



White Clover does not Increase Soil N₂O Emissions Compared to Ryegrass in Non-Frozen Winter, but Increases CH₄ Uptake

Guodong Shao¹ · Juanjuan Ai¹ · Michaela A. Dippold¹ · Lichao Fan² · Maxim Dorodnikov³ · Bo Liang⁴ · Klaus Dittert⁵ · Haitao Wang⁵

Received: 18 January 2024 / Accepted: 10 June 2024 / Published online: 24 June 2024
© The Author(s) 2024

Abstract

As one of the most important forage species in Europe, white clover (*Trifolium repens*) is a legume that is well recognized for its potential to increase productivity especially under reduced N input. It is hypothesized that legumes have the potential to decrease overwinter soil greenhouse gas (GHG) emissions due to more efficient N recycling as compared to non-legume forbs. We conducted a field experiment recording high-resolution soil nitrous oxide (N₂O) and methane (CH₄) fluxes during the winter months (December 2019 to March 2020) on a five-year-old grassland in central Germany with white clover, fertilized and unfertilized perennial ryegrass (*Lolium perenne*), and bare soil. White clover and fertilized ryegrass stimulated soil N₂O emissions by 174% and 212% as compared to bare soil, and by 36% and 56% as compared to unfertilized ryegrass, respectively, due to their greater N availability and higher water-filled pore space (WFPS). The estimated cumulative CH₄ fluxes under white clover were a net CH₄ sink, whereas ryegrass and bare soil were net CH₄ sources. Soil N₂O fluxes were predominantly regulated by both mineral N and WFPS, while CH₄ fluxes were mainly explained by WFPS. N-fertilization during the growing season did not affect off-season N₂O and CH₄ fluxes in perennial ryegrass plots. The combined non-CO₂ global warming potential highlighted the possible mitigation effect of white clover on overwinter GHG emissions. Our findings suggest that GHG emissions from legumes are not offsetting their productive benefits during the non-frozen winter seasons.

Keywords N₂O emission · CH₄ emission · Freeze–thaw · Legume · Nitrogen fertilization

1 Introduction

Grasslands occupy approximately 40% of the Earth's terrestrial surface (excluding Antarctica and Greenland; Suttie et al. 2005) and play an important role in providing essential ecosystem services, such as forage production for ruminant livestock (Boval and Dixon 2012), high biological diversity (Habel et al. 2013), water purification and retention, erosion reduction, carbon (C) sequestration as well as landscape and aesthetic values (Isselstein and Kayser 2014; Schirpke et al. 2017). In temperate Europe, managed grasslands are one of the most important land use practices, providing consumed feed sources for efficient ruminant meat and dairy products, and thus are increasingly gaining attention for their benefits to society (Kizeková et al. 2018; Nölke et al. 2021; Schils et al. 2022).

Nitrous oxide (N₂O) and methane (CH₄) are the two most important greenhouse gases (GHGs) after carbon dioxide (CO₂) and are the main contributors to stratospheric ozone

✉ Haitao Wang
haitao.wang@agr.uni-goettingen.de

¹ Geo-Biosphere Interactions, Department of Geosciences, University of Tuebingen, 72076 Tuebingen, Germany

² College of Natural Resources and Environment, Northwest A&F University, Yangling 712100, China

³ Institute of Landscape Ecology, University of Muenster, 48149 Muenster, Germany

⁴ Soil and Water Conservation Bureau of Tibet Autonomous Region, Lhasa 850000, China

⁵ Department of Crop Science, Division of Plant Nutrition and Crop Physiology, University of Goettingen, Carl-Sprengel-Weg 1, 37075 Goettingen, Germany

depletion (Jackson et al. 2019; Ravishankara et al. 2009), with global warming potentials (GWPs) that are respectively 265 and 28 times greater than CO₂ on a 100-year time scale (IPCC 2014). The global atmospheric concentrations of N₂O and CH₄ have increased steadily at rates of ~0.91 and ~6.33 ppb yr⁻¹ over the last 20 years, respectively (Lan et al. 2022). Globally, managed grasslands represent huge potential for mitigating GHG emissions and have long been recognized for nitrogen (N) and carbon (C) storage (Wiesmeier et al. 2013). Nevertheless, grassland soils can act as sources or sinks for atmospheric N₂O and CH₄, depending on changes in environmental factors and management practices (Chang et al. 2021; Dijkstra et al. 2013).

Numerous studies have addressed the responses of N₂O and CH₄ fluxes to the changes in management intensification, land use change, and N deposition in European temperate grasslands (Drewer et al. 2017; Feigenwinter et al. 2023; Garnier et al. 2019; Ma et al. 2021; Reinsch et al. 2020). Abagandura et al. (2020) reported that intercropping Kura clover (*Trifolium ambiguum* M. Bieb) with prairie cordgrass (*Spartina pectinata* link.) not only increased biomass yield but also reduced cumulative N₂O emissions and net global GWP compared with the prairie cordgrass monocultures. Most of the previous studies were conducted in controlled laboratory experiments or only during the growing seasons, but it is well documented that excluding the N₂O emissions from the non-growing season would result in a reduction of N₂O emission factors by up to 30% in agroecosystems globally (Shang et al. 2020), especially during freeze–thaw cycles in winter and early spring (Li et al. 2021; Luo et al. 2013). Furthermore, large episodic N₂O emissions emitted from thawing grassland soils have been observed (Byers et al. 2021; Wolf et al. 2010). Wang et al. (2022) reported that CH₄ uptake from a cold alpine meadow was increased under warming and the effect was stronger during unfrozen winter. Therefore, with the background of increased global warming during winter seasons, a better understanding of the responses of N₂O and CH₄ fluxes of grasslands will provide important implications for their management as well as for estimating GHG budgets.

N₂O fluxes from soil are the result of a combination of microbial nitrification and denitrification, with N₂O being produced as a by-product of nitrification and a gaseous intermediate product, evolved during the reaction processes of denitrification (Butterbach-Bahl et al. 2013). Higher microbial biomass C (MBC) and N (MBN) usually increase soil N₂O emissions in terrestrial ecosystems via nitrification (Li et al. 2020, 2022). Whether soil acts as a source or sink of CH₄ depends on the balance between the production of CH₄ by methanogenic microorganisms under anaerobic conditions and oxidation by methanotrophic microorganisms predominately under well-aerated conditions (Le Mer

and Roger 2001). Soil N₂O and CH₄ fluxes are therefore strongly regulated by environmental parameters that interact with soil water content (Dijkstra et al. 2013). In grassland systems, CH₄ fluxes may be closely associated with plant species which can directly regulate soil water content (Fischer et al. 2019), especially during unfrozen winter, as methanotrophs are highly sensitive to temperature (Conrad 2007). Moreover, peak CH₄ emissions also occurred during the thaw period, but the magnitude was controlled by soil moisture (Chen et al. 2021; Tagesson et al. 2012). Anaerobic oxidation of CH₄ has been recently reported in terrestrial ecosystems (Fan et al. 2022), however its occurrence and relevance in grassland needs further systematic approval. An alternative to N input via fertilization are legume-based grasslands, which provide high-quality forage and decrease production costs by reducing mineral fertilizer demand (Lüscher et al. 2014; Rasmussen et al. 2012). Thus, introducing legumes (e.g., white clover, *Trifolium repens*) into grasslands may offer the potential to mitigate soil N₂O emissions with no reduction in productivity (Barneze et al. 2020; Fuchs et al. 2020). However, knowledge on the different effects of legume and non-leguminous grassland species on soil N₂O and CH₄ fluxes specifically during winter seasons is still highly uncertain.

In this study, our objectives were to explore soil N₂O and CH₄ flux dynamics from grasslands of perennial ryegrass (*Lolium perenne*) and white clover throughout the winter period and to identify the relationships between N₂O and CH₄ fluxes with soil properties. We established a field study on a five-year-old experiment site in central Germany, with white clover grown as an unfertilized pure stand, N-fertilized and unfertilized perennial ryegrass as the non-leguminous pure stands, and bare soil treatment as no-vegetation reference. Related to these objectives, we hypothesized that (1) white clover and fertilized perennial ryegrass would have comparable soil N₂O emissions, but larger than the unfertilized perennial ryegrass due to higher N availability; (2) soil N₂O and CH₄ fluxes would be mainly regulated by N availability and soil moisture, respectively.

2 Materials and Methods

2.1 Study Site and Experimental Design

This study was conducted at the experimental station of the Georg-August-University Göttingen in Deppoldshausen (51°34' N, 9°58' E, 342 m a.s.l.), South Lower Saxony, Germany. The mean annual temperature and mean annual precipitation (during 2015–2019) recorded by the nearby weather stations were 9.5 ± 0.2 °C and 593.2 ± 57.8 mm, respectively (Nölke et al. 2022). The soil is classified as a

calcareous Luvisol according to the World Reference Base for Soil Resources (WRB 2014) with a silty-clay soil texture (55% clay, 2% sand, and 43% silt in the top 30 cm). The general soil characteristics determined by our previous studies at the same site were as follows: pH (7.4), total N (1.6 g kg^{-1}), total phosphorus (P) (38 mg kg^{-1}), and total potassium (K) (133 mg kg^{-1}) (Nölke et al. 2022).

Prior to the start of the experiment site in 2014, the land was cultivated by conventional farming with crop rotation of winter oilseed rape, winter wheat, and winter barley. In summer 2014, stands were established in a randomized complete block design with four replicates, each plot had a size of 15 m^2 ($5 \text{ m} \times 3 \text{ m}$). A detailed description of our study site is shown in Nölke et al. (2022). The selected four treatments were: (1) white clover pure stand, (2) perennial ryegrass with and (3) without fertilization, and (4) bare soil. For ryegrass N1, 240 kg N ha^{-1} was applied as NH_4NO_3 in four doses per year (80, 60, 60, and 40 kg N ha^{-1}), after each biomass harvest of perennial ryegrass.

2.2 Soil N_2O and CH_4 Flux Measurement

Soil N_2O and CH_4 fluxes were measured weekly during the winter period from 1 December 2019 to 14 March 2020 using the enclosed static chamber method. In each plot, two weeks before the first sampling day, a transparent chamber with the bottom removed and a resealable airtight lid equipped with a Luer-lock sampling port was inserted approximately 5 cm into the soil. The chambers were made of polyvinyl chloride, with a $22 \times 22 \text{ cm}$ area and 26 cm height above ground, with a total enclosed air volume of 12.6 L. During the gas sampling, plants growing in the plots of white clover and perennial ryegrass were included in the chamber. On each sampling day, gas samples were collected using a 30-mL polypropylene syringe at 1, 16, 31, and 46 min after the closure of the airtight lid. Each of the gas samples (25 mL) was immediately stored in a pre-evacuated Exetainer with septa (Labco Limited, Lampeter, UK). After each sampling, the collected gas samples were transported to the lab and were later analyzed for gas concentrations using a gas chromatograph (GC, Bruker SCION Model 456, Bremen, Germany), equipped with an auto-sampler, an electron capture detector (ECD; for the determination of N_2O), and a flame ionization detector (FID; for the determination of CH_4). Soil N_2O and CH_4 fluxes were calculated from the linear regression of the measured gas concentrations over time (46 min) and adjusted with the meantime measured air temperature and pressure according to the Eq. (1):

$$Flux = \frac{dC}{dt} \times \frac{VPm}{AR(T + 273.2)} \quad (1)$$

where, *Flux* is the N_2O or CH_4 flux ($\mu\text{g N}$ or $\text{C m}^{-1} \text{ h}^{-1}$), dC/dt is the slope from the linear regression analysis, V is the total enclosed air volume of the chamber (m^3), P is the actual atmospheric pressure (KPa), m is the molecular mass of the gas (g mol^{-1}), A is the soil area covered by the chamber (m^2), R is the universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), and T is the actual air temperature ($^{\circ}\text{C}$), respectively.

2.3 Cumulative Fluxes and Global Warming Potential

The cumulative fluxes of soil N_2O and CH_4 from each plot were estimated using trapezoidal interpolation between gas fluxes and sampling day intervals (Wang et al. 2021). We estimated the combined N_2O and CH_4 fluxes to non- CO_2 GWP for each treatment using the CO_2 -equivalents ($\text{CO}_2\text{-eq}$) conversion factors of 265 for N_2O and 28 for CH_4 on a 100-year time scale (IPCC, 2014).

2.4 Soil Samples Analyses

Following gas sampling, soil samples were collected near each chamber (in the top 20 cm) using a hand auger to determine soil water-filled pore space (WFPS), mineral N (NH_4^+ and NO_3^-), and MBC and MBN on the same day. The *in-situ* soil (in the top 5 cm) and air temperatures were measured using a digital thermometer (GMH 1170, Greisinger electronic GmbH, Regenstauf, Germany). Daily soil temperature and precipitation data were collected from the climate station at Göttingen (station ID: 1691) of the German Meteorological Service. Air pressure was measured using a pressure gauge (GDH 200–14, Greisinger electronic GmbH, Regenstauf, Germany). In each replicate plot, three random sub-samples ($\sim 50 \text{ g}$ each) were taken, pooled, and mixed thoroughly in the field. Each of the mixed soil samples was divided into three parts and transferred to the laboratory for analysis. The first part ($\sim 30 \text{ g}$) was oven-dried at 105°C for one day to determine the gravimetric moisture content, which was calculated for WFPS using a theoretical particle density of 2.65 g cm^{-3} for the mineral soil (Shao et al. 2023). The second part (20 g) was put into pre-prepared bottles containing 60 mL 0.05 M K_2SO_4 for mineral N extraction; the extraction bottles were shaken for 1 h and then filtered through 0.05 M K_2SO_4 pre-washed filter papers and then immediately frozen until analysis (Zang et al. 2016). The extractable NH_4^+ and NO_3^- were analyzed using a San++ continuous flow analyzer (Skalar Analytical, the Netherlands). The third part was used for the determination of soil MBC and MBN, for which about 10 g of mixed soil sample was put into pre-prepared 50 mL glass bottles and fumigated with ethanol-free chloroform for 24 h, followed by extraction with 40 mL 0.05 M K_2SO_4 . The

dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) concentrations were measured with a TOC/TIC analyzer (Multi C/N 2100, Analytik Jena, Germany). Then, MBC and MBN were calculated as the difference in the DOC and TDON between the paired fumigated and unfumigated soils, and divided by the correction factors of 0.45 for MBC and 0.54 for MBN, respectively (Brookes et al. 1985).

2.5 Statistical Analysis

The measured soil N_2O and CH_4 fluxes and soil controlling factors (temperature, WFPS, mineral NH_4^+ and NO_3^- concentrations, MBC, and MBN) were tested for normality of distribution using the Shapiro-Wilk test and homogeneity of variance using Levene's test. Non-normal distributed data were log or square root transformed (adding a constant value if negative values occurred, e.g., soil CH_4 fluxes). Linear mixed-effects (LME) models (Crawley, 2007) were used to test the differences in soil N_2O and CH_4 fluxes and soil controlling factors among treatments. In LME models, treatment was considered as the fixed effect and sampling days and replicate plots were random effects. The significant differences were evaluated using the analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test for multiple comparisons at $P < 0.05$. The cumulative fluxes and non- CO_2 GWP were not tested for statistical differences among treatments due to the calculation method (trapezoidal extrapolation). The relationships between soil N_2O and CH_4 fluxes and controlling factors (i.e., temperature, WFPS, mineral NH_4^+ and NO_3^-) were determined by the Spearman's rank correlation test, using the average of four replicates on each sampling day over the measurement period, statistical significances were considered at $P < 0.05$. All statistical analyses were performed using R version 3.6.2 (R Core Team, 2019).

3 Results

3.1 Soil N_2O and CH_4 Fluxes During Winter Season

Bare soil acted as a sink of N_2O (ranging from -0.61 to $-0.02 \mu\text{g N m}^{-2} \text{h}^{-1}$; Fig. 1a and d) when WFPS was below 50% or above 79% (Fig. 1d), whereas soils from the three types of grassland were generally N_2O sources (1.1 – $8.11 \mu\text{g N m}^{-2} \text{h}^{-1}$; Fig. 1a and d). Over the measurement period, fertilized ryegrass and white clover had higher soil N_2O emissions than bare soil ($P < 0.01$; Fig. 2a); N_2O emissions from ryegrass without fertilization did not differ significantly from the other treatments ($P > 0.12$; Fig. 2a).

Soil CH_4 fluxes did not show clear temporal patterns in December 2019 and January 2020, whereas in February and

March 2020, significant temporal variations were observed among treatments ($P < 0.05$; Fig. 1b). Over the measurement period, there were no significant statistical differences in soil CH_4 fluxes among treatments ($P > 0.05$; Fig. 2b). Bare soil and ryegrass without fertilization were net CH_4 sources (with mean values were 2.3 and $1.9 \mu\text{g C m}^{-2} \text{h}^{-1}$, respectively; Fig. 2b), whereas soils from white clover and fertilized ryegrass were net CH_4 sinks (with mean values were -3.81 and $-0.23 \mu\text{g C m}^{-2} \text{h}^{-1}$, respectively; Fig. 2b).

3.2 Soil Parameters and Their Relationships with N_2O and CH_4 Fluxes

The daily 5-cm soil temperatures were all above zero and ranged from 0.3 to 8.5°C (Fig. 1c and Fig. S1). Over the measurement period, the mean soil temperature did not differ among the treatments ($P = 0.84$; Fig. 3a). The WFPS ranged from 54 to 76%, 57–91%, 42–91%, and 52–84% in the bare soil, white clover, ryegrass with and without fertilization plots, respectively (Fig. 1d). The mean WFPS from white clover was the highest, followed by fertilized and unfertilized ryegrass, and bare soil ($P < 0.01$; Fig. 3b).

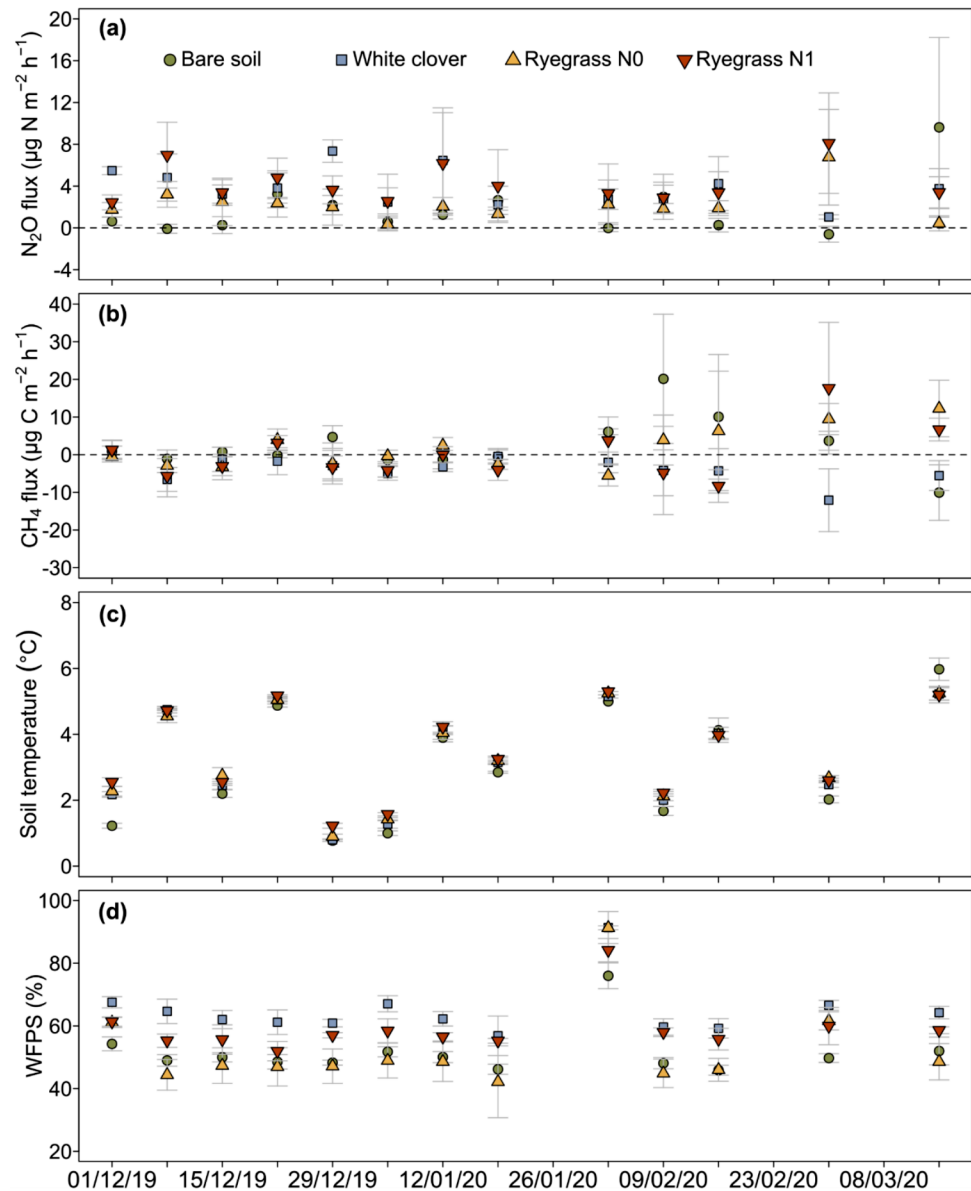
Fertilized ryegrass had highest soil NH_4^+ contents of all other treatments ($P < 0.03$; Fig. 3c). White clover and fertilized ryegrass had higher NO_3^- contents than unfertilized ryegrass ($P < 0.01$; Fig. 3d), and had higher MBC and MBN contents than bare soil ($P < 0.01$; Fig. 3e and f). Unfertilized ryegrass had similar NH_4^+ , MBC, and MBN contents as white clover ($P > 0.63$; Fig. 3c, e, and f).

Across treatments, soil N_2O fluxes showed a weak positive relationship with soil temperature and NH_4^+ (Fig. 4a and c), whereas they correlated significantly with soil WFPS and NO_3^- (Fig. 4b and d). Soil CH_4 fluxes correlated negatively with WFPS (Fig. 4f), whereas there were no significant relationships detected between soil CH_4 fluxes and any other abiotic environmental factors (Fig. 4e, g, and h).

3.3 Area-Scaled Gas Fluxes and Non- CO_2 Global Warming Potential

During the winter season, cumulative N_2O emissions indicated soils from all plots acted as N_2O sources, with values ranged from 13.9 to $43.5 \text{ kg CO}_2\text{-eq ha}^{-1}$. Specifically, fertilized ryegrass had the largest cumulative N_2O emissions, which were 56%, 14%, and 212% larger than unfertilized ryegrass, white clover, and bare soil, respectively (Fig. 5a). Cumulative CH_4 fluxes varied from -4.2 to $2.3 \text{ kg CO}_2\text{-eq ha}^{-1}$, and soil under white clover acted as a net CH_4 sink, whereas soil from the other treatments were all net CH_4 sources (Fig. 5b). The non- CO_2 GWP across the study period ranged from 16.2 to $44.2 \text{ kg CO}_2\text{-eq ha}^{-1}$. The fertilized

Fig. 1 Dynamics of mean ($\pm$ SE, $n=4$) soil N_2O (a) and CH_4 (b) fluxes, temperature in the top 5 cm (c), and water-filled pore space (WFPS; d) in the top 20 cm from bare soil, white clover, unfertilized (ryegrass N0) and fertilized (ryegrass N1) perennial ryegrass



ryegrass had the largest non- CO_2 GWP, followed by white clover, unfertilized ryegrass, and bare soil (Fig. 5c).

4 Discussion

Our results show that soil from all plots were net sources for N_2O across the winter period. The mean and cumulative N_2O fluxes in bare soil were relatively small, where both N_2O production and consumption occurred. Many earlier studies reported that overwinter N_2O emissions from managed grasslands contributed a large portion to the annual GHG budget (Byers et al. 2021; Li et al. 2021; Wolf et al. 2010), which resulted from the short-lived peak of N_2O fluxes during freeze–thaw cycles. However, we did not

detect any N_2O pulse emissions in any of our plots, probably due to the above-zero soil temperature limiting freeze–thaw events during the measurement period (Tang et al. 2022). This was in line with a previous study in an alpine grassland that observed no stimulated N_2O fluxes, not even during spring thaw, and found average winter emissions accounted for only 16.7% of the annual N_2O budget (Li et al. 2012). Contrary to our expectation, soil N_2O emissions from unfertilized perennial ryegrass were comparable with those from white clover and fertilized perennial ryegrass. Similar findings were observed by Barneze et al. (2020) in the UK, with no significant differences in soil N_2O emissions among legume pure stand, fertilized and unfertilized grass monoculture. In contrast, Byers et al. (2021) reported that red clover in pure stand had significantly higher soil

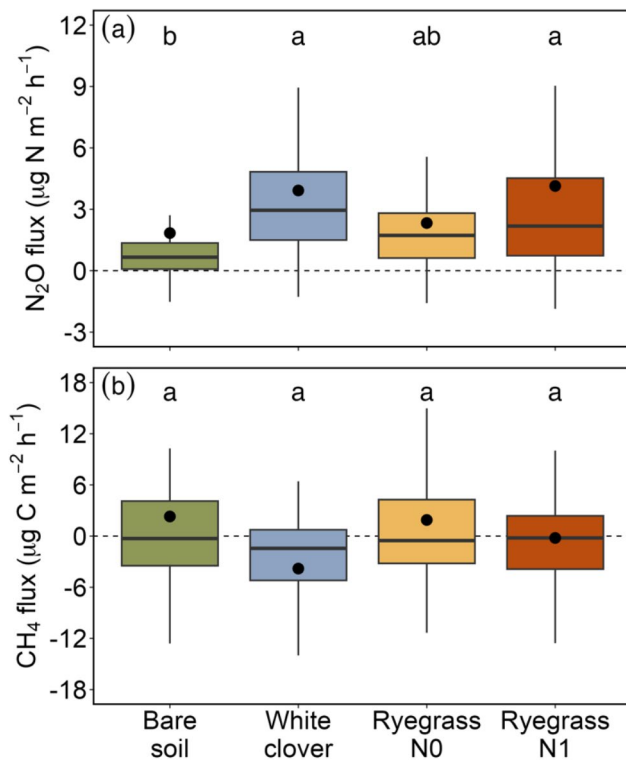


Fig. 2 Season-mean soil N₂O (a) and CH₄ (b) fluxes from bare soil, white clover, unfertilized (ryegrass N0) and fertilized (ryegrass N1) perennial ryegrass. Lowercase letters above the error bars indicate significant differences among treatments (linear mixed-effects model with Fisher's LSD test at $P < 0.05$). The black points represent mean values ($\pm$ SE, $n = 52$) over the measurement period

N₂O emissions than grass-only plots in southern Norway. Below we provide several reasons explaining why white clover's N₂O emissions may not have exceeded that of the grasses in our study.

(1) Across plots, the significant positive relationships between soil N₂O fluxes with NH₄⁺ and NO₃⁻ concentrations confirm that N availability strongly regulates N₂O emissions during unfrozen winter seasons. Although there was no significant difference in soil N₂O emissions from fertilized and unfertilized perennial ryegrass, similar trends in mineral N and N₂O emissions were observed in the fertilized plots. Similar to our findings, Röver et al. (1998) reported that N₂O emissions did not differ significantly between fertilized and unfertilized arable soils over the winter period, and the source of N₂O may be independent from the N-fertilizer applied during growing seasons. Wang et al. (2020) demonstrated that, beside soil mineral N, the availability of organic C was a more limiting factor for denitrification and N₂O production in unfrozen winter. This was also in line with our results, that the lowest N₂O emissions were recorded on bare soil, which had a much lower organic C

input from root exudation and clearly lower microbial biomass than other five-year grass species.

(2) Although soil N₂O fluxes were positively related to WFPS, nitrification activities would be limited under wetter conditions. When the WFPS was above 60%, the dominant source of N₂O emissions is linked to denitrification, and with further increases of WFPS, more N₂O is reduced to N₂ (Davidson et al. 2000). During our measurement period, most of the WFPS values ranged from 40 to 65%, except for one measurement conducted immediately after rainfall in February 2020, indicating that nitrification might contribute to most N₂O production. The unfertilized white clover had the same level of soil N₂O emissions as the fertilized perennial ryegrass, but exceeded that of bare soil, which might be explained by the following two aspects. First, compared with perennial ryegrass, white clover has a taproot and less extensive root system, which makes it possible that less water is required for above- and below-ground biomass production (Karsten and MacAdam 2001). In our study, white clover plots had the highest mean WFPS (64%), which was probably at the optimum moisture conditions favorable to both nitrification and denitrification for N₂O production (Rafique et al. 2011), and thus had higher N₂O emissions. Second, the high N₂O emissions under white clover are probably due to the increased soil mineral N (especially NO₃⁻) from legumes, released from fixed N from root decomposition and limited N uptake from soil (Niklaus et al. 2006). These results could be further supported by the higher MBC and MBN contents in white clover and fertilized perennial ryegrass plots than bare soil, which indicate greater microbial activities, that might be involved in nitrification and denitrification processes for N₂O production (Butterbach-Bahl et al. 2013; Li et al. 2020).

We found that only soil from the white clover plots was a net CH₄ sink, while the bare soil and unfertilized perennial ryegrass plots were net sources for CH₄ throughout the measurement period. Although the fertilized perennial ryegrass plots represented a negative mean soil CH₄ flux ($-0.2 \mu\text{g C m}^{-2} \text{h}^{-1}$), the large temporal variation led to a small estimated cumulative CH₄ emission. These findings are consistent with the results by Imer et al. (2013), who reported both positive and negative CH₄ fluxes occurred and showed highly temporal variations in soils from three managed Swiss grasslands, but concluded that all sites were weak CH₄ sinks because the fluxes were dominated by uptake. In this study, negative soil CH₄ fluxes from white clover occurred more frequently during the measurement period, and consequently white clover had the highest soil CH₄ uptake compared with the other treatments, which supported our second hypothesis.

Previous studies have shown that soil CH₄ fluxes could be affected by N availability. As in fertilized soils, the

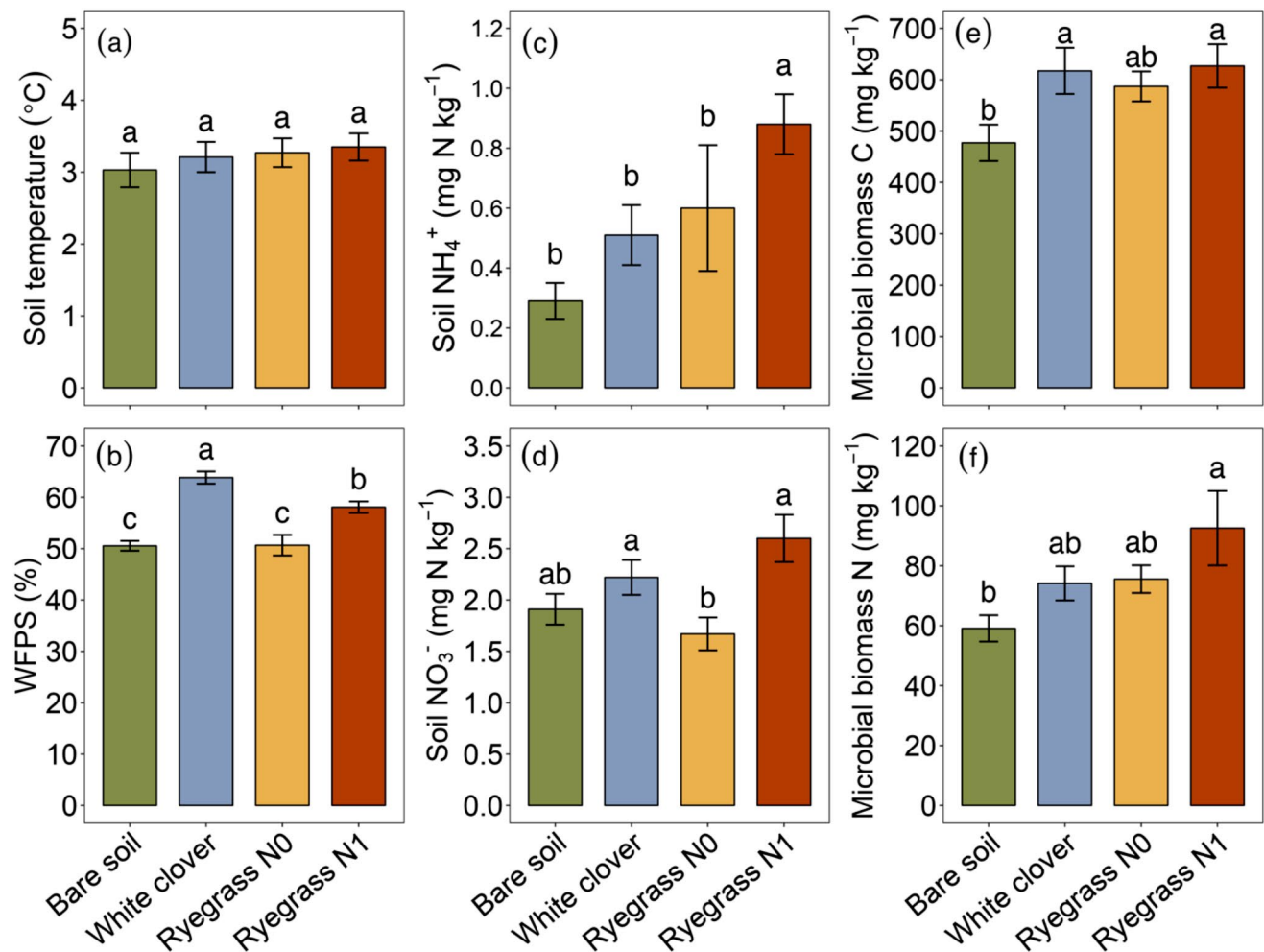


Fig. 3 Season-mean ($\pm$ SE, $n=52$) soil temperature (**a**), water-filled pore space (WFPS; **b**), NH_4^+ (**c**), NO_3^- (**d**), microbial biomass carbon (**e**), and microbial biomass N (**f**) from bare soil, white clover, unfertilized (ryegrass N0) and fertilized (ryegrass N1) perennial ryegrass.

Lowercase letters above the error bars indicate significant differences among treatments (linear mixed-effects model with Fisher's LSD test at $P < 0.05$)

increased NH_4^+ may compete with CH_4 for the same active sites of methane monooxygenase, thus leading to an inhibition of CH_4 oxidation (Steudler et al. 1989). Nonetheless, fertilization-induced high NO_3^- levels can also stimulate CH_4 uptake by limiting methanogenesis, which can be inhibited by toxic intermediates produced from denitrification (Roy and Conrad 1999). However, we did not detect any relationships between soil CH_4 fluxes with NH_4^+ and NO_3^- concentrations, which can possibly be explained by the low and small changes in N availability during the experiment period. Previous studies have shown the positive effect of soil temperature on soil CH_4 uptake (Drewer et al. 2017; Wang et al. 2022), since elevated temperature generally contributes to a lower soil water content as well as increased methanotrophic activity, and thus stimulates CH_4 consumption in soils (Dobbie and Smith 1996). There was no significant relationship between soil temperature

and CH_4 fluxes. Given the small temporal variation in soil temperature, and the fact that all treatment plots were established at the same site, current technology may not be able to detect the effect of soil temperature on CH_4 emissions. It should be underlined that soil CH_4 fluxes were negatively related to WFPS, while it was generally reported that CH_4 uptake decreased with increasing soil water content (Liu et al. 2017; van Delden et al. 2018). Other previous studies demonstrate that soil CH_4 uptake was parabolically correlated with soil moisture, that the activity of methanotrophs was inhibited under dry condition, and high soil moisture limited air diffusion into the soil (Borken et al. 2006; Dijkstra et al. 2013). Across the measurement period, the white clover plots had the highest WFPS and also the largest CH_4 uptake, which may indicate that methanotrophic activity was relatively high and soil moisture in our plots was still under the optimum soil water content for CH_4 oxidation in

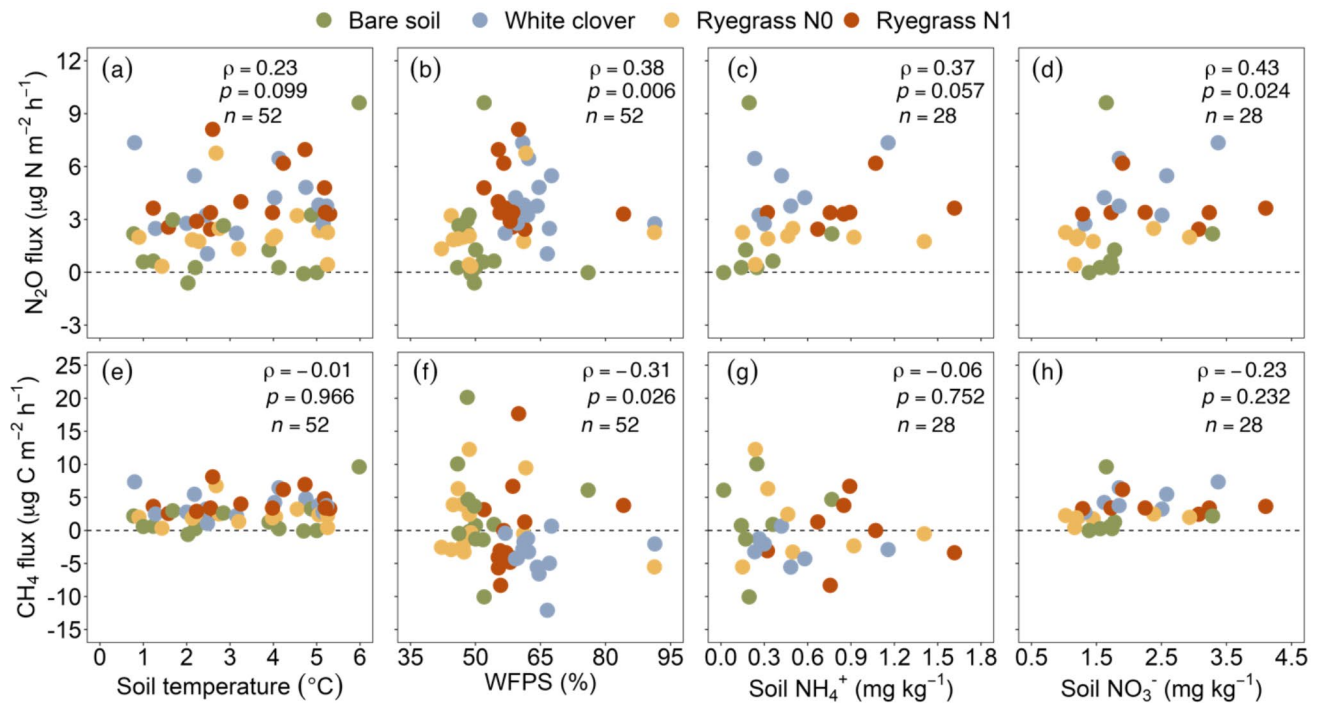


Fig. 4 Spearman rank correlations of soil N₂O and CH₄ fluxes with soil temperature (a, e), water-filled pore space (WFPS; b, f), mineral NH₄⁺ (c, g), and NO₃⁻ (d, h). Each data point represents a measurement value from each sampling day, and from bare soil, white clover, unfer-

tilized (ryegrass N0) and fertilized (ryegrass N1) perennial ryegrass. ρ (rho) indicates spearman's rank correlation coefficient, statistical significances were considered at $P < 0.05$

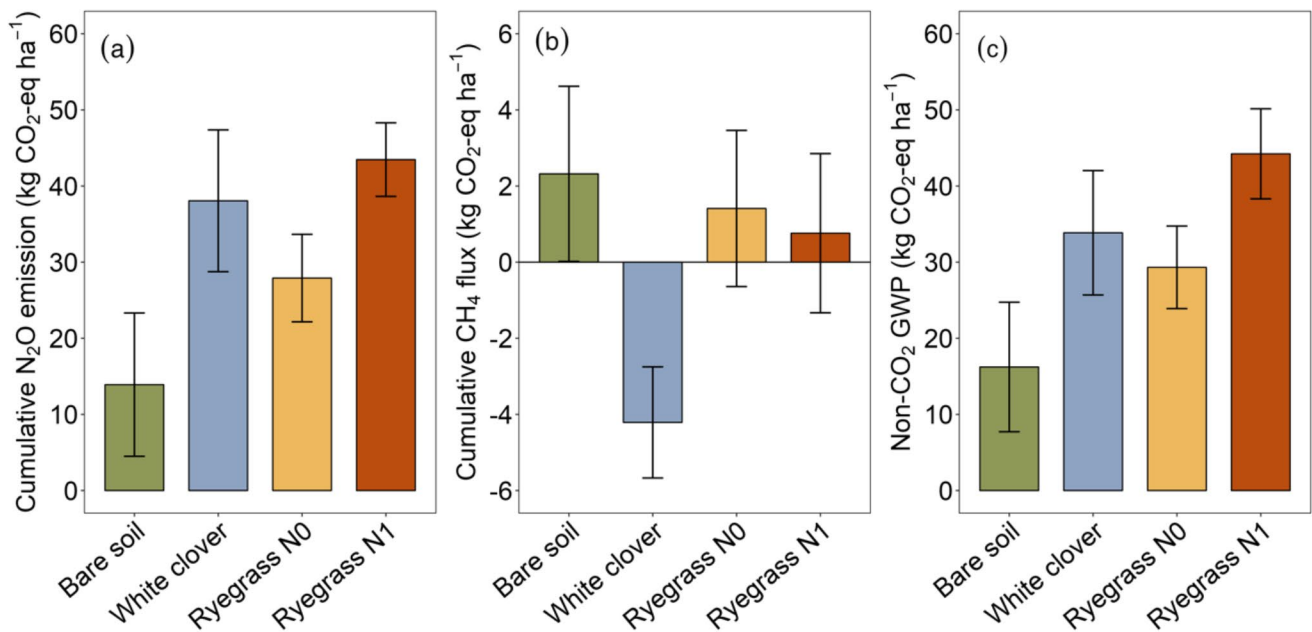


Fig. 5 Estimated areal mean (± SE, n=4) cumulative soil N₂O emissions (a), CH₄ fluxes (b), and combined N₂O and CH₄ global warming potential (non-CO₂ GWP; c) within bare soil, white clover, unfertilized

(ryegrass N0) and fertilized (ryegrass N1) perennial ryegrass. Cumulative soil N₂O emissions and CH₄ fluxes were not tested statistically since these values were calculated using the trapezoidal interpolation

the cold environment (Luo et al. 2013; Wang et al. 2022). Overall, our results suggest that the root system of white clover was able to maintain greater soil water than perennial ryegrass, which probably caused an enhancement of soil CH₄ uptake during unfrozen winter seasons. It may also suggest that the extension of white clover in rotation or as an intercrop would have a positive effect on the reduction of atmospheric CH₄, at least on a regional scale.

5 Conclusions

We investigated soil N₂O and CH₄ fluxes from three grassland types and bare soil over the unfrozen winter period on an established five-year-old grassland. Our results suggest that N₂O emissions can be maintained at low levels in both legumes and non-legumes during unfrozen winters, mainly due to limitation of mineral N, whereas methane is driven more by soil water-filled pore space, such results suggest that more developed root systems and water-holding capacity may be more advantageous for maintaining soil as a CH₄ sink under a comparable climate. Given that anthropogenic climate change (e.g., winter warming) is becoming more frequent globally, our results suggest that more attention should be paid to soil greenhouse gas emissions in grassland systems during wet and non-freezing winter seasons.

Acknowledgements We thank Susanne Koch, Simone Urstadt, Marlies Niebuhr, Ulrike Kierbaum, and Karin Schmidt for diligent and skilful assistance.

Funding This study was supported by the German Bundesministerium für Bildung und Forschung as part of the IMPAC³ project [grant number FKZ 031A351A], Program for the Promotion of Junior Researchers of the University of Tuebingen (PRO-SHAO-2022-17). Guodong-Shao was supported by China Scholarship Council. Open Access funding enabled and organized by Projekt DEAL.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Abagandura GO, Sekaran U, Singh S, Singh J, Ibrahim MA, Subramanian S, Owens VN, Kumar S (2020) Intercropping kura clover with prairie cordgrass mitigates soil greenhouse gas fluxes. *Sci Rep* 10:1–11. <https://doi.org/10.1038/s41598-020-64182-2>
- Barneze AS, Whitaker J, McNamara NP, Ostle NJ (2020) Legumes increase grassland productivity with no effect on nitrous oxide emissions. *Plant Soil* 446:163–177. <https://doi.org/10.1007/s11104-019-04338-w>
- Borken W, Davidson EA, Savage K, Sundquist ET, Steudler P (2006) Effect of summer throughfall exclusion, summer drought, and winter snow cover on methane fluxes in a temperate forest soil. *Soil Biol Biochem* 38:1388–1395. <https://doi.org/10.1016/j.soilbio.2005.10.011>
- Boval M, Dixon RM (2012) The importance of grasslands for animal production and other functions: a review on management and methodological progress in the tropics. *Animal* 6:748–762. <https://doi.org/10.1017/S1751731112000304>
- Brookes PC, Landman A, Pruden G, Jenkinson DS (1985) Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. *Soil Biol Biochem* 17:837–842. [https://doi.org/10.1016/0038-0717\(85\)90144-0](https://doi.org/10.1016/0038-0717(85)90144-0)
- Butterbach-Bahl K, Baggs EM, Dannenmann M, Kiese R, Zechmeister-Boltenstern S (2013) Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Philos Trans R Soc B Biol Sci* 368. <https://doi.org/10.1098/rstb.2013.0122>
- Byers E, Bleken MA, Dörsch P (2021) Winter n₂o accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil Ph. *Environ Res Commun* 3. <https://doi.org/10.1088/2515-7620/abd623>
- Chang J, Ciais P, Gasser T et al (2021) Climate warming from managed grasslands cancels the cooling effect of carbon sinks in sparsely grazed and natural grasslands. *Nat Commun* 12:1–10. <https://doi.org/10.1038/s41467-020-20406-7>
- Chen H, Liu X, Xue D, Zhu D, Zhan W, Li W, Wu N, Yang G (2021) Methane emissions during different freezing-thawing periods from a Fen on the Qinghai-Tibetan Plateau: four years of measurements. *Agric Meteorol* 297:108279. <https://doi.org/10.1016/j.agrformet.2020.108279>
- Conrad R (2007) Microbial Ecology of Methanogens and Methanotrophs. *Advances in Agronomy*. Academic, pp 1–63
- Contribution of Working Group III to the Fifth Assessment (2014) In: Pichs-Madruga OR, Sokona Y, Farahani E, Kadner S, Seyboth K, Adler A, Baum I, Brunner S, Eickemeier P, Kriemann B, Savolainen J, Schlömer S, von Stechow C, Zwickel T, Minx JC (eds) *Climate Change 2014: mitigation of Climate Change*. Report of the Intergovernmental Panel on Climate Change [Edenhofer. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA
- Davidson EA, Keller M, Erickson HE, Verchot LV, Veldkamp E (2000) Testing a conceptual model of soil emissions of nitrous and nitric oxides. *Bioscience* 50:667–680. [https://doi.org/10.1641/0006-3568\(2000\)050\[0667:TACMOS\]2.0.CO;2](https://doi.org/10.1641/0006-3568(2000)050[0667:TACMOS]2.0.CO;2)
- Dijkstra FA, Morgan JA, Follett RF, Lecain DR (2013) Climate change reduces the net sink of CH₄ and N₂O in a semiarid grassland. *Glob Chang Biol* 19:1816–1826. <https://doi.org/10.1111/gcb.12182>
- Dobbie KE, Smith KA (1996) Comparison of CH₄ oxidation rates in woodland, arable and set aside soils. *Soil Biol Biochem* 28:1357–1365. [https://doi.org/10.1016/S0038-0717\(96\)00152-6](https://doi.org/10.1016/S0038-0717(96)00152-6)
- Drewer J, Anderson M, Levy PE, Scholtes B, Helfter C, Parker J, Rees RM, Skiba UM (2017) The impact of ploughing

- intensively managed temperate grasslands on N₂O, CH₄ and CO₂ fluxes. *Plant Soil* 411:193–208. <https://doi.org/10.1007/s11104-016-3023-x>
- Fan L, Dippold MA, Thiel V, Ge T, Wu J, Kuzyakov Y, Dorodnikov M (2022) Temperature sensitivity of anaerobic methane oxidation versus methanogenesis in paddy soil: implications for the CH₄ balance under global warming. *Glob Chang Biol* 28:654–664. <https://doi.org/10.1111/gcb.15935>
- Feigenwinter I, Hörtnagl L, Buchmann N (2023) N₂O and CH₄ fluxes from intensively managed grassland: the importance of biological and environmental drivers vs. management. *Sci Total Environ* 903:166389. <https://doi.org/10.1016/j.scitotenv.2023.166389>
- Fischer C, Leimer S, Roscher C et al (2019) Plant species richness and functional groups have different effects on soil water content in a decade-long grassland experiment. *J Ecol* 107:127–141. <https://doi.org/10.1111/1365-2745.13046>
- Fuchs K, Merbold L, Buchmann N et al (2020) Evaluating the potential of legumes to mitigate N₂O emissions from Permanent Grassland using process-based models. <https://doi.org/10.1029/2020GB006561>. *Global Biogeochem Cycles* 34:
- Garnier J, Le Noë J, Marescaux A, Sanz-Cobena A, Lassaletta L, Silvestre M, Thieu V, Billen G (2019) Long-term changes in greenhouse gas emissions from French agriculture and livestock (1852–2014): from traditional agriculture to conventional intensive systems. *Sci Total Environ* 660:1486–1501. <https://doi.org/10.1016/j.scitotenv.2019.01.048>
- Habel JC, Dengler J, Janišová M, Török P, Wellstein C, Wiczik M (2013) European grassland ecosystems: threatened hotspots of biodiversity. *Biodivers Conserv* 22:2131–2138. <https://doi.org/10.1007/s10531-013-0537-x>
- Imer D, Merbold L, Eugster W, Buchmann N (2013) Temporal and spatial variations of soil CO₂, CH₄ and N₂O fluxes at three differently managed grasslands. *Biogeosciences* 10:5931–5945. <https://doi.org/10.5194/bg-10-5931-2013>
- Isselstein J, Kayser M (2014) Functions of grassland and their potential in delivering ecosystem services. *IBERS, Aberystwyth University, Aberystwyth*, pp 199–214
- Jackson RB, Solomon EI, Canadell JG, Cargnello M, Field CB (2019) Methane removal and atmospheric restoration. *Nat Sustain* 2:436–438. <https://doi.org/10.1038/s41893-019-0299-x>
- Karsten HD, MacAdam JW (2001) Effect of Drought on Growth, Carbohydrates, and Soil Water Use by Perennial Ryegrass, Tall Fescue, and White Clover. *Crop Sci* 41:156–166. <https://doi.org/10.2135/cropsci2001.411156x>
- Kizeková M, Hopkins A, Kanišanská R, Makovníková J, Pollák, Pálka B (2018) Changes in the area of permanent grassland and its implications for the provision of bioenergy: Slovakia as a case study. *Grass Forage Sci* 73:218–232. <https://doi.org/10.1111/gfs.12333>
- Lan X, Thoning KW, Dlugokencky EJ (2022) Trends in globally-averaged CH₄, N₂O, and SF₆ determined from NOAA Global Monitoring Laboratory measurements. <https://doi.org/10.15138/P8XG-AA10>. Version 2023-03
- Le Mer J, Roger P (2001) Production, oxidation, emission and consumption of methane by soils: a review. *Eur J Soil Biol* 37:25–50. [https://doi.org/10.1016/S1164-5563\(01\)01067-6](https://doi.org/10.1016/S1164-5563(01)01067-6)
- Li K, Gong Y, Song Y, Lv J, Chang Y, Hu Y, Tian C, Christie P, Liu X (2012) No significant nitrous oxide emissions during spring thaw under grazing and nitrogen addition in an alpine grassland. *Glob Chang Biol* 18:2546–2554. <https://doi.org/10.1111/j.1365-2486.2012.02704.x>
- Li Z, Zeng Z, Tian D, Wang J, Fu Z, Zhang F, Zhang R, Chen W, Luo Y, Niu S (2020) Global patterns and controlling factors of soil nitrification rate. *Glob Chang Biol* 26:4147–4157. <https://doi.org/10.1111/gcb.15119>
- Li J, Zhao Y, Zhang A, Song B, Hill RL (2021) Effect of grazing exclusion on nitrous oxide emissions during freeze-thaw cycles in a typical steppe of Inner Mongolia. *Agric Ecosyst Environ* 307:107217. <https://doi.org/10.1016/j.agee.2020.107217>
- Li Z, Zeng Z, Song Z, Tian D, Huang X, Nie S, Wang J, Jiang L, Luo Y, Cui J, Niu S (2022) Variance and main drivers of field nitrous oxide emissions: a global synthesis. *J Clean Prod* 353:131686. <https://doi.org/10.1016/j.jclepro.2022.131686>
- Liu J, Chen H, Yang X, Gong Y, Zheng X, Fan M, Kuzyakov Y (2017) Annual methane uptake from different land uses in an agro-pastoral ecotone of northern China. *Agric Meteorol* 236:67–77. <https://doi.org/10.1016/j.agrformet.2017.01.012>
- Luo GJ, Kiese R, Wolf B, Butterbach-Bahl K (2013) Effects of soil temperature and moisture on methane uptake and nitrous oxide emissions across three different ecosystem types. *Biogeosciences* 10:3205–3219. <https://doi.org/10.5194/bg-10-3205-2013>
- Lüscher A, Mueller-Harvey I, Soussana JF, Rees RM, Peyraud JL (2014) Potential of legume-based grassland-livestock systems in Europe: a review. *Grass Forage Sci* 69:206–228. <https://doi.org/10.1111/gfs.12124>
- Ma L, Janz B, Kiese R, Mwanake R, Wangari E, Butterbach-Bahl K (2021) Effect of Vole bioturbation on N₂O, NO, NH₃, CH₄ and CO₂ fluxes of slurry fertilized and non-fertilized montane grassland soils in Southern Germany. *Sci Total Environ* 800:149597. <https://doi.org/10.1016/j.scitotenv.2021.149597>
- Niklaus PA, Wardle DA, Tate KR (2006) Effects of plant species diversity and composition on nitrogen cycling and the trace gas balance of soils. *Plant Soil* 282:83–98. <https://doi.org/10.1007/s11104-005-5230-8>
- Nölke I, Komainda M, Tonn B, Feuerstein U, Isselstein J (2021) Including chicory and selecting white clover varieties as strategies to improve temporal stability of forage yield and quality in white-clover-based temporary grassland. *Eur J Agron* 130. <https://doi.org/10.1016/j.eja.2021.126362>
- Nölke I, Tonn B, Komainda M, Heshmati S, Isselstein J (2022) The choice of the white clover population alters overyielding of mixtures with perennial ryegrass and chicory and underlying processes. *Sci Rep* 12:1–11. <https://doi.org/10.1038/s41598-022-05100-6>
- Rafique R, Hennessy D, Kiely G (2011) Nitrous Oxide Emission from Grazed Grassland under different Management systems. *Ecosystems* 14:563–582. <https://doi.org/10.1007/s10021-011-9434-x>
- Rasmussen J, Sørensen K, Pirhofer-Walzl K, Eriksen J (2012) N₂-fixation and residual N effect of four legume species and four companion grass species. *Eur J Agron* 36:66–74. <https://doi.org/10.1016/j.eja.2011.09.003>
- Ravishankara AR, Daniel JS, Portmann RW (2009) Nitrous oxide (N₂O): the dominant ozone-depleting substance emitted in the 21st century. *Sci* (80-) 326:123–125. <https://doi.org/10.1126/science.1176985>
- Reinsch T, Malisch C, Loges R, Taube F (2020) Nitrous oxide emissions from grass-clover swards as influenced by sward age and biological nitrogen fixation. *Grass Forage Sci* 75:372–384. <https://doi.org/10.1111/gfs.12496>
- Röver M, Heinemeyer O, Kaiser E-A (1998) Microbial induced nitrous oxide emissions from an arable soil during winter. *Soil Biol Biochem* 30:1859–1865. [https://doi.org/10.1016/S0038-0717\(98\)00080-7](https://doi.org/10.1016/S0038-0717(98)00080-7)
- Roy R, Conrad R (1999) Effect of methanogenic precursors (acetate, hydrogen, propionate) on the suppression of methane production by nitrate in anoxic rice field soil. *FEMS Microbiol Ecol* 28:49–61. [https://doi.org/10.1016/S0168-6496\(98\)00092-0](https://doi.org/10.1016/S0168-6496(98)00092-0)
- Schils RLM, Bufer C, Rhymer CM et al (2022) Permanent grasslands in Europe: land use change and intensification decrease their multifunctionality. *Agric Ecosyst Environ* 330. <https://doi.org/10.1016/j.agee.2022.107891>

- Schirpke U, Kohler M, Leitinger G, Fontana V, Tasser E, Tappeiner U (2017) Future impacts of changing land-use and climate on ecosystem services of mountain grassland and their resilience. *Ecosyst Serv* 26:79–94. <https://doi.org/10.1016/j.ecoser.2017.06.008>
- Shang Z, Abdalla M, Kuhnert M, Albanito F, Zhou F, Xia L, Smith P (2020) Measurement of N₂O emissions over the whole year is necessary for estimating reliable emission factors. *Environ Pollut* 259:113864. <https://doi.org/10.1016/j.envpol.2019.113864>
- Shao G, Martinson GO, Corre MD, Luo J, Niu D, Bischel X, Veldkamp E (2023) Impacts of monoculture cropland to alley cropping agroforestry conversion on soil N₂O emissions. *GCB Bioenergy* 15:58–71. <https://doi.org/10.1111/gcbb.13007>
- Steudler PA, Bowden RD, Melillo JM, Aber JD (1989) Influence of nitrogen fertilization on methane uptake in temperate forest soils. *Nature* 341:314–316. <https://doi.org/10.1038/341314a0>
- Suttie M, Reynolds SG, Batello C (2005) Grasslands of the World. Food and Agriculture Organization of the United Nations, Rome
- Tagesson T, Mölder M, Mastepanov M, Sigsgaard C, Tamstorf MP, Lund M, Falk JM, Lindroth A, Christensen TR, Ström L (2012) Land-atmosphere exchange of methane from soil thawing to soil freezing in a high-Arctic wet tundra ecosystem. *Glob Chang Biol* 18:1928–1940. <https://doi.org/10.1111/j.1365-2486.2012.02647.x>
- Tang S, Yuan P, Tawaraya K, Tokida T, Fukuoka M, Yoshimoto M, Sakai H, Hasegawa T, Xu X, Cheng W (2022) Winter nocturnal warming affects the freeze-thaw frequency, soil aggregate distribution, and the contents and decomposability of C and N in paddy fields. *Sci Total Environ* 802:149870. <https://doi.org/10.1016/j.scitotenv.2021.149870>
- van Delden L, Rowlings DW, Scheer C, De Rosa D, Grace PR (2018) Effect of urbanization on soil methane and nitrous oxide fluxes in subtropical Australia. *Glob Chang Biol* 24:5695–5707. <https://doi.org/10.1111/gcb.14444>
- Wang H, Ma S, Dittert K (2020) Straw amendments did not induce high N₂O emissions in non-frozen wintertime conditions: a study in northern Germany. *Soil Use Manag* 36:693–703. <https://doi.org/10.1111/sum.12643>
- Wang H, Ma S, Shao G, Dittert K (2021) Use of urease and nitrification inhibitors to decrease yield-scaled N₂O emissions from winter wheat and oilseed rape fields: a two-year field experiment. *Agric Ecosyst Environ* 319:107552. <https://doi.org/10.1016/j.agee.2021.107552>
- Wang P, Wang J, Elberling B, Yang L, Chen W, Song L, Yan Y, Wang S, Pan J, He Y, Niu S (2022) Increased annual methane uptake driven by warmer winters in an alpine meadow. *Glob Chang Biol* 28:3246–3259. <https://doi.org/10.1111/gcb.16120>
- Wiesmeier M, Hübner R, Barthold F, Spörlein P, Geuß U, Hangen E, Reischl A, Schilling B, von Lütow M, Kögel-Knabner I (2013) Amount, distribution and driving factors of soil organic carbon and nitrogen in cropland and grassland soils of southeast Germany (Bavaria). *Agric Ecosyst Environ* 176:39–52. <https://doi.org/10.1016/j.agee.2013.05.012>
- Wolf B, Zheng X, Brüggemann N, Chen W, Dannenmann M, Han X, Sutton MA, Wu H, Yao Z, Butterbach-Bahl K (2010) Grazing-induced reduction of natural nitrous oxide release from continental steppe. *Nature* 464:881–884. <https://doi.org/10.1038/nature08931>
- WRB (2014) World Reference Base for Soil Resources 2014 (Update 2015). International Soil Classification System for Naming Soils and Creating Legends for Soil Maps. World Soil Resources. Food and Agriculture Organization, Rome, Italy, p. 203. Reports no. 106
- Zang H, Wang J, Kuzyakov Y (2016) N fertilization decreases soil organic matter decomposition in the rhizosphere. *Appl Soil Ecol* 108:47–53. <https://doi.org/10.1016/j.apsoil.2016.07.021>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.