



Legumes increase grassland productivity with no effect on nitrous oxide emissions

Arlete S. Barneze · Jeanette Whitaker ·
Niall P. McNamara · Nicholas J. Ostle

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Abstract

Aims Grasslands are important agricultural production systems, where ecosystem functioning is affected by land management practices. Grass-legume mixtures are commonly cultivated to increase grassland productivity while reducing the need for nitrogen (N) fertiliser. However, little is known about the effect of this increase in productivity on greenhouse gas (GHG) emissions in grass-legume mixtures. The aim of this study was to investigate interactions between the proportion of legumes in grass-legume mixtures and N-fertiliser addition on productivity and GHG emissions. We tested the hypotheses that an increase in the relative proportion of legumes would increase plant productivity and decrease GHG emissions, and the magnitude of these effects would be reduced by N-fertiliser addition.

Methods This was tested in a controlled environment mesocosm experiment with one grass and one legume species grown in mixtures in different proportions, with or without N-fertiliser. The effects on N cycling

processes were assessed by measurement of above- and below-ground biomass, shoot N uptake, soil physico-chemical properties and GHG emissions.

Results Above-ground productivity and shoot N uptake were greater in legume-grass mixtures compared to grass or legume monocultures, in fertilised and unfertilised soils. However, we found no effect of legume proportion on N₂O emissions, total soil N or mineral-N in fertilised or unfertilised soils.

Conclusions This study shows that the inclusion of legumes in grass-legume mixtures positively affected productivity, however N cycle were in the short-term unaffected and mainly affected by nitrogen fertilisation. Legumes can be used in grassland management strategies to mitigate climate change by reducing crop demand for N-fertilisers.

Keywords Grass-legume mixture · Nitrogen fertiliser · Carbon and nitrogen cycling · Plant productivity · Greenhouse gas emissions

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A. S. Barneze (✉) · N. J. Ostle
Lancaster Environment Centre, Lancaster University,
Lancaster LA1 4YQ, UK
e-mail: arletesb@gmail.com

A. S. Barneze · J. Whitaker · N. P. McNamara
Centre for Ecology & Hydrology, Lancaster Environment Centre,
Library Avenue, Bailrigg, Lancaster LA1 4AP, UK

Introduction

Grasslands provide important ecosystem services worldwide. These include sequestering carbon (C) in plant biomass and soil organic matter, and producing the majority of forage for ruminants (De Deyn et al. 2008). Grassland species composition has been shown to be an important driver of these ecosystems functions (Bardgett 2011; De Deyn et al. 2009; Loreau and Hector 2001; Tilman et al. 2001) due to plant-plant and plant-

soil interactions. The relationship between plant diversity and productivity can affect plant-soil feedbacks, e.g. through the availability of mineral nutrients in the soil. While the effects of grassland species composition has been widely studied in relation to a range of ecosystem services (Niklaus et al. 2006), its influence on greenhouse gas (GHG) emissions is relatively unexplored, especially for nitrous oxide (N_2O) and methane (CH_4) (Abalos et al. 2014; Abalos et al. 2018; Sun et al. 2013) which are important agricultural GHGs with greater warming potential.

Grass-legume mixtures are commonly used for grazing because they offer the potential to increase productivity with a lower requirement for costly and polluting nitrogen (N) fertilisers. Legumes not only provide high quality forage due to their high fibre and protein content (Mortenson et al. 2004), but also promote symbiotic N_2 fixation thereby producing more protein with less N input (Suter et al. 2015). The use of mixtures has been shown to increase plant productivity through increases in resource uptake efficiency due to: i) N transfer from legumes to grasses (Høgh-Jensen and Schjoerring 1997; Pirhofer-Walzl et al. 2012), and/or ii) species differences in root structure increasing the exploitation of soil resources (Mueller et al. 2013; van Ruijven and Berendse 2005). As a consequence, legume-based cropping can reduce N losses from soil via leaching (Drinkwater et al. 1998; Li et al. 2016) and increase C sequestration due to increased plant C inputs (De Deyn et al. 2011).

Increases in the proportion of legumes may also influence plant-soil GHG emissions through alterations to C and N cycling processes, something scarcely addressed in experimental studies. This effect is likely to differ in unfertilised and fertilised soils due to impacts on C and N availability (Bardgett et al. 1999; Oertel et al. 2016; Thomson et al. 2012). In unfertilised soils, legumes will enhance plant productivity via symbiotic N_2 fixation, which may reduce mineral-N availability in the soil and decrease soil N_2O emissions. Alternatively, increases in the proportion of legumes might increase mineral-N in soil, especially NO_3^- , due to the release of fixed N from legume roots via decomposition and the inefficiency of legumes in acquiring soil mineral-N (Niklaus et al. 2006), which could promote soil N_2O emissions. In fertilised and unfertilised soils an additional mechanism for N_2O emission from legumes could be direct N_2O production from the biological N fixation process itself but this is considered a relatively small source of N_2O emissions (Carter and Ambus 2006). In

fertilised soils, legume growth may be suppressed promoting the dominance of grasses (De Deyn et al. 2011; Ledgard 2001; Smith et al. 2008), with effects on N_2O emissions driven by the magnitude of N addition rather than legume proportion itself. Alternatively, increases in legume proportion may reduce plant N uptake (due to increase N_2 fixation), leading to higher mineral-N in the soil (Niklaus et al. 2006). Overall, the effect of increases in legume proportions on soil N_2O emissions will potentially reflect the balance between N uptake by plants and mineral-N availability in the soil. While legumes are well known to contribute to plant productivity, their effect on soil N_2O emissions is less studied.

Introducing legumes into grasslands can increase plant productivity and alter the quality and quantity of plant C and N inputs to the soil (De Deyn et al. 2009; Fornara and Tilman 2008). In fertilised grassland soils, an increase in the proportion of legumes reduced microbial and root respiration and ecosystem respiration, which was associated with increases in C and N accumulation through input of nutrients to the soil (De Deyn et al. 2011). Likewise, in unfertilised soils, the inclusion of legumes in grass-legumes mixtures might promote the reduction of ecosystem respiration due to the increase of above- and below-ground biomass, which showed a positive relationship with C and N storage in the soil (Wu et al. 2017). Given that C and N cycling are closely related, CH_4 fluxes can also be affected by grass-legume mixtures. CH_4 fluxes depend on the balance between the production by methanogenic bacteria and the consumption by methanotrophic bacteria (Chistoserdova et al. 2005; Le Mer and Roger 2001), and grassland soils generally act as a sink of CH_4 . In fertilised soils, the increase of N mineral in the soil, especially NH_4^+ , may alter the production/consumption of CH_4 in the soils, because NH_4^+ competes with CH_4 at enzymatic levels or as a competitive effect of nitrifiers and methanotrophs (Topp and Pattey 1997). In unfertilised soils, this effect is less evident than in fertilised soils as NH_4^+ concentrations will be lower. The effect of grass-legume mixtures will thus depend on intra- and inter-specific plant competition and facilitation, which in turn may affect biological N_2 fixation, community productivity (Kirwan et al. 2007; Nyfeler et al. 2011) and plant-soil GHG emissions.

In this study we investigated how the proportion of legumes in grass-legume mixtures affected plant productivity and C and N cycling in fertilised and unfertilised soils (i.e. inorganic N) without necessarily

accounting for N-fixation. The following specific hypotheses were tested: H1. In unfertilised soil, an increasing proportion of legumes in grass-legume mixtures will i) increase plant productivity and ecosystem respiration, and ii) promote plant N uptake, reducing N₂O emissions as a result of lower soil N availability. H2. In fertilised soil, N addition will iii) reduce legume productivity whilst favouring grass productivity, and reduce ecosystem respiration, and iv) with N addition causing greater N₂O emissions in all legume-grass combinations.

To test these hypotheses a factorial mesocosm experiment was conducted under controlled environment conditions with one grass and one legume species grown in mixtures with different proportions, with or without N-fertiliser. The effects of grass-legume mixtures and inorganic N addition on plant-soil C and N cycling processes were then assessed through the measurement of above- and below-ground plant productivity, soil nutrient availability and GHG emissions.

Material and methods

Experimental design

To quantify the interactive effects of N fertiliser and grass-legume mixtures on C and N cycling, a fast-growing legume species, *Trifolium pratense* L. (Tr), and a grass species, *Agrostis capillaris* L., were selected (Emorsgate Seeds, Kings Lynn, UK), both of which commonly occur in managed temperate grasslands across Europe. These plant species are known to differ in their functional traits including average root diameter, specific root length, root dry matter content, leaf N concentration, and leaf dry matter content (Table S1). Five grass-legume mixtures with 0, 25%, 50%, 75% and 100% legume abundance (percentage calculated as number of plants) were superimposed with N addition treatment resulting in 10 treatments in total. N was applied at an equivalent rate of 100 kg N ha⁻¹ as NH₄NO₃. The experiment was arranged in a fully-randomised design with five replicates per treatment.

Plastic (PVC) pots (13 cm diameter and 9 cm depth) were filled with 800 g soil at 70% water-filled pore space (WFPS). Topsoil (0–10 cm depth) was collected from a permanent temperate grassland at Hazelrigg, Lancaster University, UK (54° 1'50"N, 2.7° 46'30"W, 94.1 m a.s.l.). This site is a 61 ha area of permanent unfertilised grassland intermittently grazed by sheep and

used as a hay meadow. The soil was a silt loam (Brickfield 2 Association; Avis and Harrop (1983)) and contained 25.6 g total C kg⁻¹, 2.3 g total N kg⁻¹, and had a pH (H₂O) of 5.9. Roots and plant fragments were removed, soil was sieved through 8 mm screen, thoroughly mixed and air-dried at 20 °C to 70% WFPS. Soil moisture was gravimetrically maintained every two days in all mesocosm pots.

Prior to the start of the experiment, *T. pratense* and *A. capillaris* seeds were surface-sterilised by dipping them in diluted bleach (1% v: v) for 1 min and thoroughly rinsing them with sterile distilled water. Seeds were initially sown in sterilised potting soil (John Innes No. 2, Westland Garden Health, UK), germinated in a greenhouse and grown for four weeks, receiving water daily. The seedlings were then transplanted into pots and, after one week, dead plants were replaced, and N-fertiliser was applied. Each mesocosm pot contained a total of 12 plants. Pots were maintained in a controlled environment room (temperature, 19 °C day, 16 °C night; light between 08:00 h and 20:00 h daily).

Greenhouse gas fluxes measurements

Measurements of CO₂, N₂O and CH₄ were made on mesocosm pots using opaque plastic chambers (13 cm diameter and 12 cm depth) with septa to allow headspace gas sampling. For each gas flux measurement, the chamber was attached with clip seal to the mesocosm and a 10 mL headspace sample was taken immediately (t₀) with a gas syringe. 5 mL of the gas was then injected into a pre-evacuated 3.5 mL exetainer vial (Labco, Lampeter, UK). Further headspace samples were taken 15 (t₁₅) and 30 (t₃₀) minutes after chamber closure. Chambers were then removed. Gas samples were analysed using a PerkinElmer Autosystem XL Gas Chromatograph (GC) (PerkinElmer, Waltham, MA, USA) fitted with a methaniser with a Flame Ionisation Detector (FID) and Electron Capture Detector (ECD) operating at 130 °C. The GC was fitted with a stainless steel Porapak Q 50–80 mesh column (length 2 m, outer diameter 3.17 mm) maintained at 60 °C. Three calibration gas standards (500 ppm, 1000 ppm, 4000 ppm CO₂) (Air Products, Waltham on Thames, UK) were run every 14 samples to enable calibration and drift correction (Case et al. 2012). Gas fluxes were calculated using linear regressions through sampling time points and were corrected for temperature and barometric pressure following the ideal gas law (Holland et al. 1999).

Gases were sampled immediately after N application and daily during the first 14 days of the experiment, then three times a week up to day 40. Cumulative GHG fluxes were calculated by linear interpolation of the average GHG emissions between the measurements to integrate the fluxes over the total experimental period. Emissions of N_2O and CH_4 were converted to use the concept of global warming potential (GWP) (IPCC 2007), where the GWP value for CH_4 (based on a 100-yr time horizon) is 34 and for N_2O is 298 (IPCC 2013). The net GHG balance was calculated by the mean of GHG (N_2O and CH_4) and soil carbon stock (SOC) (Table 5).

Plant and soil analyses

At the end of the experiment (40 days), grass and legume above-ground biomass were harvested and separated, with dry matter content determined by drying in an oven at 105 °C for 24 h. Subsamples were dried at 60 °C for 24 h, then ground and analysed for total C and N content using an elemental analyser (TruSpec® CN, St. Joseph, MI) with a furnace temperature of 950 °C. Ethylenediaminetetraacetic acid (EDTA) was used as a reference. To determine shoot N uptake per plot, values of shoot N content (%) were multiplied by total dry matter content which is the shoot N content at the end of the experiment.

Soil was taken from the pots at the end of the experimental period. Gravimetric moisture content was determined after drying at 105 °C for 24 h. Mineral-N (NH_4^+ and NO_3^-) was extracted with 1 M KCl in a 1:5 (soil weight: extractant volume) ratio and analysed with a spectrophotometer (Auto Analyser 3 Digital colorimeter BRAN + LUBBE). Net mineralisation (net N production: NH_4^+ -N + NO_3^- -N) and net nitrification (net NO_3^- -N production) rate were determined by incubating the soil at 25 °C for 14 days, analysing the final mineral-N content as described above, then calculating the daily mineral-N production rate as the difference between final and initial inorganic N content, divided by the incubation period. Soil C and N were determined after dried subsamples at 60 °C at 24 h, then finely ground using an elemental analyser (TruSpec® CN, St. Joseph, MI) with furnace temperature at 950 °C. Soil pH was measured on 10 g fresh soil with 25 mL deionised water, left to stand for 30 min and then tested with 210 pH Meter (Hanna Instruments, RI, USA). Plant roots were separated from the soil from each mesocosm

pot to determine below-ground biomass/productivity after washing and drying at 105 °C for 24 h. A subset of roots was stored in the fridge with 10% ethanol solution to measure specific root length (SRL) and root dry matter content (RDMC). Root length and diameter were analysed using WinRhizo® root analysis software (Regent Instruments Inc., Sainte-Foy-Sillery-Cap-Rouge, QC, Canada) coupled to an Epson flatbed scanner.

Statistical analyses

Generalised least squares models (GLS) were used to test the significance of treatment effects on plant productivity, plant-soil properties and GHG emissions. As most variables did not fit a linear regression, quadratic and cubic regressions were included in the model with the following formulae, where LEG refers to legume proportion and NADD to N addition:

$$y \sim \text{LEG} * \text{NADD} + \text{I}(\text{LEG}^2) * \text{NADD} \\ + \text{I}(\text{LEG}^3) * \text{NADD} \quad (1)$$

Fixed effects were LEG, LEG^2 , LEG^3 , NADD and their interactions. For all GLS models, data were checked for normality and equal variances using residual plots method, and log-transformed where necessary before analysis (i.e. for cumulative CO_2 , below-ground biomass, shoot N yield, shoot C:N, shoot:root ratio, soil mineral-N, N mineralisation and nitrification). Weight functions were used to account for unequal variances following (Zuur et al. 2009). The same model parameters were found to be significant when legume proportion (%) was replaced with legume biomass. The proportion of legumes and biomass legumes were very highly correlated ($r^2 = 0.80$, Variance inflation factors (VIF) > 5). Additionally, the model with legume proportion showed the lowest Akaike information criterion (AIC). Tukey post hoc testing was performed to test the effects between grass-legume mixtures and monocultures using a pairwise comparison between different legumes proportions. All statistical analysis and graphs were made using R programming language 3.4.3 (R Development Core Team 2017) with the additional packages *nlme* (Pinheiro et al. 2019), *plyr* (Wickham 2011) and *multcomp* (Hothorn et al. 2008).

Results

Realised legume proportion

The objective of this study was to achieve grass-legume mixtures with 25%, 50% and 75% legume abundance, based on percentage calculated as number of plants. However, at the end of the experiment, the realised legumes achieved was 38%, 45% and 53% for unfertilised soils and 32%, 36% and 41% for fertilised soils based on legumes biomass (g dwt) (Fig. 1). This shows that legume plant weight was not largely different between treatments.

Plant productivity

There was a significant, non-linear relationship between the proportion of legumes and above-ground biomass in the unfertilised and fertilised soil ($p = 0.02$, $\text{LEG}^2 * \text{NADD}$, Fig. 2a, Table 1) showing that above-ground biomass was greater in fertilised compared to unfertilised soils in all treatments except for the legume monoculture (100%). Below-ground biomass decreased non-linearly with increases in legume proportion in both unfertilised and fertilised soil, with a significant interaction between legume and fertiliser treatments ($p = 0.03$, $\text{LEG}^3 * \text{NADD}$, Fig. 2b, Table 1). In unfertilised soils, below-ground biomass increased between 0 and 25% legume treatments and then decreased with subsequent increases in legume proportions (Table S2). In contrast, in the fertilised soils, below-ground biomass was consistent in the three mixture treatments (25%, 50%, 75%), and was smaller than the grass monoculture and greater than the legume monoculture (Table S2).

Shoot N uptake (g N m^{-2} , total N in grass+legumes) had a significant non-linear relationship with legume proportion ($p < 0.0001$, $\text{LEG} * \text{NADD}$, Table 2), with greater shoot N uptake in fertilised compared to unfertilised soil (Fig. 3a). In unfertilised and fertilised soils, shoot N uptake is greater in the grass-legume mixtures than in the monocultures (Table S2), and this reflects the differences found in above-ground biomass which is also greater in the mixtures soils (Fig. 2a).

The shoot N concentration (%) of the grasses and legumes decreased linearly with increases in legume proportion in the fertilised soils, but not in unfertilised soils ($p = 0.03$ and $p = 0.001$, $\text{LEG} * \text{NADD}$, in grass and legume species respectively, Fig. 3b, c, Table 2). The

shoot N concentration (%) in grass and legumes was greater in fertilised compared to unfertilised soil. Shoot CN ratio decreased with increases in legume proportion ($p < 0.0001$, $\text{LEG} * \text{NADD}$, Fig. 3d, Table 2), only in unfertilised soil, while shoot/root ratio increased with increasing legume proportion in unfertilised soils ($p = 0.01$, $\text{LEG} * \text{NADD}$, Fig. 3e, Table 2).

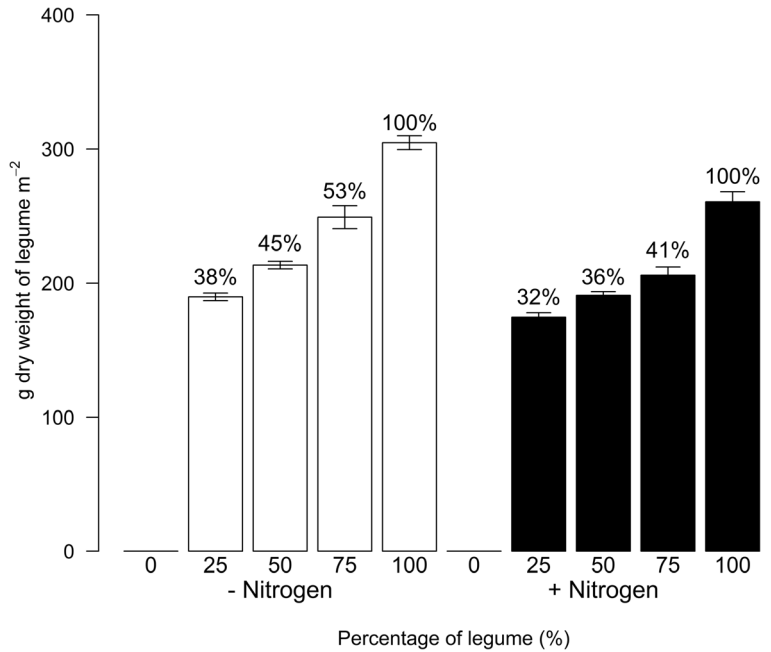
Soil parameters

Soil pH was not affected by increases in legume proportion ($p > 0.05$, Table 3) but was significantly decreased by N addition from an average of 6.1 to 5.4 ($p < 0.05$, Table 3). Total soil N, NO_3^- -N and net nitrification rates were not significantly affected by legume proportion ($p > 0.05$, Table 3), but were greater under N addition ($p < 0.05$, Table 3). Soil NH_4^+ -N had a non-linear relationship with increasing legume proportion ($p = 0.001$, $\text{LEG}^2 * \text{NADD}$, Table 3) but only in fertilised treatments. Net mineralisation rate increased non-linearly with increases in legume proportions ($p = 0.006$, $\text{LEG}^3 * \text{NADD}$, Table 3), on the fertilised soils only. Some treatments are not described in Table 3 as they are not showing any significant effects (i.e. $\text{LEG} \times \text{NADD}$, LEG^2 , LEG^3).

Greenhouse gas fluxes

Increased legume proportion did not affect cumulative N_2O emissions, regardless of N addition ($p > 0.05$, Fig. 4a, Table 4). N-fertiliser addition stimulated N_2O emissions by approximately four times compared to unfertilised soils ($p < 0.0001$, Fig. 4a, Table 5). Cumulative CO_2 fluxes (ecosystem respiration) decreased with increasing legume proportion ($p < 0.0001$, $\text{LEG} * \text{NADD}$, Fig. 4b, Table 5) in fertilised soils, but were consistent in unfertilised soils. Cumulative CH_4 emissions were affected by legume proportion with a significant cubic regression ($p = 0.01$, Table 5), which means that there were significant differences between treatments but no consistent trend with increasing legume proportion. In unfertilised soils, CH_4 emissions were greater with 25% of legumes than 50% of legumes, while in fertilised soil 25% of legumes CH_4 fluxes were greater with than 75% of legumes (Table S2). N addition significantly increased CH_4 emissions by up to 50 times compared to unfertilised soils ($p < 0.0001$, Fig. 4c).

Fig. 1 Legume above-ground biomass (g dwt m^{-2}) within grass-legume mixtures at the end of the controlled temperature mesocosm experiment. Percentage above bars represent realised legume proportion. Data represent mean \pm SE ($n = 5$)



Discussion

The aim of this study was to investigate how grassland legume proportion and N addition interact to affect plant-soil properties with feedback consequences for ecosystem GHG emissions. Legume proportions in

grass-legume mixtures increased total plant productivity although there was no effect on ecosystem respiration or N_2O emissions from the soil. Greater GHG emission differences were observed between unfertilised and fertilised soils. Below, we will discuss the effect of grass-legume mixtures on plant productivity and shoot

Fig. 2 Total above- (a) and below-ground (b) biomass (g dwt m^{-2}) in response to legume proportion and nitrogen addition treatments after six weeks growth under controlled environment conditions. Data represent mean \pm SE ($n = 5$)

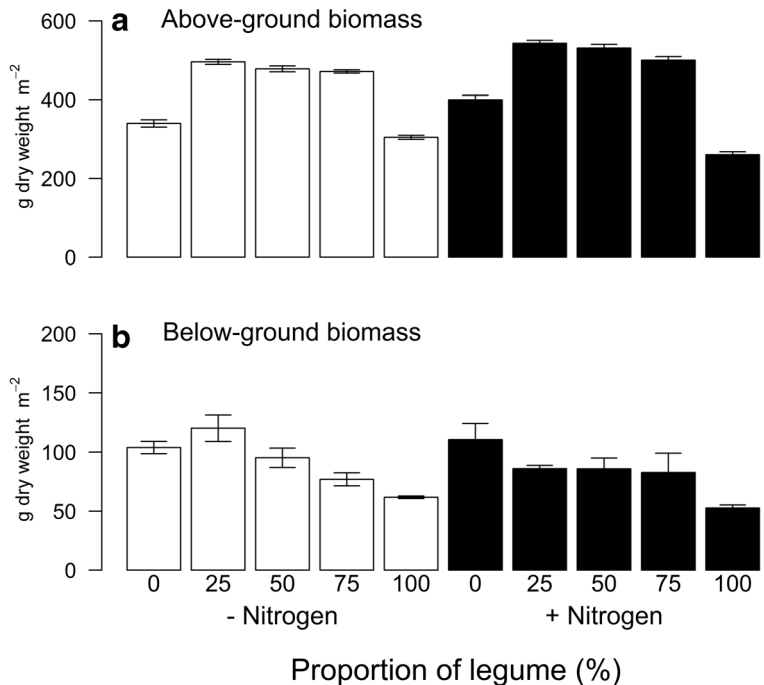


Table 1 The effect of legume proportion (LEG), nitrogen addition (NADD) and interactions on above- and below-ground biomass

	d.f.	Above-ground biomass g dry weight m ⁻²		Below-ground biomass g dry weight m ⁻²	
		F value	P	F value	P
LEG linear (LEG)	1	61.70	<0.0001	58.96	<0.0001
NADD	1	14.93	0.0004	3.77	0.06
LEG quadratic (LEG ²)	1	511.44	<0.0001	5.19	0.03
LEG cubic (LEG ³)	1	0.57	0.45	0.15	0.70
LEG x NADD	1	18.29	0.0001	0.03	0.85
LEG ² x NADD	1	5.77	0.02	0.37	0.54
LEG ³ x NADD	1	1.60	0.21	4.97	0.03

Above-ground biomass corresponds to the legume+grass biomass. Significant effects ($P < 0.05$) are shown in bold

N uptake, and how legume proportions affect GHG emissions in the presence or absence of N, linking them with plant productivity and plant-soil properties.

Effect of grass-legumes mixtures and N addition on plant productivity and shoot N uptake

According to our hypothesis, in unfertilised soils increasing legumes would increase productivity overall, but our second hypothesis states that grasses would be favoured over legumes in fertilised soils. Interestingly, our results showed that grass-legumes mixtures had higher plant productivity (above-ground biomass) compared to monocultures in both fertilised and unfertilised soils regardless of the proportion of legumes, but in fertilised soils, mixtures showed greater productivity (Fig. 2). Shoot N uptake followed the same pattern (Fig. 3a), indicating that the presence of legumes in

mixtures facilitates increased N uptake and therefore increased plants growth.

The increase in plant productivity in grass-legume mixtures can be explained due to the ability of legumes to fix atmospheric N₂ in the soil providing N rich organic matter (Mulder et al. 2002; Spehn et al. 2002). Leguminous species can then significantly affect grassland biomass production (Spehn et al. 2000) and produce three times greater biomass compared to mixtures without legumes (Spehn et al. 2000). Legumes in grass-legume mixtures in this study have contributed to an increase of 40–60% plant productivity. In addition to N₂ fixation, legumes can increase plant productivity in mixed communities by spatial or temporal complementarity of soil resource use. For instance, grasses and legumes have different root morphology, which may be responsible for different strategies of resources use (Table S1). Clovers (*Trifolium* spp) have thicker, shorter,

Table 2 Effects of legume proportion (LEG), nitrogen addition (NADD) and interaction on shoot N uptake, grass and legume N content, shoot CN ratio and shoot/root ratio

	d.f.	Shoot N uptake (g m ⁻²)		Grass N content (%)		Legume N content (%)		Shoot CN ratio		Shoot/Root biomass ratio	
		F value	P	F value	P	F value	P	F value	P	F value	P
LEG linear (LEG)	1	21.04	<0.0001	1.10	0.30	0.21	0.65	8.35	0.006	14.0	0.0005
NADD	1	1401.12	<0.0001	2238.94	<0.0001	1124.8	<0.0001	1948.7	<0.0001	4.12	0.05
LEG quadratic (LEG ²)	1	307.83	<0.0001	0.10	0.76	0.70	0.41	0.84	0.36	32.75	<0.0001
LEG cubic (LEG ³)	1	12.53	0.001	0.50	0.48	0.02	0.88	1.72	0.20	0.47	0.50
LEG x NADD	1	70.77	<0.0001	5.30	0.03	12.94	0.001	36.95	<0.0001	6.41	0.01
LEG ² x NADD	1	8.17	0.007	0.17	0.68	0.01	0.91	0.00	0.99	1.94	0.17
LEG ³ x NADD	1	1.87	0.18	0.04	0.85	0.55	0.46	0.33	0.57	1.22	0.14

Significant effects ($P < 0.05$) are shown in bold

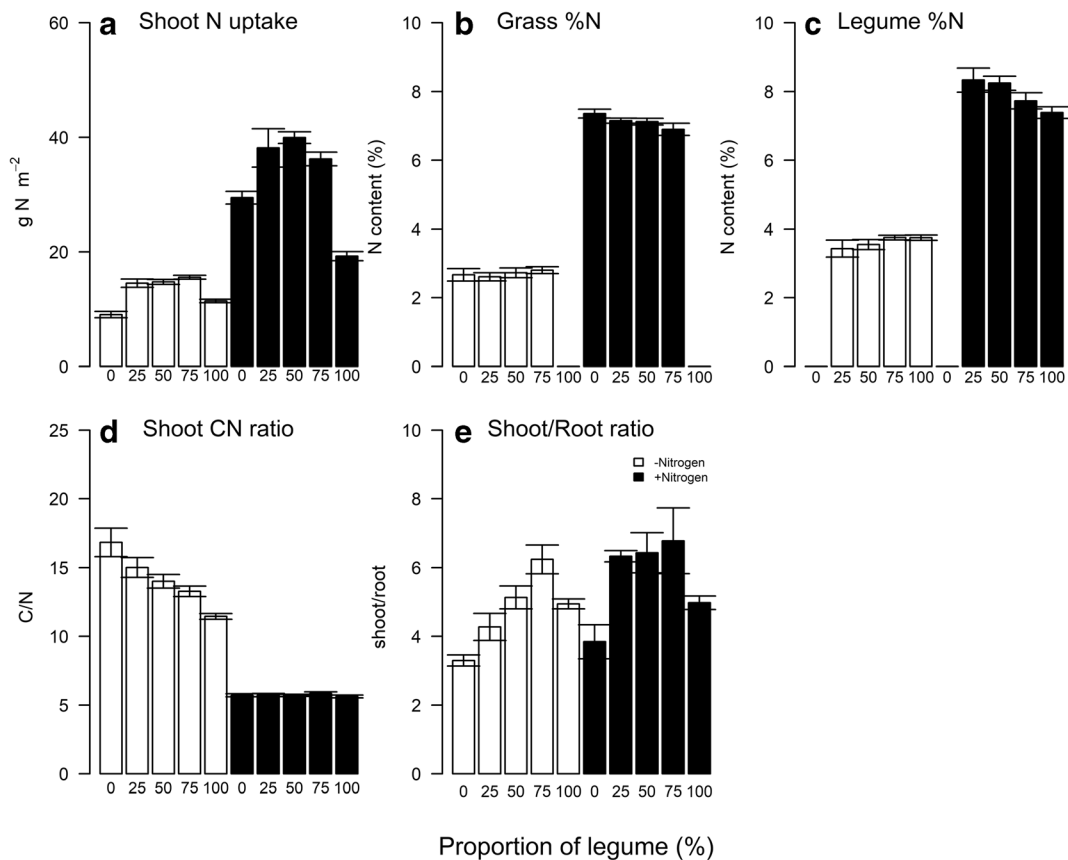


Fig. 3 Interactive effects of legume proportion and nitrogen addition on a) total shoot N uptake (g N m⁻²), b) Grass N content (%), c) Legume N content (%), d) shoot CN ratio, and e) shoot/root

ratio (dwt biomass) after six weeks growth under controlled environment conditions. Data represent mean \pm SE (n = 5)

and less branched root systems (Haynes 1980) i.e. lower SRL, and exhibit weak competition for nutrients with low mobility in the soil. While grasses (*Agrostis* spp) are more soil exploitative species, which invest more energy in the development of roots to acquire nutrients from the soil (De Deyn et al. 2008; Maire et al. 2009). Increased productivity of mixtures may, therefore, be due to different species functional traits and complementarity in resource use. This might also increase N use efficiency (van Ruijven and Berendse 2005) and facilitate N transfer from legumes to grasses (Høgh-Jensen and Schjoerring 1997; Laidlaw et al. 1996). In unfertilised soils, shoot CN ratio declined with increases in legume proportion (Fig. 3a), showing that mixtures with legumes are acquiring more N and increasing N use efficiency of the sward.

The increase of N transfer from legumes to grasses (McElroy et al. 2017) is another important process that can promote greater productivity. As shown by the N

concentration in the grass in monoculture and mixtures, N transfer to grasses from legumes in mixtures was not significantly different (Fig. 3b, c), however shoot N uptake was higher in mixtures compared to monoculture indicating that when legumes are present, companion plants can access more nutrients. Grassland field experiments suggest the benefits of legumes to overall plant productivity are evident at around 30–50% of realised legume proportion based on above-ground biomass (Suter et al. 2015). This corresponds well with our findings, where legume proportions between 25 and 75% (which correspond to 33–50% realised legume proportion based on above-ground biomass at the end of the experiment) showed higher above-ground productivity and shoot N uptake, and were significantly greater than either grass or legume monocultures in both unfertilised and fertilised soils (Fig. 2a, b, 3a). There were no significant differences in productivity between the three mixture treatments, which is explained by

Table 3 Effects of legume proportion (LEG), nitrogen addition (NADD) and interaction on soil parameters. Data are mean \pm SE ($n = 5$). Significant effects ($P < 0.05$) are shown in bold

	Soil pH	Total soil N mg kg ⁻¹	Soil NH ₄ ⁺ -N	Soil NO ₃ ⁻ -N	Net mineralisation rate mg kg ⁻¹ d ⁻¹	Net nitrification rate
<i>Without nitrogen</i>						
0% Legume	6.04 \pm 0.17	1.89 \pm 0.20	1.00 \pm 0.09	0.43 \pm 0.22	0.48 \pm 0.08	0.39 \pm 0.07
25% Legume	6.2 \pm 0.10	2.20 \pm 0.25	0.84 \pm 0.04	0.45 \pm 0.67	0.54 \pm 0.14	0.44 \pm 0.13
50% Legume	6.2 \pm 0.04	2.10 \pm 0.11	0.92 \pm 0.10	0.30 \pm 0.12	0.60 \pm 0.09	0.52 \pm 0.08
75% Legume	6.0 \pm 0.14	1.96 \pm 0.19	0.87 \pm 0.05	0.46 \pm 0.18	0.50 \pm 0.05	0.42 \pm 0.04
100% Legume	6.2 \pm 0.07	2.25 \pm 0.15	1.20 \pm 0.11	0.83 \pm 0.29	0.56 \pm 0.07	0.51 \pm 0.06
<i>With nitrogen</i>						
0% Legume	5.4 \pm 0.11	2.41 \pm 0.06	296.5 \pm 9.40	109.20 \pm 8.80	2.50 \pm 0.39	2.25 \pm 0.29
25% Legume	5.5 \pm 0.77	2.57 \pm 0.07	307.9 \pm 6.57	102.92 \pm 9.78	1.75 \pm 0.92	2.94 \pm 0.60
50% Legume	5.5 \pm 0.95	2.48 \pm 0.19	312.9 \pm 21.90	114.25 \pm 21.33	2.71 \pm 0.89	2.43 \pm 0.66
75% Legume	5.0 \pm 0.05	2.45 \pm 0.15	328.7 \pm 23.50	131.40 \pm 26.17	3.75 \pm 0.78	2.81 \pm 0.61
100% Legume	5.5 \pm 0.09	2.49 \pm 0.12	260.5 \pm 28.03	78.16 \pm 6.59	1.11 \pm 0.34	2.90 \pm 0.32
LEG linear (LEG)	F = 0.45, $p = 0.50$	F = 0.29, $p = 0.59$	F = 5.39, $p = 0.02$	F = 0.79, $p = 0.38$	F = 0.20, $p = 0.66$	F = 0.49, $p = 0.48$
NADD	F = 110, $p < 0.0001$	F = 16.10, $p = 0.0002$	F = 27.724, $p < 0.0001$	F = 1068, $p < 0.0001$	F = 70.54, $p < 0.0001$	F = 172, $p < 0.0001$
LEG ² x NADD	F = 0.01, $p = 0.92$	F = 0.02, $p = 0.87$	F = 11.98, $p = 0.001$	F = 2.90, $p = 0.09$	F = 2.76, $p = 0.15$	F = 0.15, $p = 0.70$
LEG ³ x NADD	F = 0.01, $p = 0.94$	F = 0.59, $p = 0.44$	F = 0.88, $p = 0.35$	F = 1.27, $p = 0.27$	F = 8.45, $p = 0.006$	F = 0.19, $p = 0.66$

Some parameters not reported as non-significant.

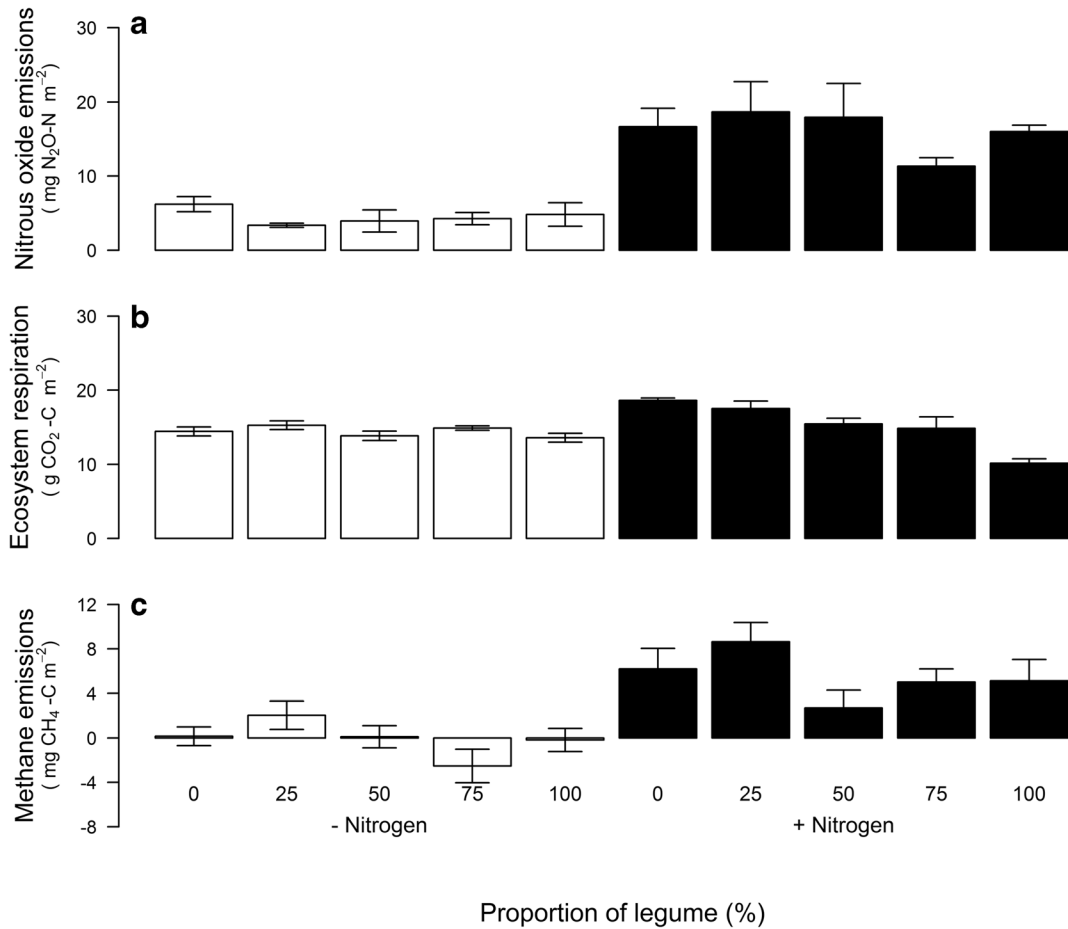


Fig. 4 Cumulative N₂O (mg N₂O-N m⁻²) (a), CO₂ (g CO₂-C m⁻²) (b) and CH₄ (mg CH₄-C m⁻²) (c) emissions in response to legume proportion and nitrogen addition in the controlled temperature

mesocosm experiment. Data are mean of cumulative emissions for all sampling dates \pm SE ($n = 18$)

similar realised legume proportions (Fig. 1). As a result our study was unable to determine the legume proportion which would maximise plant productivity and

mitigate N₂O emissions. Studies have found that over-sowing clover without any fertilisation compared to low clover proportion with organic fertilisation reduced N₂O

Table 4 Effects of legume proportion (LEG) and nitrogen addition (NADD) on cumulative emissions of N₂O, CO₂, CH₄

	d.f.	N ₂ O emissions mg N ₂ O-N m ⁻²		CO ₂ emissions g CO ₂ -C m ⁻²		CH ₄ emissions mg CH ₄ -C m ⁻²	
		F value	<i>P</i>	F value	<i>P</i>	F value	<i>P</i>
LEG linear (LEG)	1	0.93	0.34	21.3	<0.0001	3.19	0.08
NADD	1	65.22	<0.0001	1.1	0.30	35.54	<0.0001
LEG quadratic (LEG ²)	1	1.81	0.18	3.4	0.07	0.11	0.74
LEG cubic (LEG ³)	1	0.03	0.86	0.6	0.45	7.12	0.01
LEG x NADD	1	0.43	0.52	26.2	<0.0001	0.006	0.94
LEG ² x NADD	1	0.33	0.57	1.9	0.17	0.18	0.67
LEG ³ x NADD	1	2.83	0.10	0.9	0.35	0.15	0.69

Significant effects ($P < 0.05$) are shown in bold

Table 5 Net GHG balance for each grass-legume mixture without and with N fertilisation

Treatments	Net GHG balance g CO ₂ eq m ⁻²
Without N	
0% Legume	27.7
25% Legume	27.8
50% Legume	30.6
75% Legume	26.8
100% Legume	31.6
With N	
0% Legume	33.8
25% Legume	35.8
50% Legume	33.2
75% Legume	32.8
100% Legume	33.6

Net GHG balance was calculate by the mean of GHG (N₂O, CH₄ emissions) and soil carbon stock

emissions by 54% and 39% in year 1 and 2 of a field experiment (Fuchs et al. 2018). However, this reduction in emissions is more likely to be related to the difference in fertilisation between treatments rather than clover proportions per se. A study from Migliorati et al. (2015) found a 50% reduction in N₂O emissions from soil which had sorghum grown in legume history compared to sorghum grown in grass crop history without affecting plant productivity. This shows the importance of determining the optimum amount of legume needed in mixtures without compromising plant productivity and the environment.

Effects of legume proportion on GHG emissions in unfertilised soils

In unfertilised soils, despite the increase in plant productivity and total shoot N uptake with increasing legume proportion, below-ground biomass was reduced at higher legume proportions and there was no significant effect of legume proportion on ecosystem respiration or N₂O emissions but there was a significant effect on CH₄ emissions. N₂O emissions may have been unaffected under N-limiting conditions, as it has been suggested that the flux rate through N pools (Jones et al. 2005) is more important than the actual size of the relative N pools. Fast-growing species such as *T. pratense* and *A. capillaris* increase N mineralisation (Fig. S1) from

soil organic matter to a greater extent than more conservative species, with consequent increases in their growth (Personeni and Loiseau 2005; Van Der Krift and Berendse 2001). This higher N availability may temporarily enhance N₂O emissions, given that soil microbes such as nitrifiers and denitrifiers are good competitors for both NH₄⁺ and NO₃⁻ (Hodge et al. 2000). However, as found in many studies, the rate of net N uptake is four- to six-fold higher for faster growing species compared to slower growing plant species (Poorter et al. 1991). This fast flux between soil N supply and N use by both plants and microorganisms (Jackson et al. 1989) would result in no build-up of a soil mineral-N pool (Abalos et al. 2014), and would explain the decoupling between biomass production and N₂O emissions. As a consequence, under N-limiting conditions, the acceleration of the N cycle caused by these two fast-growing species (Orwin et al. 2010) is likely to offset the production-induced reduction in N₂O emissions that we hypothesised. In this context, the presence of these two fast-growing species might increase the input of C and N root exudates especially from legumes, which typically stimulate the growth of soil microbes (Denton et al. 1998; Mawdsley and Bardgett 1997). Increases in microbial biomass may have increased microbial respiration and offset the decrease in ecosystem respiration, and therefore not be affected by increases in legume proportion (Fig. 4b).

Our results showed that grass-legume mixtures have increased above-ground biomass and decreased below-ground biomass (Fig. 2). Legumes in N limiting conditions (i.e. with no N-fertilisation) might have reduced root growth, potentially increasing specific root length (SRL); which is well-known to be increased in N limiting ecosystem. This increase in SRL is correlated with plant investment into structures structure to acquire N in the soil (Du et al. 2013; Ostonen et al. 2007) reducing N-available thus reducing N₂O emissions. Low N concentration may decrease root growth, providing a smaller sink for inorganic and legume-derived N (Ofosubudu et al. 1995; Paynel et al. 2008).

Increases in legume proportion affected CH₄ uptake and emissions in both unfertilised and fertilised soils (Fig. 4c) but there was no consistent trend between legume proportion treatments (Fig. 4c). Many different and overlapping mechanisms might explain these effects. Species interactions might alter root densities and architecture, which may lead to changes in soil aeration, spatio-temporal organic C deposition, or N

use pattern (Niklaus et al. 2016). These factors could contribute to the increased productivity of mixtures, augmenting the supply of organic substrates to the soil microbial community (Suwanwaree and Robertson 2005), and increasing oxygen consumption and soil diffusive conductance (Grosso et al. 2000; Smith et al. 2003). These will in turn change the biomass and activity of methanotrophs and methanogens in the soil, determining rates of net CH_4 fluxes from soil (Ball et al. 1999).

Grass monocultures and grass-legumes mixtures in this study had higher soil NH_4^+ concentrations compared to unfertilised legume monocultures (Table 3, Fig. S1). Unsurprisingly, this shows that NH_4^+ concentration was strongly affected by N-fertilisation. Although, in this study, it was possible to observe changes in NH_4^+ concentration due to legume proportions, there was no clear correlation with CH_4 uptake. Many studies suggest that NH_4^+ may inhibit CH_4 oxidation (Hütsch 1998; Rime and Niklaus 2017), due to competitive enzymatic process of methanotrophy and nitrification in soils (Baggs and Blum 2004). However CH_4 oxidation may not be affected by NH_4^+ if it is present in a different spatial niche to active methanotrophs (Hartmann et al. 2011). Therefore, this is most likely related to the interaction between grasses and legumes specifically and their impacts on N cycling. These results show that plant composition can affect CH_4 fluxes, however, the short duration of the experiment, the low level of plant diversity and the lower realised legume proportion (Fig. 1) is likely to have contributed to the limited effects on N_2O and CO_2 emissions from the soil. Other experiments should be done to confirm the findings of this study in field conditions which will be important to evaluate the real effect of legume proportions on GHG emissions from soils.

Effects of legume proportion on GHG emissions in fertilised soils

In fertilised soils, agreeing with our hypothesis, N_2O emissions were most affected by the mineral-N applied as N-fertiliser, and its availability in the soil was irrespective of legume proportion in the sward. N_2O emissions were not affected by increases in legume proportion, which can be largely explained by legume productivity. The presence of N in the system was a determining factor for a reduction and/or suppression of legume biomass (De Deyn et al. 2011; Smith et al. 2008), and

probably to a decrease of N_2 fixation (Ledgard et al. 2001). It may also affect the nodule production or induce a reduction of N fixation, which is shown, by the reduction of N concentration in legume species with increasing legume proportion (Naudin et al. 2010; Rasmussen et al. 2013). Different abiotic (e.g. water stress, microclimate temperature) and biotic (e.g. legume-grass plant density, root contact) factors may be changing ecosystem processes (Thilakarathna et al. 2016) that relate indirectly to N_2O emissions.

In agreement with our hypothesis, ecosystem respiration was decreased by increases in legume proportion under non-N-limiting conditions. N addition might promote a reduction of legume biomass (favouring grasses) (McElroy et al. 2017), which may be the reason for the decrease in ecosystem respiration. Nyfeler et al. (2011) also showed that grasses benefited from N transfer from legumes in fertilised soil (150 kg N ha^{-1}). Additionally, some research suggests that root biomass is reduced in fertiliser treatments compared to unfertilised (Chirinda et al. 2012), due to higher root exploitation of soil to overcome the low nutrient availability in unfertilised soils. Although in our study root biomass was only slightly reduced in fertilised soil (Fig. 2). This reduction in root biomass might also decrease root respiration and overall ecosystem respiration. In addition, the reduction in ecosystem respiration may be related to less energy needed (especially by legumes) to acquire N in grass-legume mixtures (Drinkwater et al. 1998). Although we did not find a reduction in N_2O emissions in this short-term experiment, in the long-term including legumes and reducing N-fertiliser inputs would result in a more closed efficient N cycle with environmental benefits. Other studies are needed to confirm the findings in this study, specifically regarding the interaction of legume proportion and N addition on the N cycle in grasslands and its potential effect on climate change.

Implications for grassland management and climate change

Incubation experiments offer a valuable means to investigate processes and mechanisms but need to be interpreted with caution as they cannot replicate field scale experiments. Furthermore, our observed effects may be different as a function of soil type and moisture content. The use of the grass-legume mixture increased plant productivity in unfertilised and N fertilised soils without affecting nitrous oxide emissions.

This raises a potential strategy to reduce N-fertiliser use in the field, and an opportunity for climate change mitigation. The overall GHG budget calculated for this experiment showed that N-fertilisation was the driver of increased GHG emissions independently of the mixtures (Table 5). Lower GHG emissions were found from soil which had 75% legumes grown in the grass-legumes mixtures without N-fertiliser ($26.8 \text{ g CO}_2\text{eq m}^{-2}$), followed by 0 and 25% (27.7 and $27.8 \text{ g CO}_2\text{eq m}^{-2}$, respectively) (Table 5). It will be important to evaluate the productivity legume-grass mixtures under field conditions, since legumes in excess could result in a reduction in plant productivity (Suter et al. 2015). Including legumes in grasslands may deliver the equivalent productivity as fertiliser, and although we did not find a reduction in N_2O emissions in this short-term experiment, in the long-term the inclusion of legumes and reduced N-fertiliser inputs would result in a more closed and efficient N cycle with environmental benefits.

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