours (Jones et al., 2005; Kuzyakov & Xu, 2013). Moreover, it is reasonable to expect a greater DOC content in soils with grass than in bare soil given the estimation that 5% of the assimilated C is sequestrated in the rhizosphere (Pausch & Kuzyakov, 2018). However, no such increase was found in our study. Zhang, Li, Wang, & Huang (2018) reported that heavy grazing lowered the C input and decreased C accumulation and total soil organic C contents, due to reduced aboveground tissue (Schönbach et al., 2011), more exposure of the soil surface, and thus increased loss of soil moisture (Y. Zhao et al., 2007) and stimulated compensatory growth of new leaves (W. Zhao, Chen, & Lin, 2008). Therefore, the intensive cutting throughout our experiment is likely to have contributed to the lack of increased soil DOC, and the limitation of available C may restrict denitrification activity and therefore reduce soil N_2O emissions in soil with grass.

4.2 | Soil mineral N and N_2O emissions

The N_2O emissions followed a pattern that was similar to that for soil NO_3^- concentrations. For example, in each of

the eight treatments, the second N_2O emissions peak, which followed the second fertilizer application, was greater than the first peak (Figure 3a,b). Additionally, in contrast to soil with grass, bare soil treatments showed considerably greater N_2O emissions, which lasted over the entire study period. In soil with grass, N_2O emissions fell to nearly zero 2 weeks after each fertilizer application, which was associated with exploited soil NO_3^-. The relationships between soil NO_3^- and N_2O emissions indicate that, under these conditions, soil NO_3^- is the predominant factor controlling soil N_2O emissions (Dong et al., 2018; Ji et al., 2018; Zhou, Zhu, Wang, & Wang, 2017).

At 60% WFPS, both nitrification and denitrification are expected to be important contributors to soil N_2O emissions, as this moisture level is seen as the threshold between aerobic and anaerobic conditions (Köbke et al., 2018; Menéndez, Barrena, Setien, González-Murua, & Estavillo, 2012; Volpi, Laville, Bonari, o di Nasso, & Bosco, 2017). One week post the first fertilization, only 0.2 g N NH_4^+ was found and 7.9 g N NO_3^- were detected in the fertilized bare soil treatment at 20 g N m^{-2} (Figure 1c,e). Because the fertilizer was calcium ammonium nitrate at a ratio of NH_4^+ and NO_3^- N of 1:1, we assume that the vast majority of NH_4^+ was converted to NO_3^- through nitrification. Additionally, we assume that a certain proportion of the added NH_4^+ was released as N_2O during nitrification (Bremner, 1997). After 1 week, nitrification was unlikely to happen because the amount of NH_4^+ (0.2 g N) was low. Although nitrification was not investigated in this study, our observations agree with other incubation studies that more than 50% of the NH_4^+ is converted to NO_3^- within the first week after fertilizer application (Senbayram, Chen, Mühling, & Dittert, 2009; Wu et al., 2017). We anticipate that, in the first week after fertilization, both pathways (nitrification and denitrification) contributed to the observed N_2O emissions. In the following weeks, denitrification is likely to have become the predominant process contributing to N_2O emissions owing to NH_4^+ removal (Figure 1e,f) and lowered oxygen partial pressure induced by root O_2 consumption (Klemedtsson et al., 1987).

Due to root activity, two opposing effects on denitrification are likely to have occurred: (i) O_2 consumption by aerobic root activity (root respiration consuming O_2) (Kuzyakov & Razavi, 2019); and (ii) plant transpiration, leading to drainage of coarse soil pores and thus increased air-filled pore space, which will result in increased oxygen availability. In our study, although water content was adjusted every 1–2 days, soil with grass had about 5% lower WFPS than bare soil prior to irrigation. The loss of water was due to plant transpiration. Therefore, lower soil moisture due to plant transpiration may increase oxygen diffusion into the soil and thereby suppress denitrification (Menéndez et al., 2012; Volpi et al., 2017). Several previous studies reported that the presence of plants would increase denitrification (Guyonnet et al., 2017; Klemedtsson et al., 1987; Mahmood et al., 1997). However, our study was not a perfect proof of the opposite, but at least it provides evidence that the earlier reported promotion of denitrification does not always happen; at least, plants do not always induce higher N_2O emissions.

4.3 | Influence of the presence of plants on soil microbial abundances

The population size and diversity of microbial communities have repeatedly been shown to increase in the presence of plants (Guyonnet et al., 2018; Haichar et al., 2008; Li et al., 2018). In line with this, our results showed that population densities of both soil bacteria and fungi increased with the presence of ryegrass. Considering C-limited conditions in unplanted soil, we assume that root-derived input of easily available C promoted these microbial populations.

Plant root exudates are known to modulate both microbial biomass and community composition (Benizri, Nguyen, Piutti, Slezack-Deschaumes, & Philippot, 2007; Henry et al., 2008; Langarica-Fuentes, Manrubia, Giles, Mitchell, & Daniell, 2018; Zhalnina et al., 2018). However, a limited number of studies have explored how plants influence genes involved in denitrification (Henry et al., 2008; Pivato et al., 2017). We found that, with the exception of *nosZ* clade II, all denitrification genes were promoted in the presence of ryegrass, which may be due to the root exudation of easily available C. Graf (2015) proposed a greater affinity of *nosZ* clade I-carrying microorganisms to root exudates than for those carrying *nosZ* clade II. Our findings agree with the suggestions of Graf (2015): there was a trend showing that *nosZ* clade I genes were greater in soil with grass, whereas *nosZ* clade II showed no preference for bare versus planted soil.

4.4 | Relationship of reduced N_2O emissions and increased denitrifying gene abundances in soil with grass

In our study, N_2O emissions were reduced even though denitrification genes increased under grass. Recovery of N by crops is usually somewhat less than 50% (Fageria & Baligar, 2005). In our study, the high N recovery rate of ryegrass (~50% ARN) indicates that the incubation conditions (60% WFPS, 25°C day temperature and 12°C night temperature) were favourable for plant growth. The

ARN agrees well with the emission factors of bare soil and soil with ryegrass, which were 1.4%–1.8% and 0.5%–0.8%, respectively. Our results indicate that for soil N_2O production, the availability of mineral N was a more important factor than the population size of denitrifiers. It should be mentioned that, due to the limitation of the experimental design, soil samples and gas samples were not taken from the same pots, and N_2O emissions were highly variable. Therefore, it was not possible to correlate N_2O emissions and NO_3^- concentrations in this study.

In soil with grass at the 20 g N m^{-2} fertilizer level, soil NO_3^- concentrations were not depleted by plant uptake and, concurrently, cumulative N_2O emissions at this level were more than twice as high as those at the 10 g N m^{-2} fertilizer level, suggesting that an N input that exceeds the plant's needs can exponentially increase soil N_2O emissions. Previous field studies have shown congruent results (Groenigen, Velthof, Oenema, Groenigen, & Kessel, 2010; Philibert, Loyce, & Makowski, 2012; Shcherbak et al., 2014). The much lower cumulative N_2O emission levels in soil with grass, as compared to bare soil, at the 5 and 10 g N m^{-2} fertilizer levels, were most likely to be due to plant uptake of soil mineral N.

It was recently suggested that soil NO_3^- availability affects denitrifying communities (Deiglmayr et al., 2006; Saggar et al., 2013; Tang et al., 2016). However, our data revealed no link between fertilizer level and denitrification genes. The reason may be the limitation of available C in both bare soil and planted soil. In planted soil, intensive cutting may have limited C input from root exudates into the soil. It should be noted, however, that this observation requires further study, because denitrifiers were only quantified at the end of our experiment. Therefore, potential changes in microbial communities during the course of our experiment may have remained undetected. Our study aimed to explore the potential of ryegrass to reduce soil N_2O emissions under laboratory conditions. We considered the homogenization of the soil as important for a comparable starting point for the development of the soil microbial community. Our incubation study used sieved soil, which altered the soil structure and is likely to have affected the microbial community as compared to the field conditions. The incubation temperature used in the present study was higher than that expected under field conditions, which may have caused greater ammonia (NH_3) volatilization (Bremner, 2007; Forrestal et al., 2016) and higher nitrification and denitrification rates (Bremner, 1997; Saggar et al., 2013). Furthermore, NO_3^- loss by leaching was absent in our study because the incubation pots were not drained. These methodological drawbacks may have led to an overestimation of soil N_2O emissions in our study as compared to field conditions. Follow-up field studies should be carried out to explore the full potential of ryegrass under field conditions and in different soils.

5 | CONCLUSION

Our incubation experiment compared N_2O emissions and population sizes of denitrifying bacteria in soil planted with ryegrass and in bare soil under different N fertilizer levels. We found that 50% of fertilized N was recovered in plant tissues and emissions of N_2O were lower in soil with grass than in bare soil, although the proliferation of denitrifying bacteria in soil with grass was stimulated. We infer that soil mineral N is more related to N_2O emissions than soil denitrifying genes. However, because of the higher potential of denitrification in soil with grass, the risk of high N_2O emissions should also be noted, especially when N fertilizer exceeds the requirements of plants. Altogether, we conclude that ryegrass serving as a cover crop holds the potential to mitigate soil N_2O emissions in soils with moderate or high NO_3^- concentrations. Future studies should focus on how different plant species and their root exudates affect soil N_2O emissions and related soil microorganisms under field conditions and in different soils.

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AUTHORSHIP STATEMENTS

K.D., B.P., S.M. and H.W. designed the experiment, H.W. carried out the incubation experiment, L.B. performed qPCR and the analysis of the gene abundance data, H.W. drafted the manuscript, L.B., S.M., P.K., H.Z., K.D. and B.P. contributed to the final version of manuscript. K.D. supervised the project.

CONFLICT OF INTERESTS

None.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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