# *Beneficial effects of multi-species mixtures on N2O emissions from intensively managed grassland swards*

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Title Page

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

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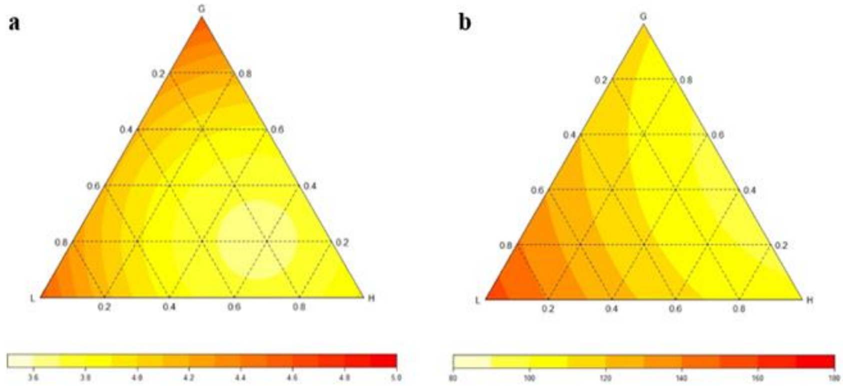
**KEY WORDS**

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).

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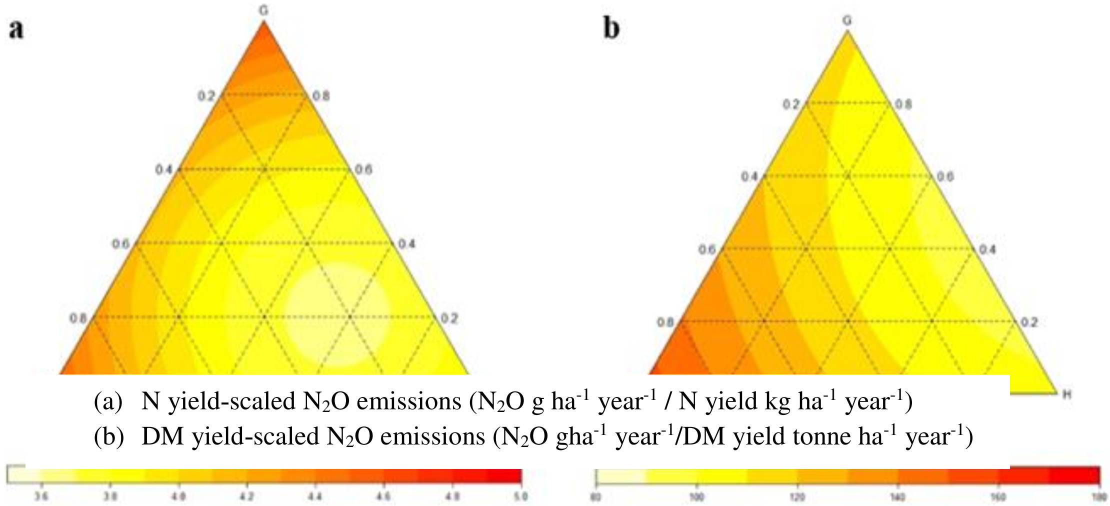


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# ABSTRACT

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 13$^{th}$ March 2018 – 21$^{st}$ 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 $\beta_1$ to $\beta_6$ coefficient are the identity effects of each species (under 150N fertiliser); if $P_i = 1$, the $\beta_i$ 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 ($\beta_7$) 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 $\delta$). 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 $\sigma^2$. 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 18$^{th}$ 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 $\delta$ 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 ($\beta$) and interaction estimates ($\delta$)

**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 |

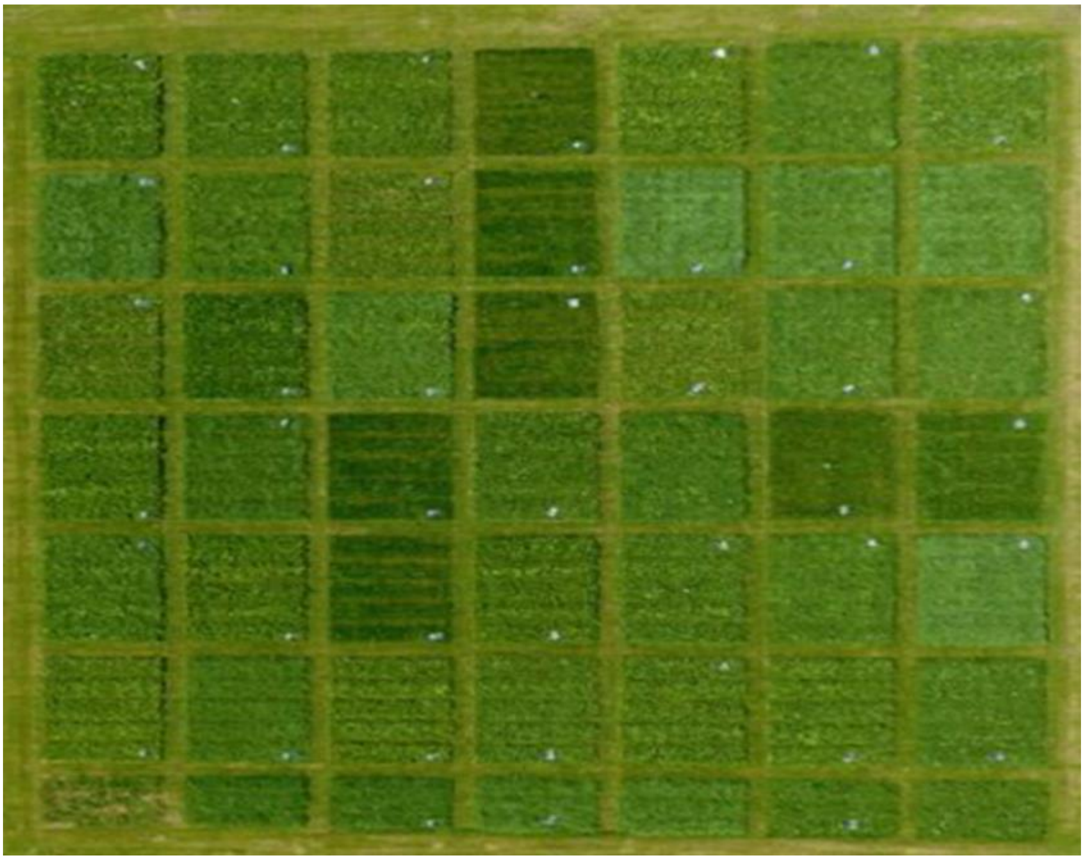
**Appendix B** Experimental design indicating the composition and relative abundance of the sown communities. Also indicated are functional group richness (FGs), species richness (Species) and number of replicates (Reps). Species include *L. perenne* (Lp) *P. pratense* (Pp), *T. pratense* (Tr), *T. repens*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

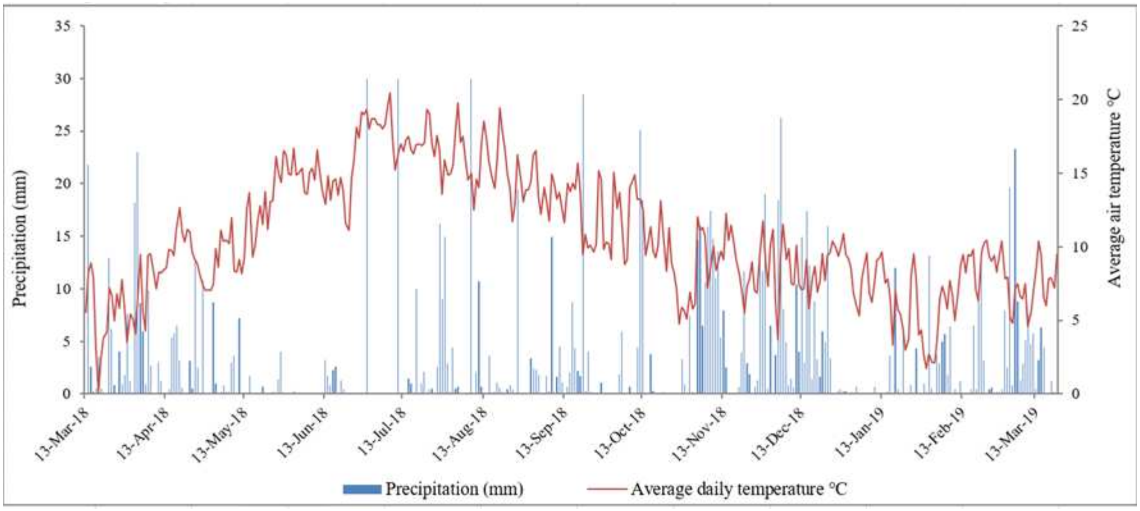
**Fig. 3.** Comparison of predicted $N_2O$-N emissions (kg $ha^{-1}$ $year^{-1}$) from monocultures and the 6-species mixture. Values that share the same letter are not significantly different ($\alpha = 0.05$).

**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

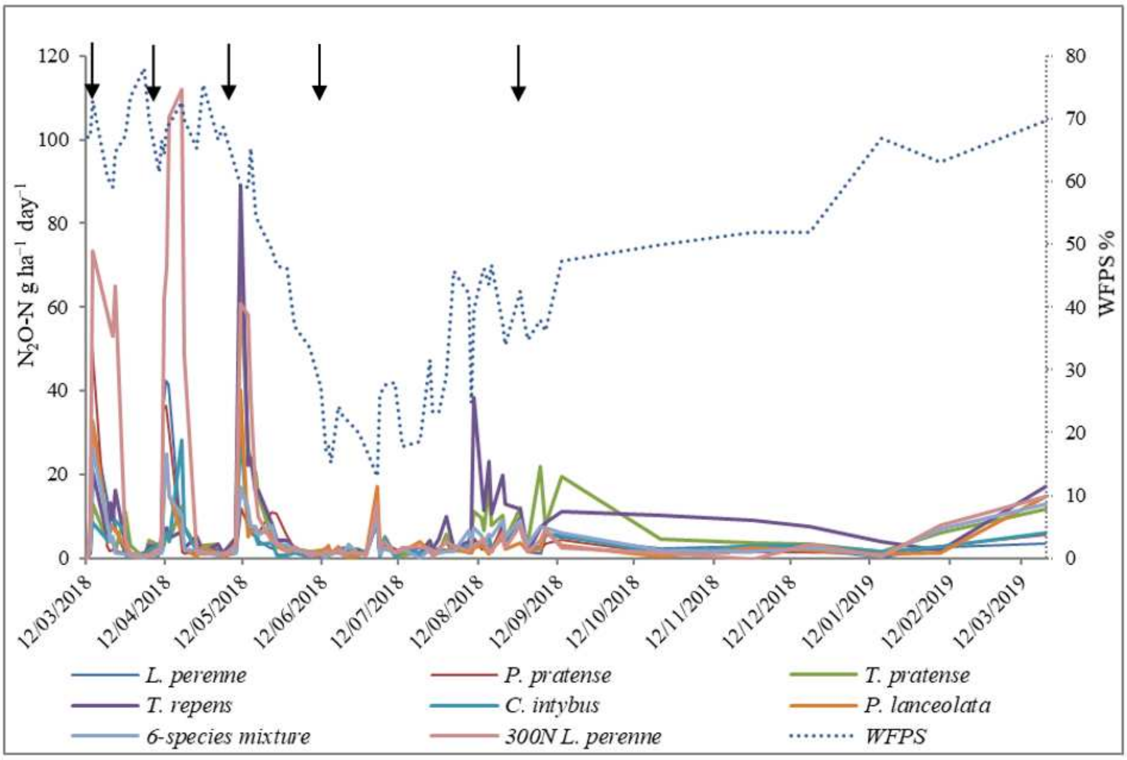
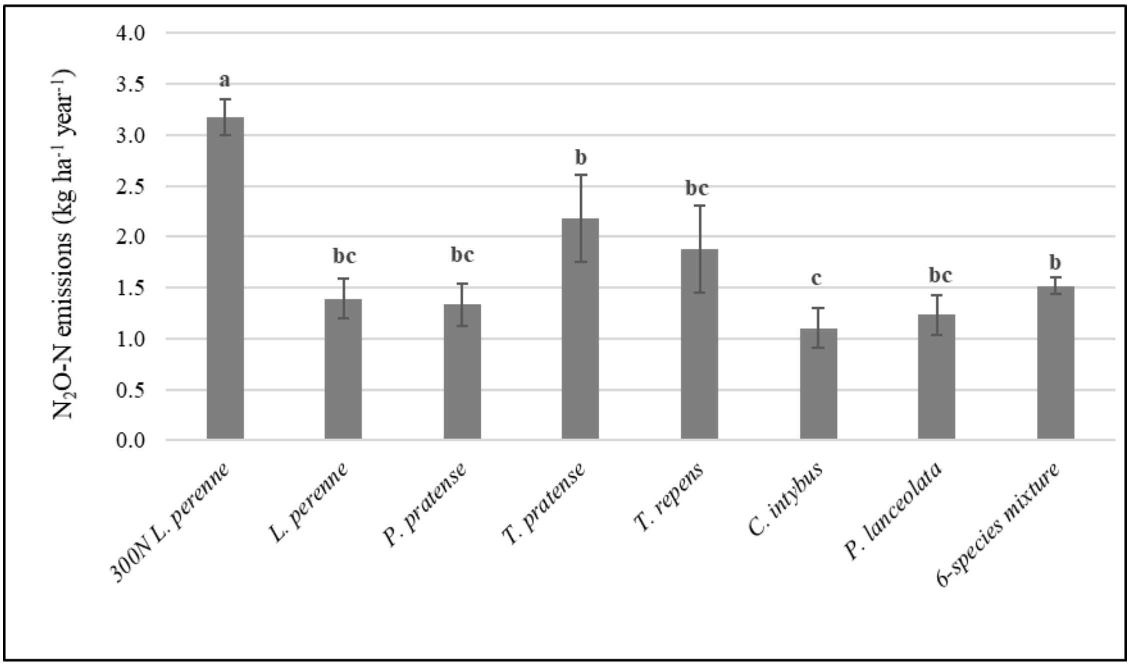

120
100
80
60
40
20
0
N2O-N g ha-1 day-1
80
70
60
50
40
30
20
10
0
WFPS %
12/03/2018
12/04/2018
12/05/2018
12/06/2018
12/07/2018
12/08/2018
12/09/2018
12/10/2018
12/11/2018
12/12/2018
12/01/2019
12/02/2019
12/03/2019
L. perenne
P. pratense
T. pratense
T. repens
C. intybus
P. lanceolata
6-species mixture
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**

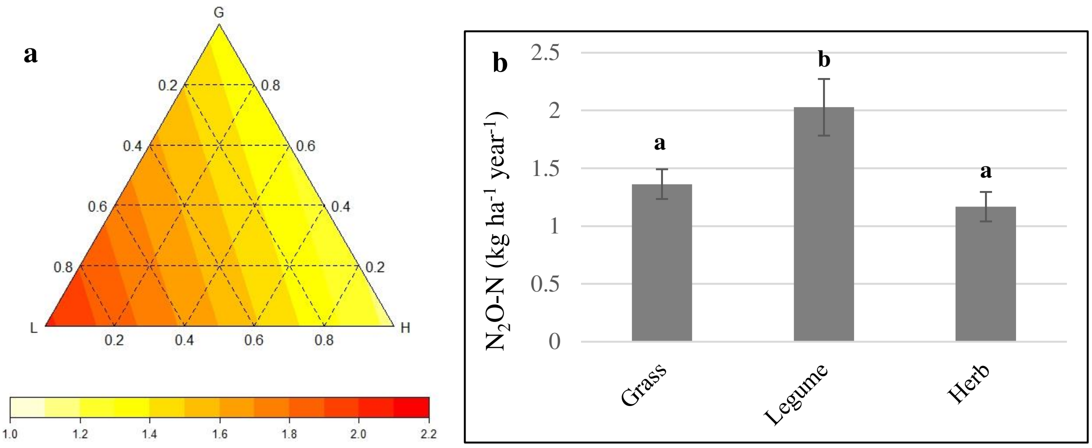

a
G
L
H
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
b
$N_2O$-N (kg ha$^{-1}$ year$^{-1}$)
0
0.5
1
1.5
2
2.5
a
b
a
Grass
Legume
Herb

Fig. 4

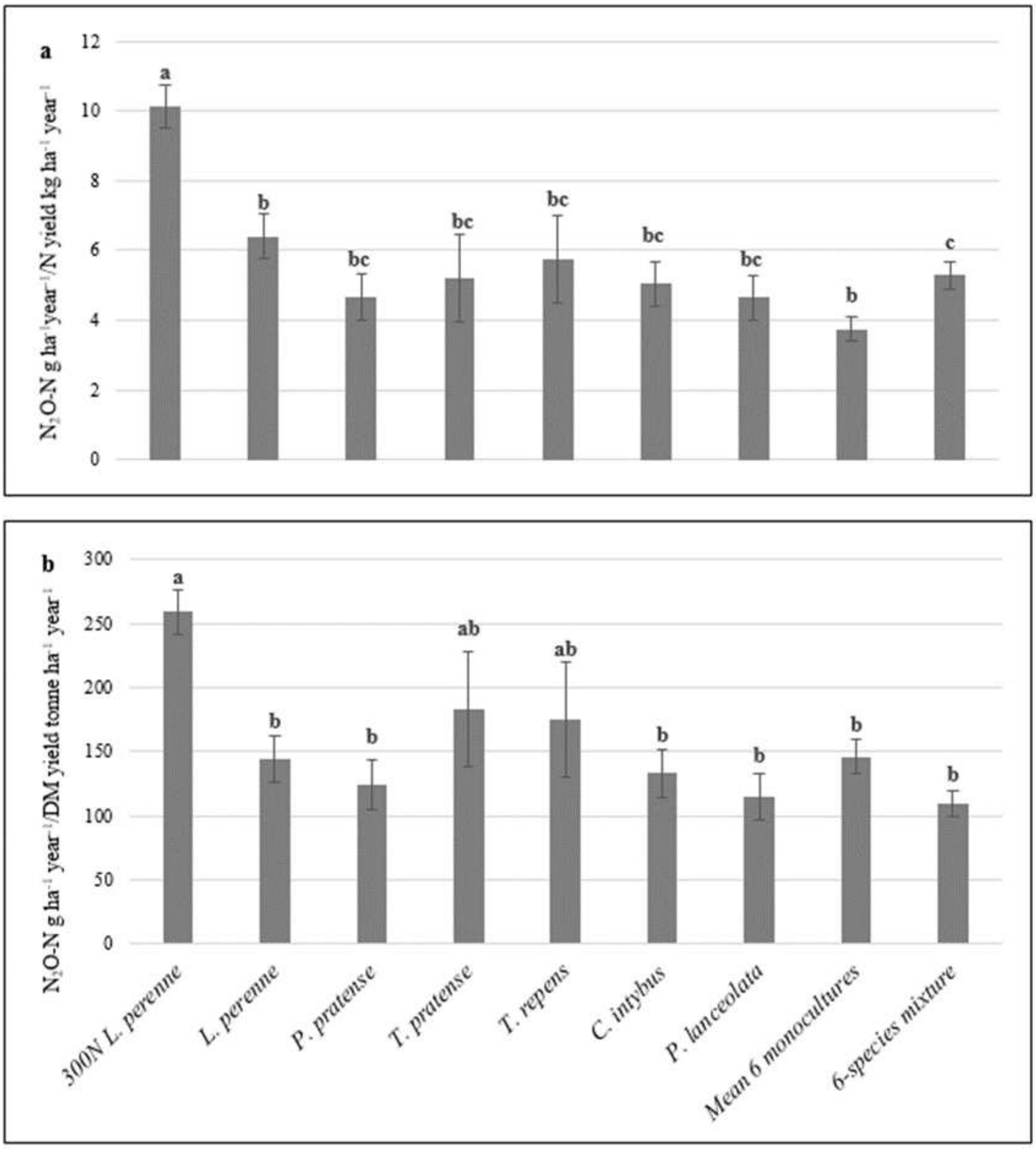

a
N$_2$O-N g ha$^{-1}$ year$^{-1}$/N yield kg ha$^{-1}$ year$^{-1}$
0
2
4
6
8
10
12
a
b
bc
bc
bc
bc
bc
b
c
b
N$_2$O-N g ha$^{-1}$ year$^{-1}$/DM yield tonne ha$^{-1}$ year$^{-1}$
0
50
100
150
200
250
300
a
b
b
ab
ab
b
b
b
b
300N L. perenne
L. perenne
P. pratense
T. pratense
T. repens
C. intybus
P. lanceolata
Mean 6 monocultures
6-species mixture

Fig. 5

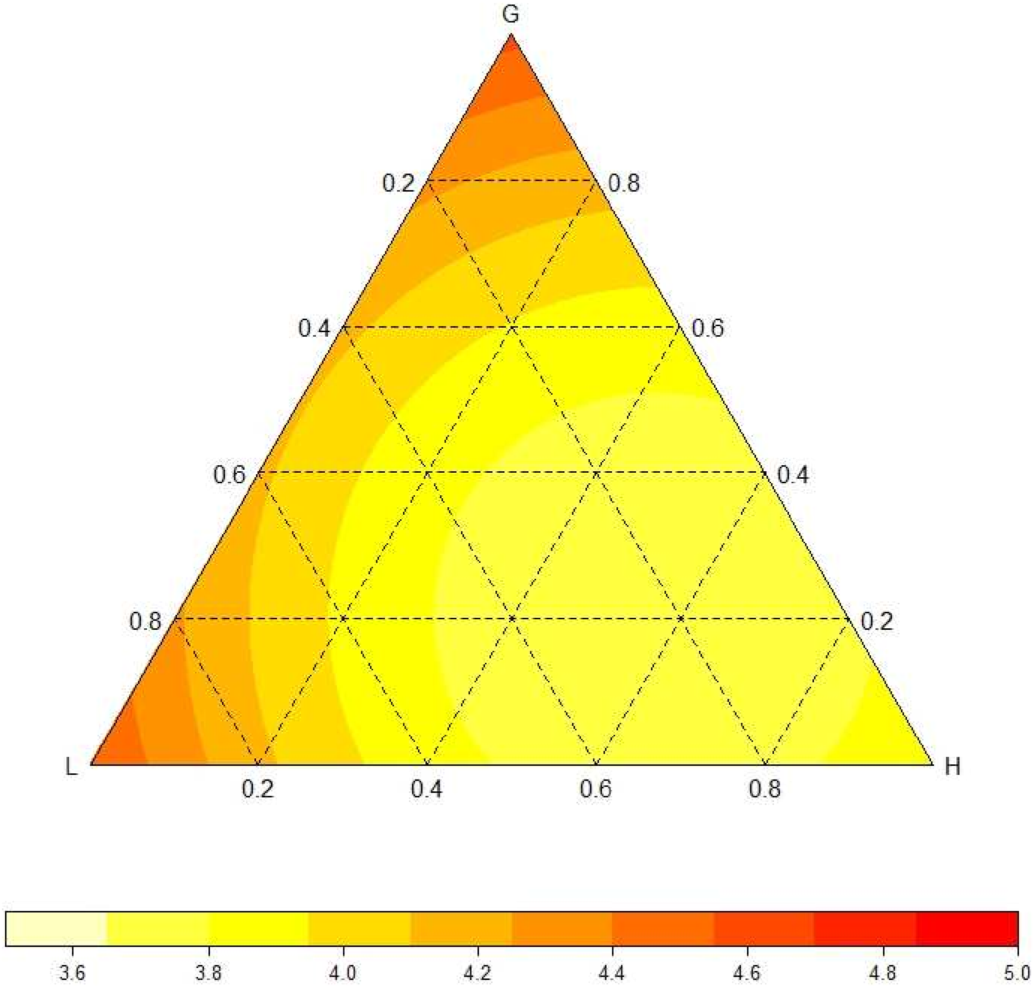



Fig 6a

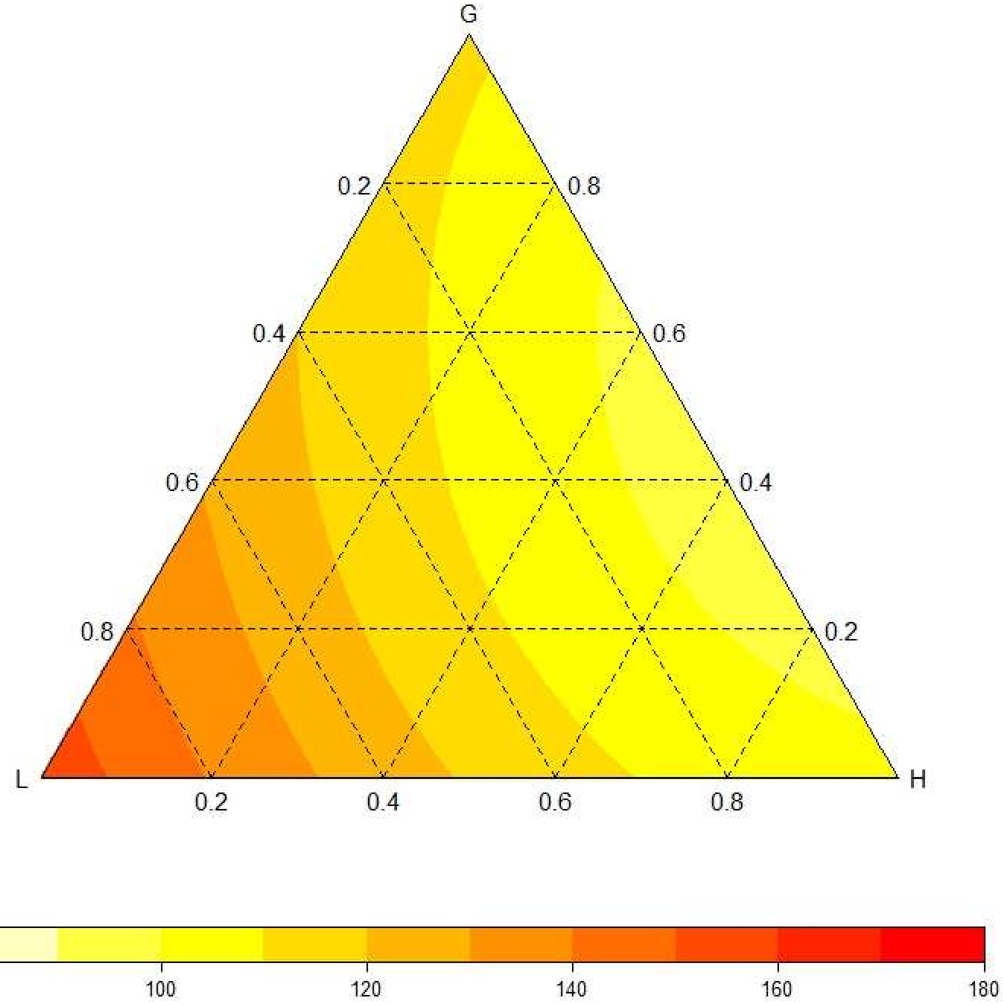



**Fig. 6b**

**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 |

**Saoirse Cummins:** data curation (nitrous oxide), formal analysis, visualization, writing – original draft preparation.

**John A. Finn:** supervision, methodology, formal analysis, visualization, writing- reviewing and editing

**Karl G. Richards:** supervision, methodology, writing- reviewing and editing

**Gary J**. **Lanigan:** supervision, methodology, writing- reviewing and editing

**Guylain Grange:** data curation (yield)

**Caroline Brophy**: methodology, formal analysis

**Laura M. Cardenas:** methodology, writing- reviewing and editing

**Tom H. Misselbrook:** methodology, writing- reviewing and editing

**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: