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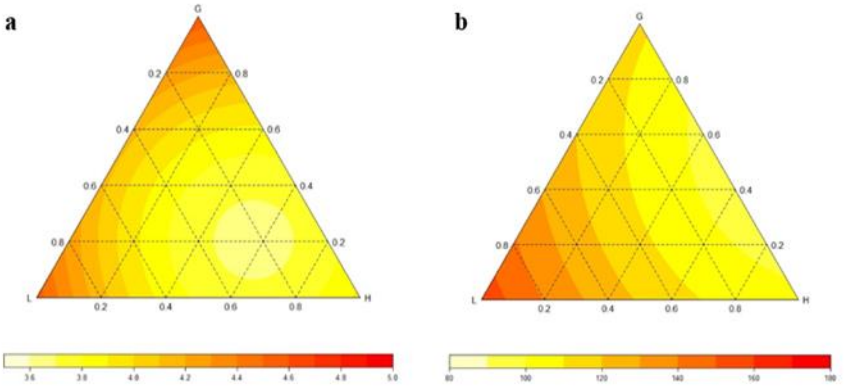
KEY WORDS

Grasslands, GHG emissions, nitrous oxide, yield-scaled N_2O emissions, multi-species mixtures, grassland production.

GRAPHICAL ABSTRACT



(a) N yield-scaled N_2O emissions (N_2O g ha^{-1} $year^{-1}$ / N yield kg ha^{-1} $year^{-1}$)

(b) DM yield-scaled N_2O emissions (N_2O gha^{-1} $year^{-1}$/DM yield tonne ha^{-1} $year^{-1}$)

Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

- We assessed annual N_2O emissions from field plots sown with multi-species grassland communities (1-6 species)
- N_2O emissions in mixtures were best predicted from a linear combination of species' identity effects (equivalent to species' performances in monoculture), with no additional suppressive effect due to interspecific interactions.
- Based on emissions intensities, the same N yield or DM yield from the 6-species mixture and *L. perenne* monoculture could have been produced while reducing N_2O losses by 41% and 24% respectively (at 150 kg ha^{-1} year^{-1} of nitrogen fertiliser).

Beneficial effects of multi-species mixtures on N_2O emissions from intensively managed grassland swards.

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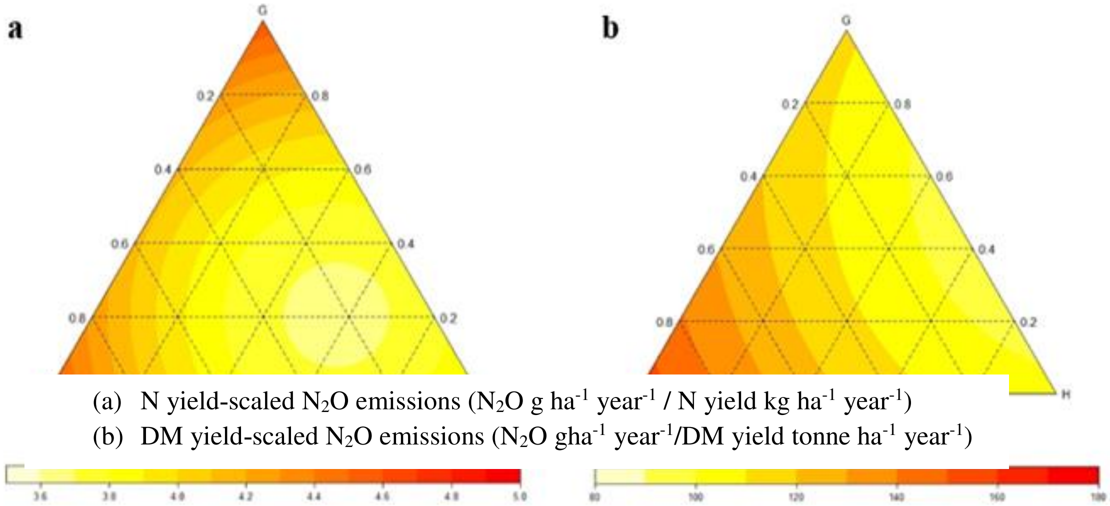

(a) N yield-scaled N_2O emissions (N_2O g ha^{-1} year^{-1} / N yield kg ha^{-1} year^{-1})
(b) DM yield-scaled N_2O emissions (N_2O gha^{-1} year^{-1}/DM yield tonne ha^{-1} year^{-1})

ABSTRACT

In a field experiment, annual N_2O emissions and grassland yield were measured across different plant communities, comprising systematically varying combinations of monocultures and mixtures of three functional groups (FG): grasses (*Lolium perenne, Phleum pratense),* legumes *(Trifolium pratense, Trifolium repens)* and herbs *(Cichorium intybus*, *Plantago lanceolata*). Plots received 150 kg ha^{-1} year^{-1} N (150N), except *L. perenne* monocultures which received two N levels: 150N and 300N. The effect of plant diversity on N_2O emissions was derived from linear combinations of species performances' in monoculture (species identity) and not from strong interactions between species in mixtures. Increasing from 150N to 300N in *L. perenne* resulted in a highly significant increase in cumulative N_2O emissions from 1.39 to 3.18 kg N_2O-N ha^{-1} year^{-1}. Higher N_2O emissions were also associated with the legume FG. Emissions intensities (yield-scaled N_2O emissions) from multi-species mixture communities around the equi-proportional mixture were lowered due to interactions among species. For N_2O emissions scaled by nitrogen yield in forage, the 6-species mixture was significantly lower than *L. perenne* at both 300N and 150N. In comparison to 300N *L. perenne*, the same N yield or DM yield could have been produced with the equi-proportional 6-species mixture (150N) while reducing N_2O losses by 63% and 58% respectively. Compared to 150N *L. perenne*, the same N yield or DM yield could have been produced with the 6-species mixture while reducing N_2O losses by 41% and 24% respectively. Overall, this study found that multi-species grasslands can potentially reduce both N_2O emissions and emissions intensities, contributing to the sustainability of grassland production.

1. INTRODUCTION

Nitrous oxide (N_2O) is a potent greenhouse gas (GHG) (Ravishankara et al., 2009) with 265 times the global warming potential of carbon dioxide (CO_2) (IPCC, 2014). Large N_2O losses result from both (N) fertiliser application to grasslands (Harty et al., 2016; Krol et al., 2020) and the N fertiliser production process itself (Wood and Cowie, 2004). Although conventional grassland systems for livestock production tend to use high levels of N fertiliser, they are not heavily reliant on imported concentrate feeds which have a high carbon footprint (O'Brien et al., 2011). Therefore, temperate grassland production systems have the potential to curtail N_2O losses through the displacement of N fertiliser for symbiotically produced plant-available N.

Multi-species mixtures composed of grasses, legumes and herbs provide a range of agronomic and environmental benefits in grass-based production systems. These include: increased dry matter (DM) yield production (greater biomass production from species in mixtures relative to the best performing monoculture – transgressive overyielding)

(Nyfeler et al., 2009; Finn et al., 2013; Moloney et al., 2020), improved animal performance (for both cattle and sheep) (Cranston et al., 2015; Roca-Fernández et al., 2016; Bryant et al., 2017; Grace et al., 2019; Jerrentrup et al., 2020), increased N use efficiency (NUE) (Hooper et al., 2005, Suter et al., 2015), weed suppression (Suter et al., 2017; Connolly., 2018), and greater yield stability during drought events (Hofer et al., 2016; Haughey et al., 2019). Although these benefits are well established, less is known about how multi-species grasslands influence the soil N cycle and therefore N_2O fluxes.

Multi-species grasslands may affect N_2O fluxes in several ways (Gardiner et al., 2016; De Klein et al., 2019). Nitrous oxide is mainly lost during the soil-based processes of nitrification and denitrification (Bremner et al., 1997). Different plant species can influence these processes through differential niche occupation of the rhizosphere which can affect plant water uptake (Holtham et al., 2007) and soil gas diffusivity. These processes determine the nitrification and denitrification pathways and the final N_2O:N_2 ratio of the denitrification process (Balaine et al., 2016). Biochemical reactions may also affect N_2O production. *Plantago* species contain biological nitrification inhibition (BNI) compounds within the plant that prevent ammonium (NH_4^+) transformation to nitrate (NO_3^-); this results in stability of the soil mineral N pool and increased plant N uptake (Chapman et al., 2006; Cantarel et al., 2015). Legume inclusion within grassland swards and multi-species mixtures allows for reduced fertiliser application without adversely affecting yields (Egan et al., 2018). This is due to biological nitrogen fixation (BNF) and the transfer of N from legumes to non-legumes within a multi-species sward (Nyfeler et al., 2011; Pirhofer-Walzl et al., 2012). Legume inclusion in grasslands can increase N_2O emissions when N fertiliser is not reduced to account for symbiotically fixed N (Hakala et al., 2012; Burchill et al., 2016; Luo et al, 2018).

There can be species-specific effects of plants on N_2O emissions. A laboratory incubation by Abalos et al. (2014) quantified the N_2O fluxes from mixtures with up to four grass species including *L. perenne, Festuca arundinacea*, *P. pratense* and *Poa trivialis.* No relationship was found between plant species richness and N_2O emissions; however, there was a significant reduction in N_2O emissions when certain plant species were combined. In a field study, Luo et al. (2018) applied cattle urine and compared the N_2O fluxes from monocultures of *P. lanceolata* and *Medicago sativa* with a *T. repens* and *L. perenne* mixture. Despite seasonal variation, *P. lanceolata* had lower N_2O emissions than *L. perenne* throughout the year. Although these studies showed potential for multi-species swards to reduce N_2O emissions, the various experiments were either not monitored on an annual basis, across a range of plant communities or did not apply different N fertiliser levels. These three considerations are each important to properly understand the application of multi-species swards in livestock production systems.

Fuchs et al. (2020) modelled the N2O mitigation potential and productivity of various combinations of legume proportions and fertilizer rates for five temperate grassland sites using two different biogeochemical models. They recommended further study of the effect of clover proportions ranging from 30–50% receiving ≤ 150 kg N ha-1 yr-1 input, as these were identified as best-bet climate smart agricultural practices. Our study directly responds to those research recommendations. In a year-long experiment, we investigated N_2O fluxes from six different forage types including two species from each of three FGs: grasses (*L. perenne, P. pratense)*, legumes (*T. pratense, T. repens)* and herbs (*C. intybus* and *P. lanceolata*) in monocultures and mixtures of systematically varying proportions. In addition, emission intensity was calculated for all treatments (van Groenigen et al., 2010) as N yield-scaled N_2O emissions (N_2O-N g ha^{-1} year^{-1}/ kg N yield ha^{-1} year^{-1}) and DM yield-scaled N_2O emissions (N_2O-N g ha^{-1} year^{-1}/ tonne DM yield ha^{-1} year^{-1}). The experimental design and analyses allowed quantification of species identity effects and species interaction effects on each of the three responses (N_2O emissions, N yield-scaled emissions and DM yield-scaled emissions). There was also a 300 kg N ha^{-1} year^{-1} *L. perenne* treatment (300N *L. perenne*) to allow for a comparison between all treatments and conventional agricultural practice. The specific aims of this study were to:

1) Investigate the effect of systematically varying species and FG proportions within grassland communities on annual N_2O emissions, N yield-scaled N_2O emissions and DM yield-scaled N_2O emissions.

2) Compare annual N_2O emissions and yield-scaled N_2O emissions from 150N forage communities with the 300N *L. perenne* community.

2. MATERIALS & METHODS

2.1 Experimental site

The year-long field experiment took place at Teagasc, Johnstown Castle, Co. Wexford, Ireland 52°18′27 N between March 2018 – March 2019. The climate is temperate maritime and meteorological data (precipitation, air and soil temperature) was recorded at the Johnstown Castle weather station. The soil type at the field site was a stagnic brown podzolic. Soil texture was sandy loam, pH was 5.7 and the average bulk density of the plots on the trial site at 5-10 cm depth was 1.35 g cm^{-3}.

2.2 Experimental design

The experimental site was treated with herbicide, ploughed, and reseeded in spring 2017. The experiment followed a simplex design (Scheffe, 1963) for use in conjunction with the statistical modelling described in Section 2.5. Experimental plots, each measuring 5 m x 7 m were sown with grassland communities (*Appendix A and D*) comprising one to six species that systematically varied FG composition and relative abundance. The six species comprised two species from each of three FGs: two grasses (*L. perenne* and *P. pratense),* two clovers (*T. repens* and *T. pratense)* and two deep-rooting herbs (*C. intybus* and *P. lanceolata*). There were 20 different communities with between one to four replicates per treatment (*Appendix B)* resulting in 43 experimental plots in total. Each main plot was divided into two 5 m x 3.5 m sub plots, and two water supply treatments were applied at random to the two halves. One split plot (randomly chosen) received natural water supply over the year ('rain fed'), while a two-month summer drought was simulated on the other half, using rainout shelters ('drought'). Here, we only report the measured N_2O emissions from the rain fed sub plots. Due to the natural drought conditions during the summer of 2018, the rain fed sub plots were irrigated on three occasions with 30mm of water, to match historical rainfall records (Met Éireann, 2020). Fertiliser N application was divided into five applications of varying rate from March-September 2018 *(Appendix C)*. Maintenance levels of P and K fertilisers were applied in line with soil test recommendations at the beginning of the growing season. Calcium ammonium nitrate (CAN) fertiliser was applied at rates of 150 kg ha^{-1} year^{-1} (150N; communities 1-19*, Appendix B)* and 300 kg ha^{-1} year^{-1} (300N *L. perenne;* community 20, *Appendix B*).

2.3 Nitrous oxide measurements

Nitrous oxide emissions were monitored from 13th March 2018 – 21st March 2019. To capture fertiliser-induced effects on N_2O fluxes, a high resolution N_2O sampling strategy was put in place for six months (March to September 2018) in order to coincide with fertiliser application - the time that emissions were expected to be highest. Sampling took place four days a week for two weeks immediately following each fertiliser application, two days a week in the next two weeks (weeks three and four) and once per week up until the next fertiliser application date. High-resolution N_2O sampling was followed by six months of low-resolution sampling at a frequency of once a month (October 2018 to March 2019). The less intensive sampling approach is reflective of the low N_2O fluxes expected during this period due to a combination of no N fertiliser application and low soil temperature (Maire et al., 2020).

Nitrous oxide was measured using static chamber methodology (De Klein and Harvey, 2012), with a single chamber placed in each plot giving a total of 43 chambers. Chambers consisted of square, stainless steel collars 40 cm (length) × 40 cm (breadth) x 10cm (height) lined with a neoprene strip and inserted to 5 cm soil depth with matching steel covers creating an approximately 16 litre headspace. A 10 kg weight was placed on top of the covers at sampling times to ensure an airtight headspace for an enclosure time of 40 minutes. A 10 ml air sample was removed through a 16 mm rubber septum using a 10 ml polypropylene syringe and hypodermic needle. The syringe was filled and emptied twice within the chamber to mix the headspace air prior to sampling. The gas samples were injected into pre-evacuated (–1,000 mbar) 7 ml screw-cap septum glass vials. Gas samples were taken from each chamber at 0, 20 and 40 minutes to measure N_2O concentration over time. Sampling events took place between the hours of 10:00 and 13:00 to obtain measurements representative of the average hourly flux of the day (De Klein & Harvey, 2012). Nitrous oxide concentrations were analysed using a gas chromatograph (GC, Varian CP 3800 GC, Varian, USA) fitted with an electron capture detector using high-purity helium as the carrier gas. Quality control N_2O standards, which were representative of the upper N_2O concentration limit expected, were analysed alongside N_2O field samples. Linear regression of the increase in N_2O gas concentrations over time (0, 20 and 40 minutes) was used to calculate daily fluxes (g N ha^{-1} day^{-1}). A single annual cumulative N_2O value was calculated per plot by integration of daily fluxes and linear interpolation between measurements (Burchill et al., 2014; De Klein and Harvey, 2012). Yield scaled-N_2O emissions (van Groenigen et al., 2010; Sanz-Cobena et al., 2014) were calculated by dividing annual cumulative g N_2O-N (g ha^{-1} $year^{-1}$) by 1) aboveground N yield (kg ha^{-1} $year^{-1}$) and 2) DM yield (tonnes ha^{-1} $year^{-1}$).

2.4 Ancillary measurements

An area of the experimental plots was designated for ancillary measurements. A meteorological station was located approximately 500 m from the experimental site. Air temperature and atmospheric pressure were noted at each N_2O sampling occasion along with volumetric soil water content using a Theta probe (type ML2; Delta-T Devices, Cambridge, UK). Soil moisture measurements were used to calculate the water filled pore space % (WFPS) (Equation 1):

Equation 1. Where SWC = volumetric soil water content, BD = bulk density and PD = particle density (Fichtner et al., 2019).

$$WFPS\,\% = \frac{SWC}{1 - \frac{BD}{PD}} \times 100$$

2.5 Data analyses

The three response variables (y) were: N_2O-N emissions (kg ha^{-1} $year^{-1}$), N yield-scaled N_2O emissions (N_2O-N g ha^{-1} $year^{-1}$/ kg N yield ha^{-1} $year^{-1}$) and DM yield-scaled N_2O emissions (N_2O-N g ha^{-1} $year^{-1}$/ tonne DM yield ha^{-1} $year^{-1}$). Using the regression-based Diversity-Interactions modelling approach (Kirwan et al., 2009), we regressed responses on the sown proportional contributions of the six species as follows:

Equation 2. Regression model equation

$$y = \sum_{i=1}^{6} \beta_i P_i + \beta_7 P_{Lp300N} + \delta \sum_{\substack{i,j=1 \\ i<j}}^{6} P_i P_j + \varepsilon$$

Where y is the response variable (model fitted separately to each of our three responses), P_i represents the sown proportion of a species in a community (for i: 1 = *L. perenne*, 2 = *P. pratense*, 3 = *T. pratense,* 4 = *T. repens*, 5 = *C. intybus* and 6 = *P. lanceolata*). The β_1 to β_6 coefficient are the identity effects of each species (under 150N fertiliser); if $P_i = 1$, the β_i coefficient is the expected monoculture response of species i, while if $P_i < 1$, the expected contribution of that species to the mixture is $\beta_i P_i$. An extra term (β_7) was included for the 300N *L. perenne* monoculture plots ($P_{Lp300N} = 1$ for these plots and 0 otherwise). Equation 2 assumes that all pairs of species interact in the same way (captured by the coefficient δ). We tested various forms of the interactions, including no interaction effects and whether pairwise interactions were determined by FG membership (Kirwan et al., 2009). The error term ε was initially assumed to be normally distributed with zero mean and constant variance σ^2. However, exploratory analysis indicated that responses from plots with 100% legume were considerably more variable than all other plots, therefore we assumed that the error was normally distributed with zero mean and with two variance terms depending on the sown proportion of legume (100% or <100%). The Diversity-Interactions modelling approach allows prediction of the response for a wide range of communities from this six-species pool, based on the relative proportions of the component species. The overall response is based on the linear combinations of the identity effects, plus the sum of the interaction effects as required. Thus, for example, for a 50:50 grass-legume mixture of *L. perenne* and *T. pratense*, the expected response is $(\beta_1)0.5 + (\beta_3)0.5 + (\delta)(0.5*0.5)$. We predicted from the final fitted model to assess the effects on our three response variables across the monocultures and selected communities, which included the 6-species mixture and 300N *L. perenne* monoculture. The analysis was performed using SAS software version the software package SAS version 9.4 (SAS Institute, Cary, North Carolina, USA).

3. RESULTS

3.1 Climatic conditions

The highest average daily temperature (Fig. 1) recorded at the field site was in July at 20.4°C. The lowest daily average temperature was 18th March 2018 at 0.1°C. These are in contrast to long-term climatic averages recorded at the Rosslare Co. Wexford station (10 km away). Between 1978 and 2007, on average the highest daily temperature was 13.1°C and the lowest was 8.1°C. The long term mean annual rainfall for Johnstown Castle was 905.5 mm, with 49.9 mm for July, whereas the total annual rainfall for 2018-2019 (Fig. 1) was 1089.4 mm, with the average monthly rainfall for July 2018 being 1.7 mm (not including irrigation). The WFPS of soil at the experimental site averaged 48% over the experimental year (Fig. 2). Following high levels of precipitation in early 2018 (Fig. 1), the WFPS stayed at ~70% until early May, whereas during June, July and August the WFPS declined to 20-30%.

Fig. 1. Precipitation (mm) and temperature (°C)

3.2 Nitrous oxide emissions

3.2.1 Seasonal patterns in N_2O emissions

High N_2O fluxes were measured in April 2018, coinciding with high soil WFPS during March 2018 (Fig. 2). Conversely, low daily average N_2O fluxes were recorded during the drought period from June-August 2018 (Fig. 2). The highest daily average N_2O flux recorded (cumulative flux/number of days within the period) during the experimental year was from 300N *L. perenne* at 17.85 g ha^{-1} day^{-1} and the lowest was from *P. lanceolata* at 4.07 g N_2O ha^{-1} day^{-1}. The 150N *L. perenne* had a daily average N_2O flux of 5.40 g ha^{-1} day^{-1}. The highest individual N_2O measurement was from 300N *L. perenne* at 112 g ha^{-1} day^{-1} (Fig. 2) on 18th April. From April to May, (Fig. 2) the daily average N_2O flux of 300N *L. perenne* was nine times higher than the 6-species mixture and five times greater than 150N *L. perenne*. No N fertiliser was applied after September 2018 (in line with the Nitrates Directive), resulting in little to no N_2O fluxes during the autumn and winter period, except for the legume monocultures. Both clover species continued to produce N_2O emissions in the autumn/winter period (Fig. 2) with 36% of legume N_2O emissions occurring from August 2018 to January 2019, a time usually associated with low emissions.

Fig. 2. Nitrous oxide emissions and corresponding water filled pore space (WFPS %)

3.2.2 Effects of plant diversity on cumulative N_2O emissions

According to model comparisons, the best model for the N_2O emissions included species identity effects for each species and a term for 300N *L. perenne*, but no effects of species interactions were detected (model coefficient estimates shown in Table 1, first column). Thus, the effects of plant diversity on N_2O emissions were derived from species identity effects and their linear combination in mixtures, rather than from synergistic or antagonistic species interaction effects in mixtures (as shown in equation 2, but with the last term involving δ omitted). There was no significant difference between the 6-species mixture and any of the 150N monocultures, with the exception of the *C. intybus* monoculture (Fig. 3). The N_2O emissions from 300N *L. perenne* (3.18 kg ha^{-1} year^{-1}) were over twice that of the 6-species mixture (1.52 kg ha^{-1} year^{-1}) (Table 1, Fig. 3).

Table 1. (a) Coefficient estimates ± standard errors for the identity effects (β) and interaction estimates (δ)

Fig. 3. Comparison of predicted N_2O-N emissions

The highest N_2O emissions were from 300N *L. perenne* (significantly higher than all other treatments). Increasing fertiliser application to a *L. perenne* monoculture from 150N to 300N increased ($P < 0.001$) cumulative N_2O emissions from 1.39 to 3.18 N_2O-N kg ha^{-1} $year^{-1}$ (Table 1, Fig. 3). N_2O emissions from 300N *L. perenne* were nearly three times higher than those from the *C. intybus* monoculture, the latter having the lowest estimated annual emissions at 1.1 kg ha^{-1} $year^{-1}$.

The ternary diagram (Fig. 4a) displays how variation in FG proportion (grass, herb and legume) affected the predicted annual N_2O emissions; higher N_2O emissions resulted from increased legume proportion while lower emissions were associated with communities dominated by grasses and/or herbs. The annual N_2O emissions from the community comprising 100% legume FG (50% *T. pratense* and 50% *T. repens*) were significantly higher ($P = 0.033$ and $P = 0.007$) than those from the equi-proportional community of either the grass (150N *L. perenne* and *P. pratense*) or herb (*C. intybus* and *P. lanceolata*) FGs, respectively (Fig. 4b).

Fig. 4 a) Predicted annual N_2O emissions (N_2O-N kg ha^{-1} $year^{-1}$) in response to variation within grassland communities. **b)** Predicted annual N_2O emissions (N_2O-N kg ha^{-1} $year^{-1}$ from each FG: grass, legume and herb.

3.3 Yield-scaled N_2O analyses (emissions intensity)

For 2018, the average N-yield in harvested forage of the 6-species mixture, 150N *L. perenne* and 300N *L. perenne* was 40.5 kg ha^{-1} $year^{-1}$, 19.9 kg ha^{-1} $year^{-1}$ and 28.5 kg ha-

^{1} year^{-1} respectively. The 6-species mixture produced an average DM yield of 12.4 tonnes DM ha^{-1} year^{-1}, and *L. perenne* at 150N and 300N produced 9.2 and 10.7 tonnes DM ha^{-1} year^{-1} respectively (Grange et al., in review). This yield data was combined with N_2O data to calculate two measures of yield-scaled N_2O emissions; as outlined in section 2, the two measures of emission intensity analysed were N yield-scaled N_2O emissions (N_2O-N g ha^{-1} year^{-1}/ kg N yield ha^{-1} year^{-1}) and DM yield-scaled N_2O emissions (N_2O-N g ha^{-1} year^{-1}/ tonne DM yield ha^{-1} year^{-1}).

For both measures of emission intensity, the best model included species identity effects for each species, and a negative average pairwise interaction effect that resulted in an additional suppressive effect on yield-scaled N_2O emissions in mixtures (as in equation 2, with model estimates in Table 1). The suppressive interaction term was only borderline significant for the DM yield-scaled emissions ($P = 0.056$), however, diagnostic analysis of the model with and without the interaction term indicated a superior fit when the interaction term was included and it was kept in the final model. Emissions from the 6-species mixture were lower than the mean of the six 150N monocultures for N yield-scaled emissions ($P = 0.012$) and there was a similar indication for DM yield-scaled emissions ($P = 0.056$), demonstrating that increasing species diversity in multi-species mixtures suppressed yield-scaled N_2O emissions (Fig. 5a and 5b). The 300N *L. perenne* treatment had higher N yield-scaled emissions (Fig. 5a) than all other 150N communities. This result was similar for DM yield-scaled emissions (Fig. 5b), with the exception of both legume monocultures.

As the proportions of grasses, legumes and herbs change, communities with high proportions of legumes and/or grasses showed an increase in N yield-scaled N_2O emissions (Fig. 6a). Communities with high legume proportion showed an increase in DM yield-scaled N_2O emissions (Fig. 6b) compared with herbs and grasses.

Fig. 5. a and b Comparison of yield-scaled N_2O emissions.

Fig. 6. a and b Estimated emission intensity analyses (ternary diagrams).

4. DISCUSSION

4.1 N_2O emissions

Nitrous oxide emissions from multi-species mixtures were best explained as a linear combination of the identity effects, showing that there was no net synergy or antagonism attributable to interspecific interactions. Given the large magnitude of differences among the species' identity effects, emissions from mixture compositions at and around the equi-proportional 6-species mixture tended to be considerably lower than those from the highest-emitting communities (Fig. 4), e.g. those dominated by legumes. There were no net synergistic or antagonistic effects due to interspecific interactions; nevertheless, there was still a benefit from multi-species mixtures through a reduction in the proportions of higher-emitting species in the mixture, i.e., legumes (Fig. 4a and b).

Our study highlights the potential for the multi-functional benefits associated with diverse grassland communities (e.g., see Introduction) to be gained without an associated increase in N_2O emissions. Comparing N_2O emissions from the 6-species mixture and 300N *L. perenne*, greater DM yield and total N yield can be obtained from the six-species mixture while halving N_2O emissions (1.52 vs 3.18 N_2O-N kg ha^{-1} $year^{-1}$) (Table 1). The strong effect of higher fertilizer application (300N vs 150N) on N_2O emissions probably reflects non-plant uptake of excess mineral N in the soil, and these results align with the previous findings of Harty et al., (2016), Krol et al. (2016) and Cardenas et al (2020). Our results reiterate the reduced nitrogen use efficiency associated with conventional grassland *L. perenne* monocultures receiving high N fertiliser application.

Overall, increasing the proportion of both grasses and herbs within a grassland community resulted in lower N_2O emissions than in legume-dominated communities (Fig. 4a). When comparing FGs, legumes had significantly higher N_2O emissions than both herb and grass communities (Fig. 4b). When looking at selected monocultures, there was no significant difference in N_2O emissions between *P. lanceolata* and 150N *L. perenne* (Fig. 3). This is surprising given that many studies have found that swards dominated by *Plantago* species can directly reduce N_2O emissions in comparison with *L. perenne* monocultures (Gardiner et al., 2018; Luo et al., 2018). Bracken et al. (2020) found evidence that *P. lanceolata* potentially inhibits nitrification when included in a mixed sward. In contrast to aforementioned studies, the lowest N_2O emissions in our experiment were from the *C. intybus* monoculture rather than *P. lanceolata.*

When looking at annual N_2O emissions from selected individual monocultures (Fig. 3), each of *T. repens* and *T. pratense* did not have significantly higher N_2O emissions than other monocultures (with the exception of *T. pratense* being higher than *C. intybus* (Fig. 3)). This is strongly related to the much higher variability associated with legume monocultures in this experiment, compared to the other species (Table 1). If this is a general occurrence, legume monocultures may need more replication to have sufficient power to test their difference from other monocultures. When averaging over the species-to-species variation within each functional group, an effect of the legume FG on N_2O emissions of N_2O was evident (Fig. 4a, b). Many studies report high N_2O emissions from legume-based pastures due to the accumulation of nitrate following mineralization of biologically fixed organic N (e.g., Dalal et al., 2003; Burchill et al., 2014).

Greater variation in N_2O emissions from legumes may stem from the high levels of variation associated with BNF (and therefore N_2O emissions from legume stands) (Rochette et al., 2004; Unkovich et al., 2008; Evers., 2011; Nyfeler et al., 2011;). Across studies, the rate of BNF can range from 100 to 380 kg N ha^{-1} $year^{-1}$ in northern temperate pastures and is dependent on multiple factors including environmental conditions, grassland management practices and legume species/cultivar type and proportion (Ledgard and Steele 1992; Hansen and Vinther 2001; Fox et al., 2019). Nitrous oxide fluxes associated with legumes can be attributed to N release from root exudates and from crop residue decomposition, rather than from the BNF process itself (Rochette and Jansen., 2005). The latter results were pivotal in the removal of the BNF process from the Intergovernmental Panel on Climate Change (IPCC) N_2O inventory methodology. Legume inclusion within a grassland community allows for N addition into the soil system by BNF, lowering reliance on N fertilizer application without compromising on yield (Egan et al., 2018). Legume residues can improve the quality and quantity of soil organic matter over time, providing benefits for following crops (termed ‘legacy effect’). Fox et al. (2019) assessed legacy effects over a range of legume proportions and N application levels, and found maximum legacy benefits on a *L. multiflorum* crop from a prior grassland ley comprising 50% legume proportion and receiving 150N. Our study should be considered when using legumes as ley cover crops as environmental benefits of N addition to soil (i.e., less fertilizer N requirement) may be compromised by high N_2O losses.

4.2 Yield-scaled N_2O emissions (emissions intensity)

The effects of multi-species swards were more pronounced when considering yield-scaled N_2O emissions (expressed as either N_2O-N g ha^{-1} $year^{-1}$ / N yield kg ha^{-1} $year^{-1}$ or N_2O-N g ha^{-1} $year^{-1}$ / DM yield tonne ha^{-1} $year^{-1}$). Looking at both responses, the performance of mixtures was best explained as a linear combination of the identity effects, and an additional antagonistic interaction between species that acted to suppress emissions intensity. Thus, compared to the yield-scaled N_2O emissions predicted from the average of the six monocultures, the yield-scaled N_2O emissions of the 6-species mixture were 29.1 % lower ($P = 0.012$) and 24.9% lower ($P = 0.056$) for the N- and DM yield-scaled measures respectively (Table 1). Given the differences among the species, mixture compositions at and around the 6-species mixture tended to have considerably lower yield-scaled N_2O emissions (Fig. 6a and b). As both yield-scaled N_2O responses had the same numerator (N_2O emissions) the significant diversity effect must be related to a strong effect of plant diversity (interspecific interactions) on each of the denominators, total N yield and total DM yield (presented elsewhere for both the former (Grange et al., unpubl.) and latter (Grange et al., in review)). Transgressive over yielding, whereby mixtures outperform the highest performing constituent monoculture, is driven by resource use efficiency and complementarity among species in mixtures (Mason et al., 2020).

Our study confirms that reduced emissions intensity can now be considered one of the many multi-functional benefits associated with multi-species swards. The 6-species mixture was more efficient, because more yield was produced with reductions in N_2O losses to the environment. Overall, the six-species mixture significantly reduced N yield-scaled N_2O emissions compared with *L. perenne* (both 150N and 300N) and 150N legume monocultures (Fig. 5a) and lower DM yield-scaled N_2O emissions than 300N *L. perenne* (Fig. 5b). These results accord with the agronomic assessment of N_2O emissions by van Groenigen et al (2010), where yield-scaled N_2O emissions of non-leguminous crops increased rapidly at higher N application levels (>190 kg N ha^{-1} year-^{1}). As a practical consequence, in comparison to 300N *L. perenne*, the same N yield or DM yield could have been produced with the 150N six-species mixture using half the amount of fertiliser and reducing N_2O losses by 63% and 58% respectively. Similarly, in comparison to 150N *L. perenne,* the same N yield or DM yield could have been produced with the six-species mixture while reducing N_2O losses by 41% and 24% respectively.

5. Conclusion

Overall, the effect of plant diversity on N_2O emissions was derived from linear combinations of the species' performance in monoculture (species' identity). The effects of multi-species mixtures on N_2O emissions intensity included species identity effects, and a net interspecific interaction that suppressed emissions intensity. The conventional 300N *L. perenne* community produced over double the N_2O emissions as the 150N six-species mixture (3.18 vs 1.52 kg N_2O-N ha^{-1} year^{-1}). Considering emissions intensity, the same N yield and DM yield of 300N *L. perenne* could have been produced with the 6-species mixture using half the fertiliser and reduced N_2O losses of 63% and 58% respectively. In comparison to 150N *L. perenne*, the same N yield and DM yield could have been produced with a 6-species mixture while producing 41% and 24% less N_2O emissions. Communities dominated by legumes significantly increased N_2O emissions, this should be considered when using legumes as cover crops. Overall, the manipulation of grassland composition is a practical, farm-scale management action that can reduce both N_2O emissions and yield-scaled N_2O emissions, and contribute to the sustainability of grassland production.

6. Acknowledgements

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7. Appendices

Appendix A Seeding rates of the multi-species experimental field trial. Species include *L. perenne* (Lp) *P. pratense* (Pp), *T. pratense* (Tr), *T. repens* (Tr), *P. lanceolata* (Pl), and *C. intybus* (Ci).

Species	Lp	Pp	Tp	Tr	Pl	Ci
Seed (Kg ha^{-1})	28	12	12	15	10	8

Appendix B Experimental design indicating the composition and relative abundance of the sown communities. Also indicated are functional group richness (FGs), species richness (Species) and number of replicates (Reps). Species include *L. perenne* (Lp) *P. pratense* (Pp), *T. pratense* (Tr), *T. repens* (Tr), *P. lanceolata* (Pl), and *C. intybus* (Ci).

Community	Reps	FGs	Species	FG(G)	FG(H)	FG(L)	Lp	Pp	Tp	Tr	Pl	Ci
1	3	1	1	1	0	0	1	0	0	0	0	0
2	3	1	1	1	0	0	0	1	0	0	0	0
3	3	1	1	0	1	0	0	0	1	0	0	0
4	3	1	1	0	1	0	0	0	0	1	0	0
5	3	1	1	0	0	1	0	0	0	0	1	0
6	3	1	1	0	0	1	0	0	0	0	0	1
7	2	1	2	1	0	0	0.5	0.5	0	0	0	0
8	2	1	2	0	1	0	0	0	0.5	0.5	0	0
9	2	1	2	0	0	1	0	0	0	0	0.5	0.5
10	2	2	4	0.5	0.5	0	0.25	0.25	0.25	0.25	0	0
11	2	2	4	0.5	0	0.5	0.25	0.25	0	0	0.25	0.25
12	2	2	4	0	0.5	0.5	0	0	0.25	0.25	0.25	0.25
13	1	3	5	0.6	0.2	0.2	0.6	0	0.1	0.1	0.1	0.1
14	1	3	5	0.6	0.2	0.2	0	0.6	0.1	0.1	0.1	0.1
15	1	3	5	0.2	0.6	0.2	0.1	0.1	0.6	0	0.1	0.1
16	1	3	5	0.2	0.6	0.2	0.1	0.1	0	0.6	0.1	0.1
17	1	3	5	0.2	0.2	0.6	0.1	0.1	0.1	0.1	0.6	0
18	1	3	5	0.2	0.2	0.6	0.1	0.1	0.1	0.1	0	0.6
19	3	3	6	0.33	0.33	0.33	0.17	0.17	0.17	0.17	0.17	0.17
20	4	1	1	1	0	0	1	0	0	0	0	0

Appendix C Fertiliser application rate equivalents to each plot over the agronomic year. (The community numbers are as listed in Appendix B.)

Split	Date	Fertiliser application: communities 1-19	Fertiliser application: community 20
1	12-Mar-2018	30 kg N ha^{-1}	60 kg N ha^{-1}
2	09-Apr-2018	30 kg N ha^{-1}	60 kg N ha^{-1}
3	09-May-2018	30 kg N ha^{-1}	60 kg N ha^{-1}
4	11-Jun-2018	20 kg N ha^{-1}	40 kg N ha^{-1}
5	20-Aug-2018	40 kg N ha^{-1}	80 kg N ha^{-1}

Appendix D Aerial photograph of the experimental plot layout.

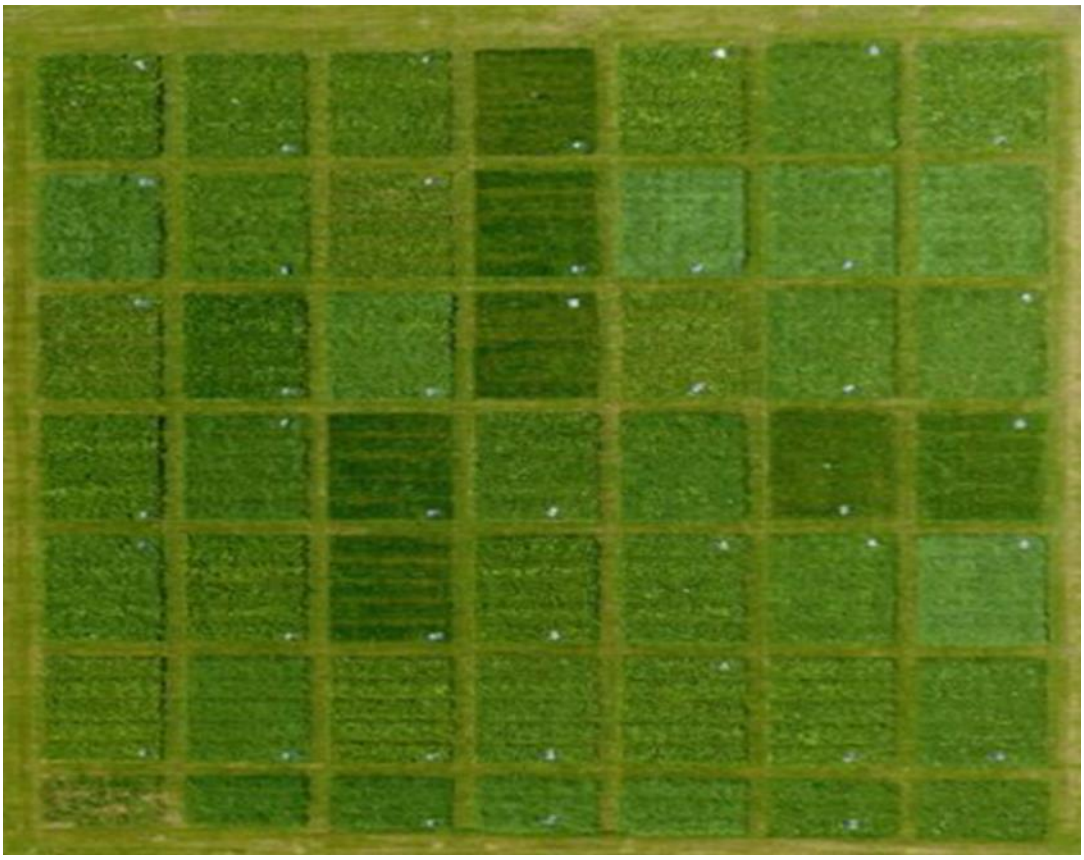

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Figure legends

Fig. 1. Precipitation (mm) and temperature (°C) meteorological data for the experimental site collected from the JC meteorological station. Graph includes 3 x 30mm irrigation events which took place during summer 2018 of (due to drought).

Fig. 2. Nitrous oxide emissions and corresponding water filled pore space (WFPS %) of the experimental site for each sampling occasion. Emissions are displayed for the 6-species mixture, monocultures of the individual species (*L. perenne, P. pratense, T. pratense, T. repens, C. intybus* and *P. lanceolate*) and the 300N *L. perenne* monoculture. All communities received 150 kg ha^{-1} year^{-1} nitrogen fertiliser, except for the 300N *L. perenne* community that received 300 kg ha^{-1} year^{-1} of inorganic nitrogen fertiliser. Arrows indicate fertiliser application dates (*Appendix C).*

Fig. 3. Comparison of predicted N_2O-N emissions (kg ha^{-1} year^{-1}) from monocultures and the 6-species mixture. Values that share the same letter are not significantly different ($\alpha = 0.05$).

Fig. 4 a) Predicted annual N_2O emissions (N_2O-N kg ha^{-1} year^{-1}) in response to variation in the proportion of the grass (G), herb (H) and legume (L) FGs within grassland communities. The communities represented in this ternary diagram are based on the equi-proportional contribution of each of the two species within a FG. Thus, each vertex represents a 50:50 mixture of the two component species in the respective FG; the sides represent communities with varying proportions of two FGs (comprising four species), and the interior points represent varying proportions of three FGs (comprising six species). Thus, for example, the predicted N_2O emissions for the community comprising

10% grass, 40% legume and 50% herb is calculated from the species-level composition comprising 5% *L. perenne,* 5% *P. pratense,* 20% *T. pratense*, 20% *T. repens*, 25% *C. intybus* and 25% *P. lanceolata.* **b)** Predicted annual N_2O emissions (N_2O-N kg ha^{-1} year^{-1}) for a 50:50 mixture of the two species from each FG: grass, legume and herb (these predictions correspond to the vertices in the ternary diagram). For example, the legume FG contains 50% *T. pratense* and 50% *T. repens*. Bars that share a letter are not significantly different ($\alpha = 0.05$).

Fig. 5. a and b Comparison of yield-scaled N_2O emissions from forage monocultures, the 6-species mixture and the mean of the six 150N monocultures for **a)** N yield-scaled N_2O emissions (N_2O-N g ha^{-1}year^{-1}/N yield kg ha^{-1} year^{-1}) and **b)** DM yield-scaled N_2O emissions (N_2O-N g ha^{-1} year^{-1}/DM yield tonne ha^{-1} year^{-1}). Values that share the same letter are not significantly different ($\alpha = 0.05$).

Fig. 6. Estimated emission intensity analyses for **a)** N yield-scaled N_2O emissions (N_2O g ha^{-1} year^{-1} / N yield kg ha^{-1} year^{-1}) and **b)** DM yield-scaled N_2O emissions (N_2O gha^{-1} year^{-1}/DM yield tonne ha^{-1} year^{-1}) in response to variation in the proportion of grass (G), herb (H) and legume (L) FGs within grassland communities. The communities represented in this ternary diagram are based on an equal proportional contribution of each of the two species within a FG. Thus, each vertex indicates the average of the two component species in the respective FG; the sides represent communities with varying proportions of two FGs (comprising four species), and the interior points represent varying proportions of three FGs (comprising six species).

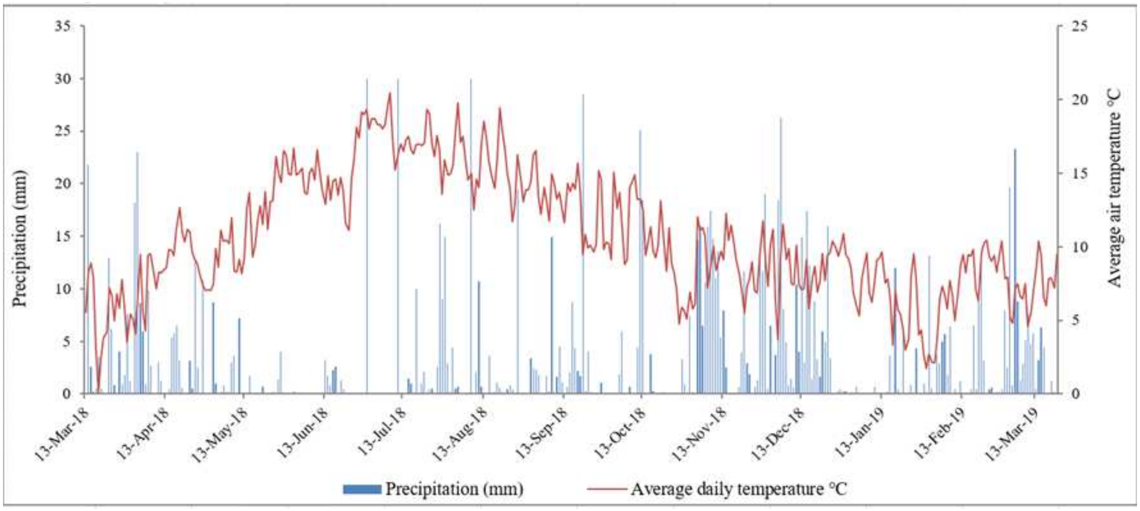
Precipitation (mm)
Average air temperature °C
35
30
25
20
15
10
5
0
25
20
15
10
5
0
13-Mar-18
13-Apr-18
13-May-18
13-Jun-18
13-Jul-18
13-Aug-18
13-Sep-18
13-Oct-18
13-Nov-18
13-Dec-18
13-Jan-19
13-Feb-19
13-Mar-19
Precipitation (mm)
Average daily temperature °C

Fig. 1

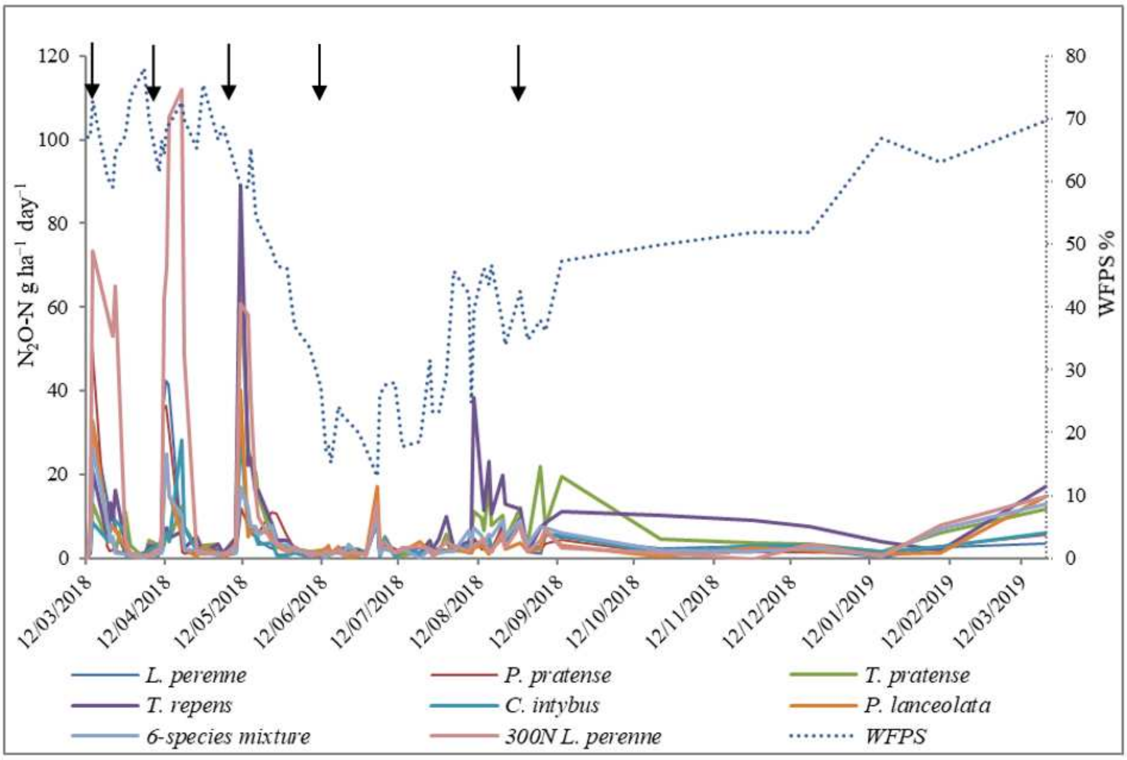
N$_2$O-N g ha^{-1} day^{-1}
WFPS %
0
20
40
60
80
100
120
0
10
20
30
40
50
60
70
80
12/03/2018
12/04/2018
12/05/2018
12/06/2018
12/07/2018
12/08/2018
12/09/2018
12/10/2018
12/11/2018
12/12/2018
12/01/2019
12/02/2019
12/03/2019
L. perenne
P. pratense
T. pratense
T. repens
C. intybus
P. lanceolata
6-species mixture
300N L. perenne
WFPS

Fig. 2

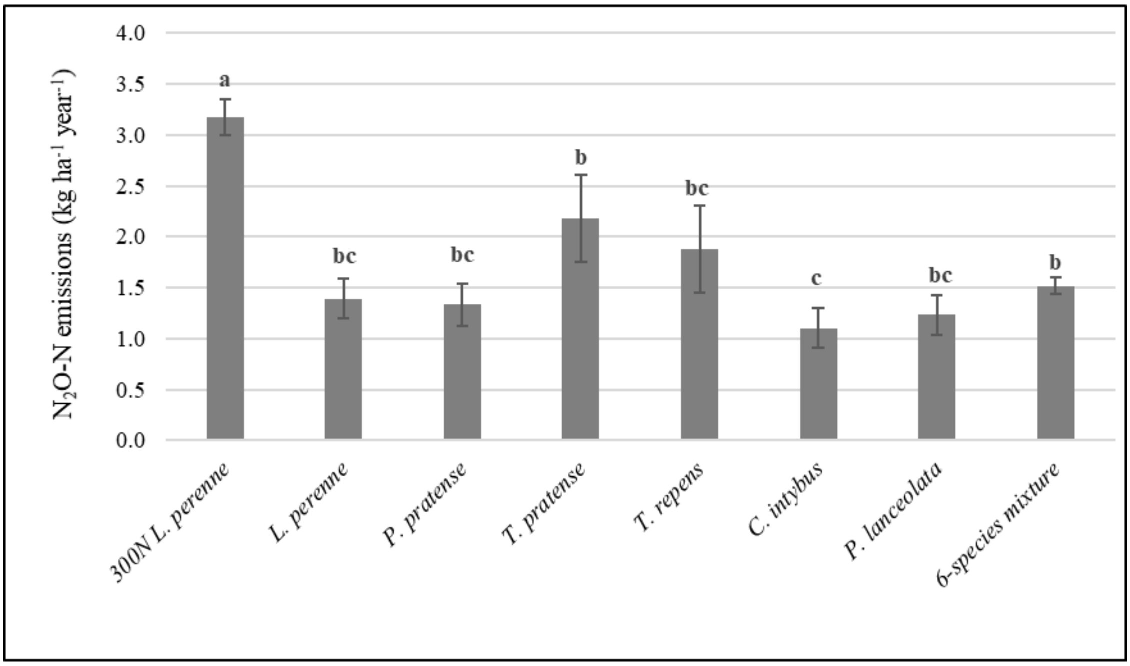
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
N_2O-N emissions (kg ha^{-1} year^{-1})
a
bc
bc
b
bc
c
bc
b
300N L. perenne
L. perenne
P. pratense
T. pratense
T. repens
C. intybus
P. lanceolata
6-species mixture

Fig. 3

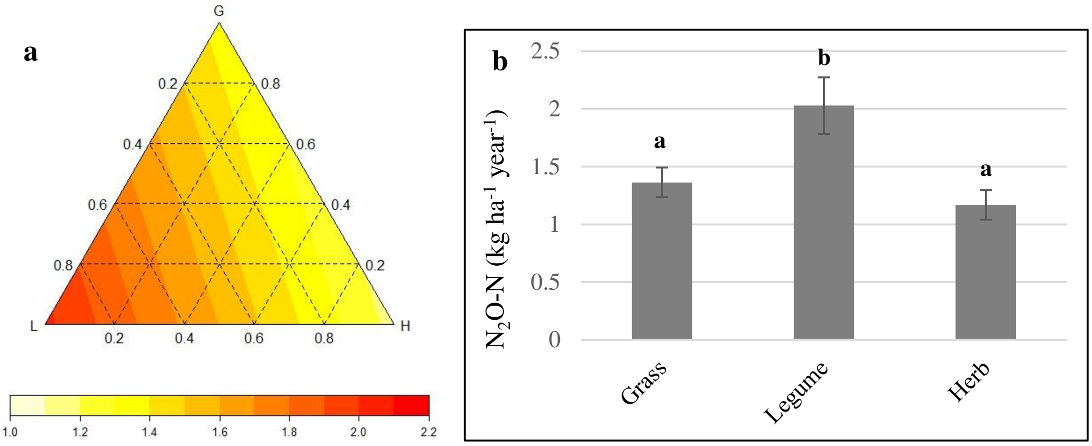
a
G
L
H
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
b
N_2O-N (kg ha^{-1} year^{-1})
0
0.5
1
1.5
2
2.5
a
b
a
Grass
Legume
Herb

Fig. 4

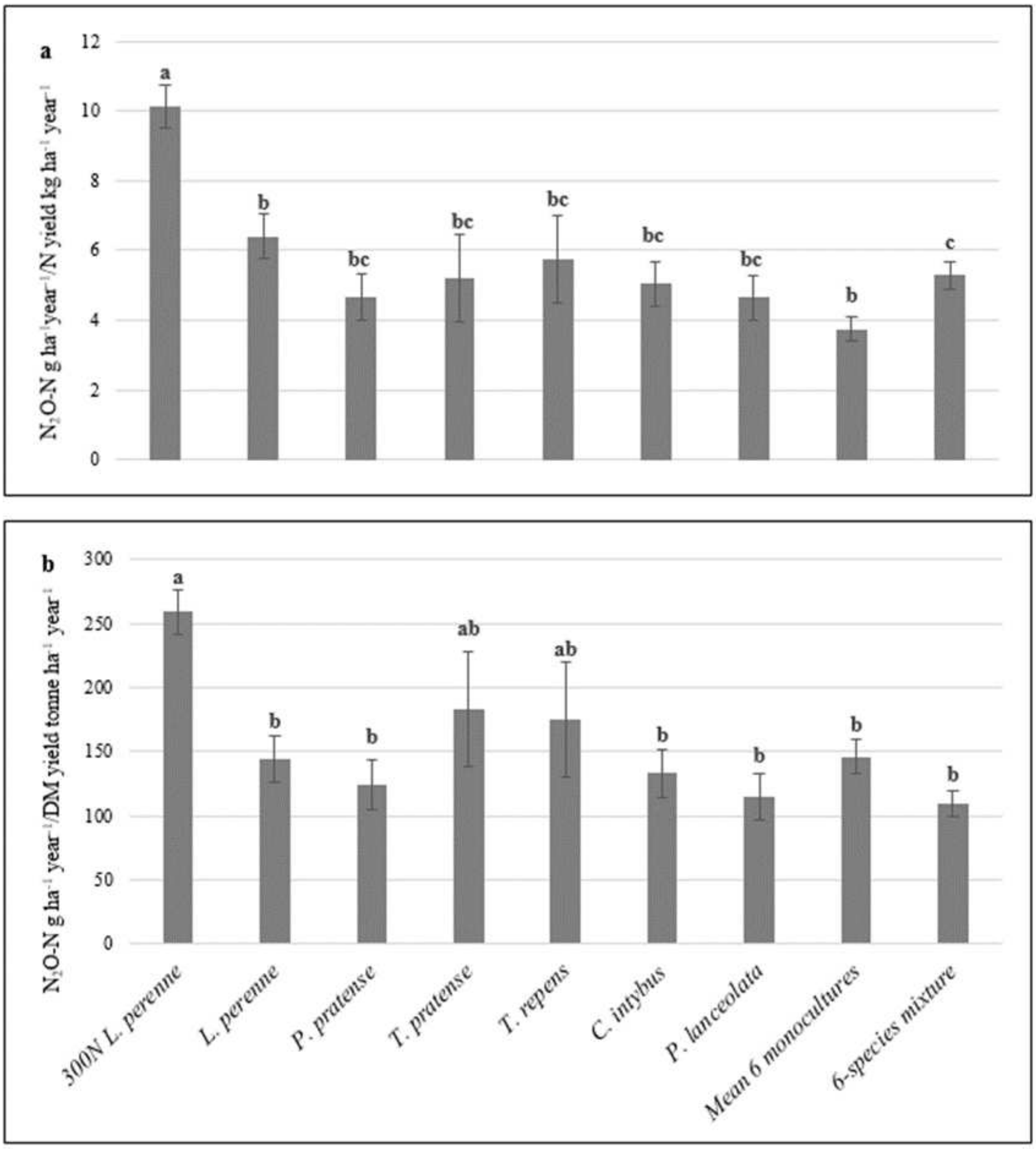
a
N2O-N g ha-1 year-1/N yield kg ha-1 year-1
0
2
4
6
8
10
12
a
b
bc
bc
bc
bc
bc
b
c
b
N2O-N g ha-1 year-1/DM yield tonne ha-1 year-1
0
50
100
150
200
250
300
a
b
b
ab
ab
b
b
b
b
300N L. perenne
L. perenne
P. pratense
T. pratense
T. repens
C. intybus
P. lanceolata
Mean 6 monocultures
6-species mixture

Fig. 5

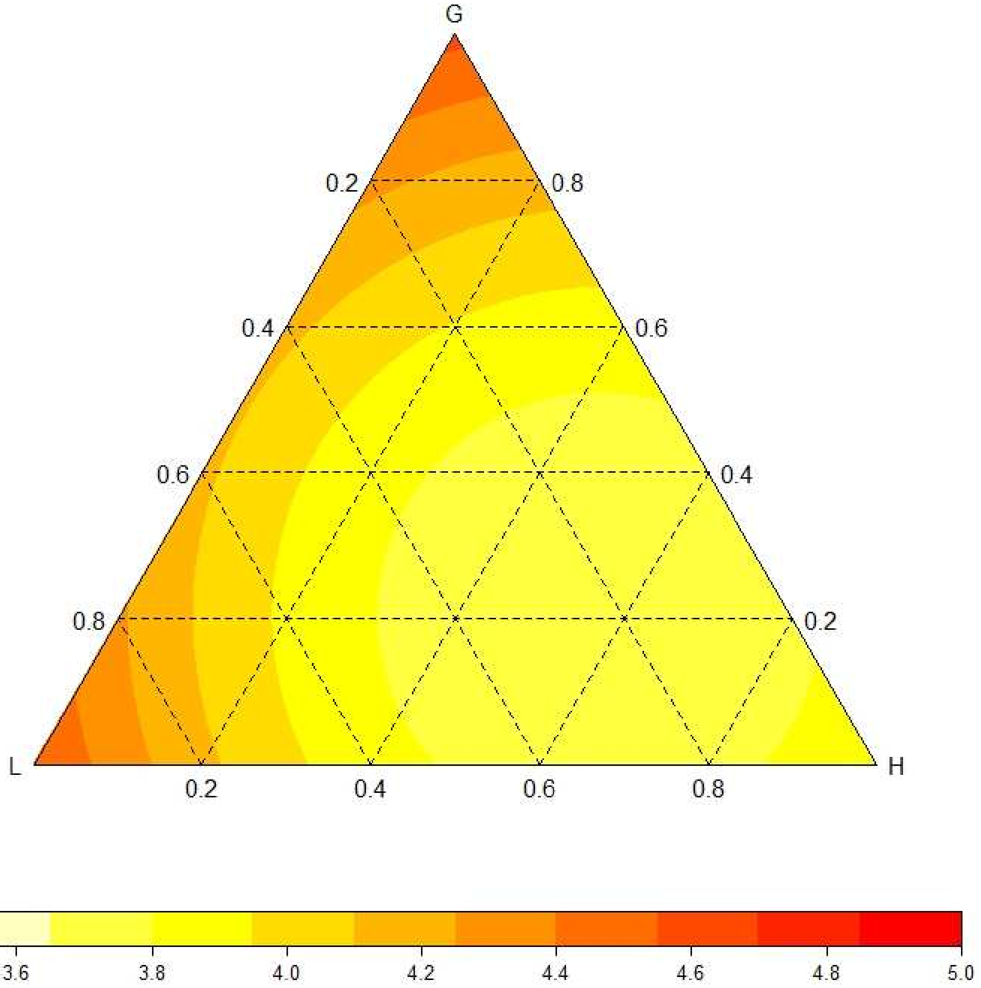

Fig 6a

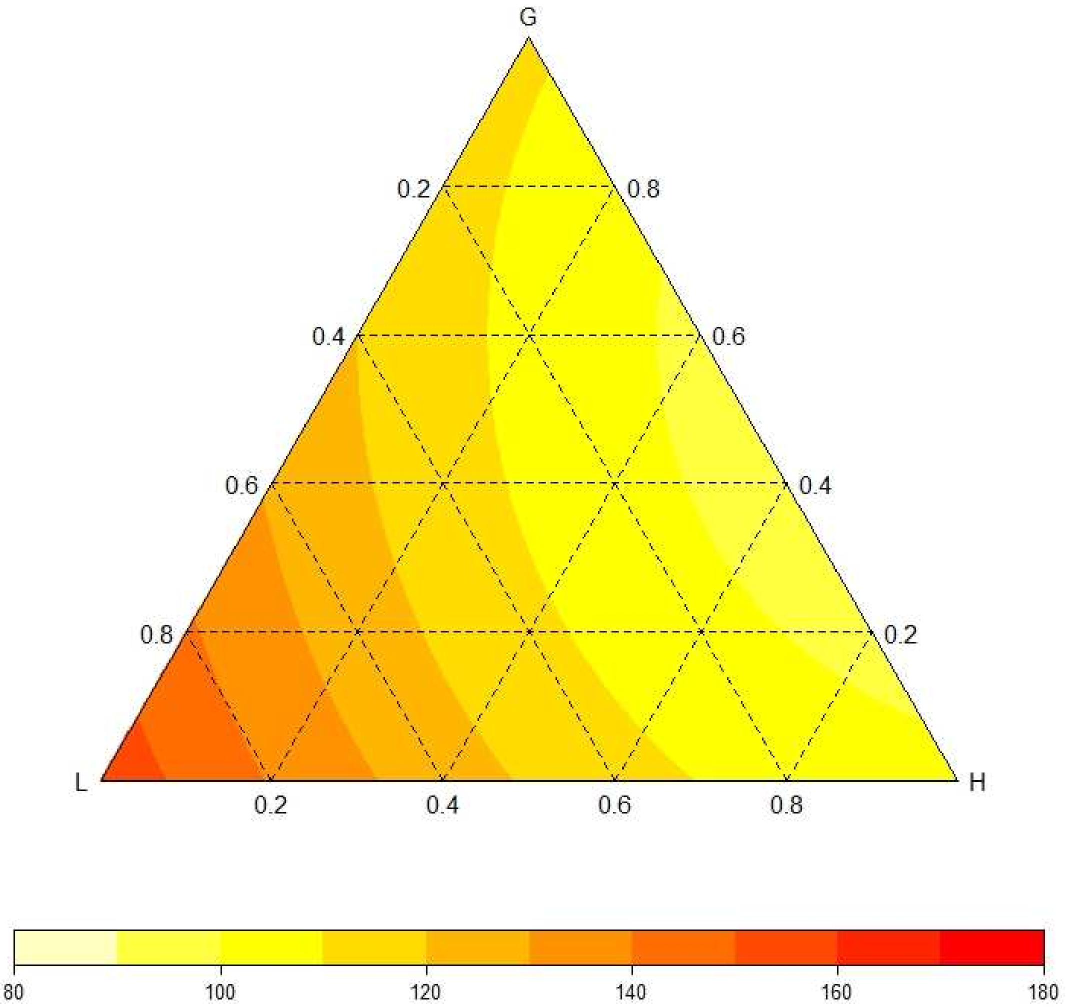

Fig. 6b

Table 1. (a) Coefficient estimates ± standard errors for the identity effects (β) and interaction estimates (δ) from equation 2, and (b) predictions for the average monoculture and the equi-proportional 6-species mixtures. These are presented for the models fitted to each of the three responses: N_2O-N emissions, N yield-scaled N_2O emissions and DM yield-scaled N_2O emissions.

		Modelled estimates		
		N_2O emissions (N_2O-N kg ha^{-1} year^{-1})	**N yield-scaled N_2O emissions** (N_2O-N g ha^{-1} year^{-1}/N yield kg ha^{-1} year^{-1})	**Dm yield-scaled N_2O emissions** (N_2O-N g ha^{-1} year^{-1}/DM yield tonne ha^{-1} year^{-1})
(a)	300N *L. perenne*	3.18 ± 0.196	10.14 ± 0.603	259.3 ± 17.46
	150N *L. perenne*	1.39 ± 0.198	6.39 ± 0.644	144.4 ± 18.68
	P. pratense	1.33 ± 0.206	4.65 ± 0.659	124.1 ± 19.11
	T. pratense	2.18 ± 0.428	5.21 ± 1.269	183.6 ± 44.95
	T. repens	1.87 ± 0.428	5.76 ± 1.269	174.6 ± 44.95
	C. intybus	1.10 ± 0.197	5.04 ± 0.640	133.2 ± 18.57
	P. lanceolata	1.23 ± 0.197	4.66 ± 0.640	115.0 ± 18.57
	Species interaction effect δ	n/a	-3.69 ± 1.394	-87.1 ± 43.98
(b)	6-species mixture	1.52 ± 0.083	3.75 ± 0.356	109.5 ± 10.33
	Mean of 6 monocultures	1.52 ± 0.083	5.29 ± 0.401	145.8 ± 13.51

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Chris K. Reynolds: methodology, writing- reviewing and editing

Dominika J. Krol: supervision, methodology, writing- reviewing and editing

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: