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Title: Sward composition and soil moisture conditions affect nitrous oxide emissions and soil nitrogen dynamics following urea-nitrogen application

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Abstract

Increased emissions of N_2O, a potent greenhouse gas (GHG), from agricultural soils is a major concern for the sustainability of grassland agriculture. Emissions of N_2O are closely associated with the rates and forms of N fertilisers applied as well as prevailing weather and soil conditions. Evidence suggests that multispecies swards require less fertiliser N input, and may cycle N differently, thus reducing N loss to the environment. This study used a restricted simplex-centroid experimental design to investigate N_2O emissions and soil N cycling following application of urea-N (40 kg N ha^{-1}) to eight experimental swards (7.8 m^2) with differing proportions of three plant functional groups (grass, legume, herb) represented by perennial ryegrass (PRG, *Lolium perenne*), white clover (WC, *Trifolium repens*) and ribwort plantain (PLAN, *Plantago lanceolata*), respectively. Swards were maintained under two contrasting soil moisture conditions to examine the balance between nitrification and denitrification. Two N_2O peaks coincided with fertiliser application and heavy rainfall events; 13.4 and 17.7 g N_2O-N ha^{-1} day^{-1} (ambient soil moisture) and 39.8 and 86.9 g N_2O-N ha^{-1} day^{-1} (wet soil moisture). Overall, cumulative N_2O emissions post-fertiliser application were higher under wet soil conditions. Increasing legume (WC) proportions from 0% to 60% in multispecies swards resulted in model predicted N_2O emissions increasing from 22.3 to 96.2 g N_2O-N ha^{-1} (ambient soil conditions) and from 59.0 to 219.3 g N_2O-N ha^{-1} (wet soil conditions), after a uniform N application rate. Soil N dynamics support denitrification as the dominant source of N_2O especially under wet soil conditions. Significant interactions of PRG or WC with PLAN on soil mineral N concentrations indicated that multispecies swards containing PLAN potentially inhibit nitrification and could be a useful mitigation strategy for N loss to the environment from grassland agriculture.

Keywords: Nitrous oxide, soil nitrogen cycling, multispecies swards, perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*), ribwort plantain (*Plantago lanceolata*).

Highlights

- Measurement of N_2O emissions and N cycling from varying sward compositions.
- Post N application (40 kg N ha^{-1}) N_2O loss increased with white clover proportion.
- N_2O emissions from PRG were 2.5 fold higher in wet soil (WFPS >60%) compared to ambient.
- Soil N dynamics suggest denitrification as dominant N_2O source when WFPS >60%.
- *Plantago lanceolata* (forage herb) potentially regulates N cycling pathways.

1. Introduction

Improving the sustainability of food production systems, while also reducing associated GHG emissions, is a major global challenge (IPCC 2019). Nitrous oxide (N_2O), is a potent GHG, with the tropospheric concentration continuing to increase (Thompson et al. 2019; Makowski, 2019). Anthropogenic soil N_2O emissions are governed by the rate and form of N applied as well as other biotic and abiotic factors, such as microbial community composition and activity, soil texture, and climatic conditions (Braker and Conrad, 2011; Butterbach-Bahl et al. 2013). Soil N loss is therefore a significant economic and environmental barrier to achieving sustainable food production.

Nitrogen can be lost from agricultural soils in a number of ways that include nitrate (NO_3^-) leaching, and gaseous N forms such as nitric oxide (NO), ammonia (NH_3), dinitrogen (N_2) and N_2O. Despite over a century of research into the N cycle, there are still numerous questions regarding N transformations and losses from terrestrial ecosystems (Müller and Clough, 2014). Thus, it is imperative to continue research into soil N cycling; developing food production systems that are N-use efficient while mitigating the threats posed by N loss to the environment.

1.1 N_2O losses from agricultural grassland soils

Several N transformation pathways can lead to N_2O production from soil (Butterbach-Bahl et al. 2013; Müller et al. 2014; Zhang et al. 2015). Nitrification is the oxidative conversion of NH_4^+ to NO_3^- during which N_2O can be lost to the atmosphere (Davidson and Verchot, 2000). Denitrification reduces NO_3^- to N_2O and finally to N_2 (Arnold, 1954; Gayon and Dupetit, 1882). Microbial activity (fungal and bacterial) regulates N_2O production from nitrification and denitrification in soil (Baggs, 2011). With conditions conducive to nitrification, Conrad et al. (1983), found that soil N_2O emissions associated with NH_4^+-N fertiliser application increased when compared to those associated with NO_3^--N. Egginton and Smith (1986) showed that when conditions favoured denitrification, soil N_2O emissions associated with NO_3^--N fertiliser application increased compared to those associated with NH_4^+-N. This contrast emphasizes the importance of selecting the appropriate N fertiliser type and rate, to suit the land management practices, to align with antecedent soil and weather conditions, in order to reduce N loss as N_2O. Increased availability of NO_3^--N, together with wetter soil conditions, has been shown to increase soil denitrification rates producing higher N_2O emissions (Arnold, 1954; De Klein and van Logtestijn, 1994; Dobbie and Smith, 2003a, 2003b; Harty et al., 2017). Dobbie and Smith (2003b) and Krol et al. (2016) observed that rainfall around the time of N application and its effect on water-filled pore space (WFPS) was a key driver of N_2O emissions.

1.2 The influence of soil WFPS on N_2O production

Soil moisture is a major contributory factor to N_2O emissions via its influence on N transformation pathways. Below 60 – 70% WFPS, Nõmmik (1956) found that the microbial activity resulting in denitrification was negligible. Davidson (1991) stated that nitrification is the dominant source of N_2O when WFPS < 70%. However, even in a predominantly aerobic soil (conducive to nitrification), Burford and Stefanson, (1973) found anaerobic microsites within the soil that gave rise to N_2O produced by denitrification. The dynamic and heterogeneous nature of soil moisture means that conditions promoting nitrification and denitrification can often occur simultaneously (Abbasi and Adams, 2000). Furthermore, soils with an increased soil organic matter (SOM) content often show N_2O production associated with the turnover or organic N (Zhang et al., 2015). The interaction between N inputs, microbial activity and soil WFPS is complex. Improved knowledge of these interacting factors under different agricultural systems is essential for the development of N_2O mitigation options and improving N fertiliser use efficiency.

1.3 Dry matter production and N recovery in multispecies swards

Multispecies swards composed of different plant functional groups (e.g. grasses, N fixing legumes and herbs) have been investigated as alternatives to PRG monocultures due to their potential to meet primary productivity needs while requiring less fertiliser N inputs (Husse et al., 2017; Nyfeler et al., 2009; Nyfeler et al., 2011; Suter *et al* 2015; Lüscher et al., 2014). Niche differentiation and complementarity resulting from differential resource use by the individual plants within mixtures, benefiting the mixture as a whole (Loreau et al., 2001), are often cited as the mechanisms by which multispecies swards produce greater dry matter (DM) yields compared to monocultures. For example; deeper rooting species grown in mixtures can improve nutrient uptake from greater soil depths (Hoekstra et al., 2015; Jumpponen et al., 2002; Massey et al., 2013). Cong et al., (2017, 2018) and Elgersma et al. (2014) found that herbs, such as *Plantago lanceolata*, have positive effects on DM yields when included in multispecies swards with Cong et al., (2017) also reporting a significant increase in root biomass of swards containing *Plantago lanceolata*.

It has been shown that the inclusion of legumes in sward mixtures for their contribution of biologically fixed N, is a suitable means to replace fertiliser N requirements and maintain or often increase DM yields and N recovery compared to PRG monocultures (Grace et al. 2019; Kirwan et al. 2007; Nyfeler et al. 2009; Nyfeler et al. 2011). Multispecies swards are also considered more resilient than monocultures to environmental stresses such as drought which may be vital for maintaining DM production and adapting to more frequent adverse weather conditions resulting from anthropogenic climate change (Finn et al., 2018; Hoekstra et al., 2015; Isbell et al., 2017).

1.4 N_2O emissions and N cycling associated with multispecies swards

How multispecies swards influence N_2O emissions is still not understood. Niklaus et al. (2006) proposed that plant community composition impacts N cycling, soil properties related to gas diffusivity and interactions of plants with soil microbial communities which influence soil N_2O emissions. They found some reduction in N_2O associated with species diversity but observed increased N_2O emissions in the presence of legumes. Allan et al. (2013) found no significant effects on N_2O emissions from multispecies swards but did find a significant legume effect on soil NO_3^-. Abalos et al. (2014) only examined grass species diversity (not plant functional group diversity) but found that certain grass mixtures led to a N_2O reduction through greater productivity and complementarity in root morphology. Niklaus et al. (2016) found that species richness reduced N_2O emissions over time except from legume containing swards when fertiliser was added. Many authors have found that multispecies swards can reduce NO_3^- leaching and propose high winter activity and differences in root system architecture to explain this reduction (Leimer et al., 2015, 2016; Malcolm et al., 2014; Scherer-Lorenzen et al., 2003). Some studies have found a reduction in N_2O emissions associated with the application of compounds extracted from *Plantago lanceolata* leaves (Dietz et al., 2013; Gardiner et al., 2018). Recently, Carlton et al. (2019) reported that swards of perennial ryegrass, white clover and ribwort plantain (*Plantago lanceolata*) had significantly lower nitrate leaching than compositions of just perennial ryegrass and white clover, proposing that root exudates from ribwort plantain had an inhibitory effect on nitrification. These authors also found a lower abundance of ammonia oxidising bacteria (AOB), highlighting the importance of the interactions between multispecies swards and soil microbial communities in regulating soil N cycling. There is a growing interest in the use of plants as mitigation options for N_2O emissions from agricultural grasslands (De Klein et al., 2019). More research is needed to determine what impact growing plants such as *Plantago lanceolata* in multispecies swards has on soil N cycling and N_2O emission over time.

We hypothesised that plant functional group (grass, legume, herb; represented by PRG, WC and PLAN) identity effects and plant functional group diversity effects (interaction between functional groups) may significantly affect N_2O emissions depending on their proportions and soil moisture conditions. To test these hypotheses, we carried out an experiment that focused on plant functional group identity and diversity effects on N_2O emissions, post N fertiliser application, from an agricultural grassland soil managed under two contrasting soil moisture conditions.

2. Materials and Methods

2.1 Experimental site

This experiment was carried out at University College Dublin (UCD) Lyons Farm (3°18’ N, 6° 32’ W, *ca.* 80 m AOL) in Co. Kildare, Eastern Ireland. The general climate is cool temperate oceanic. The mean monthly total rainfall accumulation (1981 to 2010) for July and August is 54.2 – 72.3 mm, respectively, with an annual mean total rainfall of 754.2 mm (Met Éireann, 2018). The mean temperature (1981 to 2010) for July and August is between 15.7 and 15.4 °C, respectively, with an annual mean temperature of 9.7 °C (Met Éireann, 2018). The soil type has been previously classified as a grey brown podzolic soil with a silty clay loam texture (Lalor, 2004) (a Luvisol under the World Reference Base (WRB) soil classification system (IUSS Working Group WRB, 2014)). Further details of the site’s soil characteristics are presented in Table 1.

The experimental swards used in this experiment were established in August 2013 as part of a multi-species grassland sward experiment (Grace et al., 2018). Prior to this, the site had been managed under continuous tillage, most recently in maize (*Zea mays*). Plots (1.95 x 10 m), comprising of various seed mixes, were established in August 2013 (Grace et al., 2018). From 2013 to 2016 the subset of plots used in this experiment received an annual fertiliser N rate of 90 kg N ha^{-1} yr^{-1} and herbage was cut and removed 8 times per year (Grace et al., 2018). For this experiment, subplots of 1.95 x 4 m were used. Each plot was harvested to a height of 4 cm between April - October 2017 using a Haldrup forage harvester (Løgstør, Denmark) at 21 - 30 day intervals.

2.2 Experimental design

Following the diversity-interaction modelling approach described by Kirwan et al. (2009), a constrained simplex experiment was set up by Grace et al. (2018). A subset of eight plots from the Grace et al. (2018) study were used for this experiment. The simplex experimental design treats a sward as a mixture of component species (PRG, WC, PLAN) and assumes that the measured responses depend on the relative proportions of the component species within the mixture (Cornell, 2002). The estimate of the response variables of a specific composition derives from the compositions included in the design (Lawson and Wilden, 2016). Eight plots of pasture mixtures consisting of different proportions of three plant functional groups (grasses, legumes and forage herbs, represented by PRG, WC and PLAN) were selected from the larger experiment (Figure 1). As the diversity-interaction model (Simplex model) is based on a regression approach, it does not require replication of sward mixtures (Kirwan et al. 2009).

The eight plots are referred to by the ratios of the different plant functional groups included within the original seed rates (Grace et al. 2018) e.g. grass monoculture = 100:0:0. A single species represented each functional group; the grass species was perennial ryegrass (PRG, *Lolium perenne*), the legume species was white clover (WC, *Trifolium repens*) and the forage herb species was ribwort plantain (PLAN, *Plantago lanceolata*). A practical agronomic constraint was imposed on the simplex design such that there must be a minimum of 40% grass (PRG) in each mixture.

Two stainless steel collars for static chambers to measure N_2O emissions were installed in each plot on 28 June 2017. This was approximately one week prior to the first sampling day. Chamber bases were only removed to facilitate grass harvesting and were returned to the same position immediately following this. The collars were inserted into the soil to a depth of ≥ 5 cm (De Klein and Harvey, 2012). The collars were square (40 cm × 40 cm) and 12 cm high, and had a rim lined with a neoprene foam seal to prevent gas diffusion when the chambers were closed (Minet et al., 2016). The corresponding stainless steel static chamber lid height was 10 cm. Lids were weighed down during sampling with a 5 kg weight to provide an air-tight seal.

2.3 Soil bulk density and water filled pore space (WFPS)

Six soil samples were taken from each plot using stainless steel bulk density rings on 13 June 2017. Gravimetric soil moisture and soil bulk density was measured by difference after drying for 24 hours at 105°C. The gravimetric soil moisture content and mean soil bulk density (1.16 g cm^{-3}) were used to calculate the mean water filled pore space (WFPS) of the plots, based on an assumed particle size density of 2.65 g cm^{-3} (Krol et al., 2015).

Half of each plot was kept at ambient soil moisture while the other was watered to achieve a higher WFPS. To do this, 7.5 L of water was applied in two applications using a watering can fitted with a rose head (5 L on 30 June 2017 and 2.5 L on 05 July 2017) which simulated 30 mm of rainfall in total. The estimated return period for 30 mm of rainfall in one day at this site is 1.09 years based on the available historical weather data (Met Éireann, 2018). The 30 year averages from the nearby weather station show that the greatest total daily rainfall recorded for July was 33.7 mm with a mean monthly total of 54.2 mm (Met Éireann, 2018). The target WFPS for the wet soil moisture conditions was 70 – 80 %. To maintain the desired separation of WFPS between the ambient and wet soil moisture conditions, the wet areas received a second water application of 3L (equivalent of 12 mm rainfall) on 17 July 2017. The area incorporating the other static chamber in each plot was maintained under ambient soil moisture conditions. A buffer area (≥1 m) was used to separate the ambient and wet soil moisture areas.

2.4 Fertiliser application

While the larger plot area received no fertiliser application throughout 2017, fertiliser N was applied by syringe in the form of a urea solution, at a typical rate of 40 kg N ha^{-1} for the time of year (Wall and Plunkett, 2016), to the base of each static chamber (0.16 m^2) and to an area adjacent to the chambers to be used for periodic soil sampling (0.09 m^2). The fertiliser urea solution was prepared by dissolving a total of 41.16 g of lab grade urea in 2 L of 18 mQ water. At the base of each chamber 66.67 ml of the fertiliser was applied and 37.5 ml was applied to each of the adjacent areas to be used for periodic soil sampling. No other macro or micro nutrients were applied immediately prior to or during the experimental period. Table 1 presents the most recently measured soil chemical properties of these plots. They had a mean soil pH of 7.2 and a Morgan's-extractable phosphorous (P) and potassium (K) content of 29.2 and 175.0 mg L^{-1}, indicating that P and K were non-limiting based on the Irish Soil Index System (Wall and Plunkett, 2016). Plots were harvested to 4 cm on 06 July 2017 prior to fertiliser application on 11 July 2017 (Fig 2).

2.5 Sampling N_2O emissions and calculating daily flux

Background N_2O fluxes were measured on one occasion five days prior to fertiliser application and then regularly for a two-month period post fertiliser application. Gas samples were taken by syringe, through a rubber septum port on the lids of the static chambers, four times per week for the first two weeks, twice per week for the next two weeks and then once per week for the following month (Harty et al., 2016). In general, daily N_2O fluxes are controlled by soil temperature (Livesley et al., 2008). Therefore, it is necessary to choose the most appropriate sampling time to represent the average daily flux (Laville et al., 2011). Gas samples were taken in the mornings between 09.00 and 12.00 to obtain the most representative estimate of average daily N_2O flux (Alves et al., 2012; Parkin 2008; Smith and Dobbie 2001). Headspace samples

(10 ml) were taken during a 60-minute closure period at times 0, 30 and 60 minutes after the static chambers were closed. The syringe was flushed three times with ambient air prior to each sample removal. During sample removal, the syringe was plunged three times to evenly mix the gas within chambers. Ten ml gas samples were injected into 7 ml pre-evacuated glass vials with double-wadded PTFE/silicone septa (Labco, UK) to achieve overpressure for storage.

The N_2O concentration was measured by gas chromatography (GC) using a Bruker Scion 456 GC with a ^{63}Ni electron capture detector (Bruker, Germany) in combination with a Combi-PAL xt® auto-sampler (CTC Analytics AG, Switzerland). Five calibration standards were run at the beginning of each sample batch with verification standards run after every 10 samples. Occasionally, the first air sample (T(0 min)) concentrations were higher than ambient. De Klein and Harvey (2012) discussed a number of issues that can impact T(0 min) and defined outliers. The T(0 min) outliers were substituted with the average of the T(0) concentrations for that sampling date to avoid introducing sources of additional variation, as outlined by De Klein and Harvey (2012). Daily N_2O fluxes were calculated based on the change in N_2O concentration over the three sampling time points using the following equation (De Klein and Harvey 2012):

Eq. 1: $F(daily) = (\Delta C/\Delta t) \times ((M \times P) / (R \times T)) \times (V/A)$

Whereby:

F(daily) is the daily N_2O flux (g N_2O-N ha^{-1} day^{-1});

$\Delta C/\Delta t$ is the slope of the line between the N_2O concentrations (ppm) at the three sampling time points;

M is the molar mass of N_2O-N (28 g mol^{-1});

P is the atmospheric pressure (Pa) measured at Casement Aerodrome (53°30′N, -6°44′W) meteorological station (approx. 5.8 km east of the experimental site and similar elevation) at the time and date of sampling;

T is the air temperature (K) measured at the plot at the time of sampling;

R is the ideal gas constant (8.314 J K^{-1} mol^{-1});

V is the headspace volume of the closed chamber (approx. 0.026 m^3);

and A is the area covered by the base of the gas chamber (approx. 0.1695 m^2).

Cumulative N_2O emissions for the two-month period post fertiliser application (g N_2O-N ha^{-1}) were determined by integrating the daily N_2O fluxes from Eq. 1 using the trapezoidal integration method (de Klein and Harvey, 2012; Harty et al., 2016) to interpolate between sampling dates.

2.6 Dry matter yields

Herbage was harvested on three occasions from within each chamber base, using a small handheld pruning shears, to a height of 4 cm above the soil surface (Burchill et al., 2014); at 15 days, 26 days, and 31 days post fertiliser application. Fresh herbage was separated into each plant functional group and weighed, followed by oven drying at 65°C for 48 hours (Burchill et al., 2014) and reweighing, to determine the herbage dry weight of each plant functional group within the sward mixtures. For each sward mixture, the individual plant functional group dry weights were summed to determine the total dry weight yielded for each sward mixture. Yields were expressed in units of kilograms of dry matter per hectare (kg DM ha^{-1}). Herbage yields for each sampling date were summed to get the total post fertiliser yield. The average percentage inclusion of PRG, WC and PLAN in the herbage collected during this experiment (4 harvest dates, Figure 2) for each sward mixture was determined to compare the present botanical compositions of the swards for the short duration of this experiment within the static chambers (0.16 m^2) with the ratios of the original seeding rates for the field plots (Table S1 Supplementary Information). A more representative and longer-term quantification of the persistence of each species in the experimental swards from the entire plot area (19.5 m^2) is presented by Grace et al. (2018).

2.7 Soil sampling and KCl-extractable TON and NH_4^+

Two molar potassium chloride (KCl) was used to extract mineral N from soil samples that were taken periodically. These samples were taken from the fertilised area adjacent to the static chambers to avoid any physical disturbance of the area within the chamber. This extracted soil N represents the mineral N that might potentially be found in soil solution, and thus be available for plant uptake, or be vulnerable to loss via volatilisation or leaching (Maynard and Kalra, 1993; Müller et al., 1998). The soil samples were analysed to determine the levels of soluble soil mineral N as total oxidisable N (TON; the sum of NO_3^- and NO_2^-) and NH_4^+. Soil samples were taken to a depth of 10 cm using a 2 cm diameter soil corer. On each sampling date, 4 evenly spaced cores were taken and placed in a labelled zip-lock bag and brought to the lab immediately for further processing. The first set of soil samples were taken five days prior to fertiliser application to determine the background levels of KCl-extractable TON and NH_4^+. Soil samples were taken on four subsequent occasions (6, 15, 29 and 66 days after fertiliser application). Upon completion of the N_2O sampling period (77 days after fertiliser application), 30 cm deep intact soil cores were taken from within each chamber using a 5 cm diameter corer (Eijkelkamp Soil & Water, Netherlands). Each core was split into three depths; 0 – 10 cm, 10 – 20 cm and 20 – 30 cm to assess the concentrations of KCl-extractable mineral N over depth in the soil.

Soil samples were processed within 24 hours by sieving the fresh soil through 2 mm soil sieves. Soil sieves were cleaned with deionized water and dried between each sample. Fresh sieved soil (20 g) was weighed into centrifuge containers and 50 ml of 2 M KCl solution was added (1:2.5 ratio). The remaining soil from each sample was weighed and dried at 105ºC for 24 hours and reweighed to measure the gravimetric soil moisture content. The centrifuge containers were shaken for 1 hour and soil solutions were then filtered into 50 ml plastic containers through Whatman no. 2 filter paper. Samples were immediately placed into a freezer for storage. The frozen KCl extracts were defrosted overnight prior to chemical analysis.

The methods used by Saghir et al., (1993); Stevens and Laughlin, (1995); Watson and Mills, (1998); Watson et al., (2000) were adapted to measure TON and NH_4^+ colorimetrically using a Shimadzu UV-1280 spectrophotometer with the wavelength set at 520 nm and 625 nm, respectively.

The limits of detection (LOD) for the analyses of TON and NH_4^+ concentrations, were 0.248 ppm and 0.001 ppm, respectively, based on the mean concentration + 3 x standard deviation 0 ppm calibration standard. All of the samples analysed for TON were above the LOD. However, for the final set of soil samples, to analyse NH_4^+ over depth, many of the values were below the LOD, particularly at the two lowest depths; 10 – 20 cm and 20 – 30 cm. Prior to statistical analyses the values <LOD were corrected to zero.

2.8 Meteorological and soil data

Average daily air temperature (°C) and rainfall (mm) for the study period were acquired from the Met Éireann meteorological station at Casement Aerodrome (53°30ˊN, -6°44ˊW), approximately 5.8 km east of UCD Lyons Farm and with similar elevation (80 m) above sea level (Met Éireann, 2017). Surface soil moisture (% volume, 0 – 6 cm depth) and temperature

(°C, 0 – 10 cm depth) were recorded on each sampling date using a ML2 Theta Probe (Delta-T Devices Ltd., HH2, UK) and a TinyTag View 2 with a PB-5002-1M5 Thermistor Probe (Gemini Data Loggers, UK), respectively.

2.9 Statistical analysis

Results were statistically analysed using a simplex model in R (R Core Team, 2017). Identity effects and diversity effects of the three plant functional groups (represented by PRG, WC and PLAN) were modelled as described by Connolly et al., (2009) and Kirwan et al., (2009). Functional group identity effects occur when the response associated with a monoculture of one of the plant functional groups is significantly different to the response of a monoculture of another plant functional. Functional group diversity effects occur when the response of the mixture of plant functional groups is significantly different from the response that would be expected based on the proportional composition of functional groups in the mixture. Interactions between functional group identity effects and two soil moisture levels as well as three-way interactions between functional group diversity effects and soil moisture levels were also tested. The model outputs and simplex contour plots were produced using the "lm" function and "mixexp" package in R (Lawson and Willden, 2016). Tests of significance were performed at the $P<0.05$ level. All other plots were produced using the "ggplot2" package in R (Wickham, 2009).

The effect of events such as fertiliser application and heavy rainfall which occurred during the experimental period on temporal variations of TON and NH_4^+ was analysed by fitting soil sampling dates as a time parameter to the original simplex model. A general correlation model (with any correlation possible between sampling times) was used for testing TON responses and a compound symmetry model with constant correlation between sampling times was used for testing NH_4^+ responses. Full models were initially fitted to the data followed by reduced models, removing insignificant terms and observing hierarchy. Final model selection was based on parsimony, Akaike Information Criteria (AIC) and likelihood ratio tests between the model options for incorporating the time factor. A similar approach was taken to incorporate soil depths (0 – 10 cm, 10 – 20 cm, 20 – 30 cm) into the simplex model to statistically analyse the TON and NH_4^+ concentrations measured from the final set of destructive soil cores used to determine the effect of depth on TON and NH_4^+.

3. Results

3.1. Temporal trends in rainfall, temperature, N_2O fluxes and mineral N

There was a clear initial separation in WFPS of the ambient (approx. 60 %) and wet (> 70%) chamber areas across all plots achieved by the additional water added to the wet chamber areas at the beginning of the experiment (Fig 2). Due to several days of persistent heavy rainfall (15.1 mm) in mid-July the mean WFPS for the ambient and wet chambers were within 5% of each other (Fig 2). There was a significant grass (PRG) functional group identity effect on soil bulk density (Table 2). The PRG only plot had a lower soil bulk density of 1.17 g cm^{-3}. The maximum model estimated bulk density was 1.26 g cm^{-3} at a predicted legume (WC) to grass (PRG) ratio of 56:44. The lowest estimated bulk density was 1.14 g cm^{-3} at a predicted grass (PRG) to herb (PLAN) ratio of 64:36.

Higher N_2O fluxes corresponded with fertiliser application (11 July 2019), heavy rainfall and reductions in soil and air temperatures (Figs 2 and 3). Daily N_2O fluxes initially peaked one day after fertiliser application. The highest initial peaks were 13.4 g N_2O-N ha^{-1} day^{-1} from the 40:60:0 sward mixture (ambient soil moisture) and 39.8 g N_2O-N ha^{-1} day^{-1} from the 70:0:30 sward mixture (wet soil moisture). The highest daily N_2O fluxes from both ambient and wet soil conditions occurred on 21 July 2017, coinciding with a period of high rainfall and WFPS; 17.7 g N_2O-N ha^{-1} day^{-1} from the 40:30:30 sward mixture (ambient soil moisture) and 86.9 g N_2O-N ha^{-1} day^{-1} from the 40:60:0 sward mixture (wet soil moisture) (the mixture with the highest proportion of WC) (Fig 3).

For the majority of the sward mixes the temporal trend for soil TON concentrations was to initially decline from approximately 5.0 - 10.0 mg kg^{-1} and then level out between 2.0 – 6.0 mg kg^{-1} after 20 days (Fig 3). Unlike TON concentrations, NH_4^+ concentrations, did not appear to have an obvious temporal trend (declining / increasing) over time and ranged from approximately 0 – 4.0 mg/kg for most of the sward mixes (Fig 3).

3.2 Cumulative post-fertiliser N_2O emission

The highest cumulative N_2O emission for the two-month period post-fertiliser application under ambient soil conditions was 206.4 g N_2O-N ha^{-1} from the 40:30:30 mixture. The highest cumulative N_2O emission for the two-month period post-fertiliser application under wet soil conditions was 434.3 g N_2O-N ha^{-1} from the 40:60:0 mixture. Cumulative post-fertiliser N_2O emissions ranged from 22.1 - 206.4 g N_2O-N ha^{-1} for the ambient soil and from 62.5 - 434.3 g N_2O-N ha^{-1} for the wet soil.

There was a strongly significant grass (PRG) and legume (WC) functional group identity effect ($P<0.01$) on cumulative N_2O emissions (Table 2), with emissions increasing with increasing WC proportion and decreasing with increasing PRG proportion for both wet and ambient soil moisture conditions (Figs. 4 and 5). There was a significant grass (PRG) x soil moisture interaction ($P<0.05$, Table 2), with N_2O emissions being much higher under wet soil conditions than ambient (Fig 4).

3.3 Dry matter yields

Cumulative DM yield post-fertiliser application ranged from 760 – 3060 kg DM ha^{-1}. The 70:30:0 sward mixture (ambient soil moisture) produced the highest cumulative DM yield, while the 70:0:30 sward mixture (wet soil moisture) produced the lowest cumulative DM yield. There was a significant legume (WC) and herb (PLAN) functional group identity effect on DM yields with model predictions of DM yields increasing with increasing proportions of WC and PLAN ($P<0.05$; Table 2). The proportions of PRG, WC and PLAN (kg DM ha^{-1}) within each of the mixtures at the time of the experiment is expressed as a percentage of the total DM (kg DM ha^{-1}) and provided in Table S1 (Supplementary Information).

3.4 Soil mineral nitrogen dynamics

There was a highly significant grass (PRG) functional group identity effect and time effect on TON concentrations in soil KCl extracts ($P<0.001$). There was also a strongly significant herb (PLAN) x grass (PRG) functional group diversity effect ($P<0.01$) as shown by the curved response with lower TON concentrations at the 50:50 mixed proportions of herb (PLAN) and grass (PRG) than at the 100% point of either herb (PLAN) or grass (PRG) (Fig S1, left).

There was a significant legume (WC) functional group identity effect ($P<0.05$) on NH_4^+ concentrations in soil KCl extracts, with NH_4^+ concentrations tending to increase markedly with increasing WC content under wet soil conditions but having the opposite trend under ambient soil moisture conditions (Fig S2, right). There was no significant effect of time on NH_4^+. There was a significant legume (WC) x herb (PLAN) functional group diversity effect with a curved response showing concentration predictions higher near the 50:50 mixed proportions of legume (WC) and herb (PLAN) than at the 100% point of either legume (WC) or herb (PLAN ($P<0.05$; Fig S2).

Results of the last soil sampling 77 days post-fertilisation, which included three sampling depths, showed that there was a significant effect of depth ($P<0.05$) on TON concentrations, with concentrations tending to decrease with depth. Mean concentrations across all sward mixes were 3.99 (0 – 10 cm), 3.32 (10 – 20 cm) and 3.15 (20 – 30 cm) mg kg^{-1}. There were also significant grass (PRG) ($P<0.001$) and legume (WC) functional group identity effects ($P<0.05$; Fig S3, right), with TON tending to increase in concentration with increasing PRG proportion and decrease with increasing WC proportion. There was a significant herb (PLAN) x grass (PRG) functional group diversity effect with a curved TON response showing concentrations lowest around the 50:50 herb (PLAN) to grass (PRG) ratio ($P<0.05$; Fig S3, left).

Using the corrected values for NH_4^+ over depth; there was a strongly significant effect of depth on NH_4^+ concentrations, with concentrations tending to decrease with depth. Mean concentrations across all sward mixes were 0.87 (0 – 10 cm), 0.05 (10 – 20 cm) and 0.05 (20 – 30 cm) mg kg^{-1}. There was a significant herb (PLAN) x soil moisture interaction ($P<0.05$; Fig S4, middle). There was a significant three-way interaction of the herb (PLAN) x grass (PRG) functional groups with soil moisture ($P<0.05$; Fig S4, left and middle). Around the 50:50 herb (PLAN) to grass (PRG) ratio NH_4^+ concentrations were higher under wet soil moisture conditions and lower under ambient for all three soil depths.

4. Discussion

4.1. Temporal N_2O emissions

It is clear from this study that N_2O emissions from multispecies swards were strongly impacted by fertiliser N management practices and soil moisture conditions. Higher N_2O emissions occurred directly post-fertilisation and under wetter soil conditions. N_2O emissions peaked when WFPS was above 60%, suggesting denitrification as a dominant source of N_2O emission over nitrification. In temperate grasslands, peak N_2O emissions have been related to fertiliser N application timing while also inferring that rainfall contributed to greater emissions and seasonal variability of N_2O (Jackson et al., 2015; Jones et al., 2007; Liu et al., 2015). Average daily N_2O fluxes, based on data reported by those studies as well as studies in Ireland by Harty et al. (2016) and Krol et al. (2016), range from 0 g N_2O-N ha^{-1} d^{-1} for unfertilized control plots to approximately 30 g N_2O-N ha^{-1} d^{-1} for N fertilized plots, with some large daily peaks reported > 1000 g N_2O-N ha^{-1} d^{-1}. The observed daily N_2O fluxes presented in Fig 3 fall within these previously reported ranges.

Temporal N_2O emissions appeared to be directly related to soil TON concentrations which decreased as N_2O emissions peaked, especially under wet soil conditions (Fig 3). Hatch et al. (1990; 1991) found that peak daily rates of net mineralization could range from 0.7 – 4.1 kg N ha^{-1} d^{-1} and that peak rates were related to re-wetting of soil after dryer weather. They found that total net mineralization was highest under grass/clover swards. In the current study such increased rates of daily mineralization, particularly in clover containing swards, may have increased the amounts of mineral N available to be lost as N_2O during the second large peak in Fig. 3. Krol et al. (2016) statistically related N_2O emissions with soil moisture at the time of N application and cumulative rainfall post application. Decreasing concentrations of soil TON, when WFPS is high, supports denitrification of NO_3^- in soil solution to N_2 and N_2O as the main pathway for N_2O production. The loss of N_2 was not quantified but may have accounted for a substantial amount of the N loss particularly from plots under wet soil conditions (Selbie et al., 2015).

Despite the application of N as urea the NH_4^+ concentrations were much more constant over time. This might suggest that urea was rapidly converted to NH_4^+ which was then consumed through plant uptake or rapidly nitrified to NO_3^-. Other reasons may be mineralization of organic N replacing NH_4^+-N taken up from soil solution by plants or converted to NO_3^- by nitrification (Müller and Clough 2014; Müller et al., 2004, 2011). Adsorption of NH_4^+ to organic matter and soil particles may also have occurred (Harty et al., 2017).

Plots under wet soil conditions mostly remained above 60% WFPS as planned but occasionally were below 60%. Therefore, it was considered that the greater N_2O emissions from plots under wet soil conditions were due to a contribution from both nitrification and denitrification sources (Abassi and Adams, 2000; Davidson, 1991; Nõmmik, 1956). It is also notable that the larger N_2O peaks under ambient soil conditions (Fig 3) occurred shortly after the heavy rainfall period when the WFPS for these plots was >60%. Under wet soil conditions, soils had >60% WFPS for a considerably longer period of time (~39 days; 66 % of time) compared to ambient soil conditions (~22 days; 37 % of time) and mean cumulative N_2O emissions were considerably higher (214.06 g N_2O-N ha^{-1} and 108.65 g N_2O-N ha^{-1}, respectively).

4.2 Cumulative N_2O emissions and DM yields

The model predictions for an increase in WC proportion from 0% - 60% (i.e. within the constraints of the seeding rates of Grace et al. 2018) showed an increase in cumulative N_2O emissions from 22.3 to 96.2 g N_2O-N ha^{-1} under ambient soil conditions and from 59.0 to 219.3 g N_2O-N ha^{-1} under wet soil conditions, respectively (grass to legume ratio). The PRG only plots may have been N limited whereas the plots containing higher WC proportions may not have been as N-limited due to biological N fixation. This may also explain the significant increase in DM yield with increasing proportions of legume and herb.

The significant legume (WC) functional group identity effect on cumulative N_2O emissions indicates that applying the same N application rate, to multispecies swards with high proportions of legume, compared to PRG monocultures, is an inappropriate N management practice that can result in greater N_2O emissions, particularly during periods of high WFPS. Multispecies swards with higher proportions of WC and PLAN were found to have higher DM yields than the PRG only sward. Larger proportions of legumes within the sward mixtures likely provided sufficient biologically fixed N to support DM production and the additional N applied as fertiliser was then underutilised by the plants, making it prone to loss to the environment. Mixtures containing legumes have shown potential for reducing synthetic fertiliser N requirements while maintaining or increasing DM production compared to high fertiliser N input grass monocultures (Grace et al., 2019; Kirwan et al., 2007; Nyfeler et al., 2009), but benefits associated with mixtures greatly diminish if managed at high N fertiliser rates (Nyfeler et al., 2011).

Cumulative N_2O emissions were also higher under wet soil conditions (Figs. 4 and 5). There was a significant grass (PRG) x soil moisture interaction, with 2.5 fold higher N_2O emissions from the grass monocultures under wet soil conditions than ambient. Drier soil conditions were expected to favour N_2O production from nitrification over denitrification. Interestingly, there were no significant interactions of soil moisture and the other mixture components; legumes (WC) and herbs (PLAN), nor were there any significant 3-way interactions of the functional groups with soil moisture. Perhaps, this is due to differences in the root systems of the mixtures and their effects on soil structure and porosity compared to the grass monocultures (Gould et al., 2016; Niklaus et al., 2006). The present study noted that predicted soil bulk density increased with an increasing proportion of legume (WC). A higher soil bulk density in swards with greater WC content supports the argument for greater contribution to the overall N_2O emissions from denitrification due to the soil being more compact. Harrison-Kirk et al. (2015) found that N_2O and N_2 production ratios indicated that denitrification was more dominant under conditions of compaction and reduced porosity, after successive saturation and drying cycles. In the present study, the lowest model-predicted bulk density was 1.14 g cm^{-3} resulting from a simulated 64:36 ratio of grass (PRG) to herb (PLAN). Plots were established in 2013 providing four years of plant growth and root establishment. Consequently, PLAN roots over time may have transformed the porous architecture of the soil, thus reducing the soil bulk density and altering soil gas diffusivity properties as outlined by Friedl et al. (2018). The effects of different plants on long-term soil structure and subsequent N_2O emissions and N cycling is an area of growing research interest that requires further investigation (De Klein et al., 2019).

4.3 Soil TON and NH_4^+ concentrations over time and depth

The changes in TON concentrations over time may be attributed to disturbances such as the fertiliser application and heavy rainfall. Assuming that TON is largely NO_3^-, which is very mobile in soil, the decrease in TON concentration over time, despite fertiliser N application, might suggest that NO_3^- was being removed from the soil solution through plant uptake, through a combination of plant uptake, immobilisation, conversion to N_2 or N_2O, and leaching (Müller and Clough 2014; Müller et al., 2004, 2011). The fact TON concentrations decreased significantly over time, while NH_4^+ concentrations did not, indicates that denitrification was likely the most dominant N_2O production pathway during this experiment. Given the relatively restricted drainage of the plots used, additional residual soil N from swards with higher WC proportions, that may have been leached from more freely draining soils (Leimer et al., 2015, 2016; Scherer-Lorenzen et al., 2003), would have been available for conversion to N_2O by denitrification particularly under the wet soil moisture conditions. This would be consistent with the higher N_2O emissions observed under wet soil moisture conditions.

The significant diversity effect of herb (PLAN) x grass (PRG) on TON concentrations analysed over time and the significant diversity effect of legume (WC) x herb (PLAN) on NH_4^+ analysed over time suggest that perhaps NH_4^+ was slower to convert to NO_3^- under multispecies swards with PLAN. Carlton et al. (2019) found significantly lower NO_3^- losses from mixtures containing *Plantago lanceolata* (PLAN) compared to those of just PRG and WC, associated with nitrification inhibition and a reduction in ammonia oxidizing bacteria. The long-term establishment of the plots used here may have allowed biological nitrification inhibitors from root exudates or leaf litter of PLAN (Dietz et al., 2013; Gardiner et al., 2018) to build up prior to this experiment or may have led to the differential soil microbial population development.

The current study indicates that PLAN growing in multispecies swards could potentially be an alternative biological nitrification inhibition option (as opposed to synthetic inhibitors, Di et al., 2014; Harty et al., 2016; Zaman et al., 2008) to be used as a mitigation strategy for both N_2O emissions and nitrate leaching, while improving N use efficiency and the sustainability of grassland based agricultural systems (Carlton et al., 2019; De Klein et al., 2019). However, this study made it clear that future research should take a balanced N fertiliser management approach, when comparing N_2O emissions from multispecies swards, accounting for biological N fixation from legumes. There is a need to consider the effects of all components of multispecies swards as part of long term systems experiments to ensure that differences in N fertiliser management (including rate and timing) and species composition are quantified. This would enable appropriate management advice options to suit prevailing weather and soil conditions.

5. Conclusion

Increasing legume (WC) proportions from 0% to 60% in multispecies swards resulted in model predicted N_2O emissions increasing from 22.3 g N_2O-N ha^{-1} to 96.2 g N_2O-N ha^{-1} (ambient soil conditions) and from 59.0 g N_2O-N ha^{-1} to 219.3 g N_2O-N ha^{-1} (wet soil conditions), after a uniform N application rate. Appropriate timing and application of lower quantities of fertiliser N to multispecies swards containing WC compared to PRG monocultures is important to mitigating N_2O emissions, particularly in wet soil conditions. Consideration of biologically fixed N from WC and mineralization of organic N under multispecies swards is necessary to develop appropriate N fertiliser management strategies for multispecies swards.

Soil moisture had a significant interaction with PRG resulting in over 2.5 times higher cumulative N_2O emissions under wet conditions compared to ambient from the PRG monoculture. Soil mineral N dynamics suggested denitrification was the dominant production pathway of N_2O, particularly under wet soil conditions, but that nitrification may also have contributed, particularly when WFPS dropped below 60%. Future ^{15}N tracing studies could provide clearer insights on the effect of multispecies swards and soil moisture conditions on different N transformation pathways resulting in N_2O production. Multispecies swards could help reduce reliance on feriliser N inputs, while maintaining DM production needs. Swards containing *Plantago lanceolata* (PLAN) showed potential for regulating soil N cycling (biological nitrification inhibition) which could be a useful strategy for mitigating N losses to the environment, either as N_2O or leached NO_3^- and improving the sustainability of grassland agriculture.

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Tables

Table 1: Site description and summary of soil properties.

Site

Location: UCD Lyons Research Farm (3°18’ N, 6° 32’ W) **Elevation:** 80 m above sea level

Soil Physical Properties

Soil Type	Soil Texture	Sand (%)	Silt (%)	Clay (%)	Bulk Density (g cm^{-3})
Luvisol	Clay Loam	24.83 (± 0.65)	43.57 (± 0.76)	31.63 (± 0.21)	1.20 (± 0.05)

Soil Chemical Properties

pH	SOM (LOI%)	TN (%)	TC (%)	P (mg L^{-1} soil)	K (mg L^{-1} soil)	Mg (mg L^{-1} soil)	Ca (mg L^{-1} soil)	S (mg L^{-1} soil)
7.24 (± 0.10)	5.71 (± 0.26)	0.306 (± 0.01)	3.083 (± 0.26)	29.20 (± 4.66)	175.00 (± 16.46)	87.33 (± 6.81)	3606.00 (± 100.54)	10.23 (± 0.72)
				Index 4	**Index 4**	**Index 3**		

(SOM: Soil Organic Matter, LOI: Loss on Ignition, TN: Total Nitrogen, TC: Total Carbon, P: Phosphorus, K: Potassium, Mg: Magnesium, Ca: Calcium, S: Sulphur). **Index** refers to 1 - 4 scale (3 is adequate, 4 is high); Irish Soil Index (Wall and Plunkett, 2016).

Table 2: Statistical significance for the functional group identity and diversity effects and soil moisture interactions for soil bulk density (g cm^{-3}) cumulative N_2O loss (g N_2O-N ha^{-1}) and cumulative DM yield (kg DM ha^{-1}).

Effect Type	**Parameter**	**Bulk Density**	**N_2O**	**DM Yield**
Functional Group Identity Effects	Grass Intercept	2.39^{e-12}***	1.26^{e-06} ***	NS
	Legume	NS	0.00356 **	0.0301*
	Herb	NS	NS	0.0144*
Functional Group Diversity Effects	Grass x Legume	NS	NS	NS
	Grass x Herb	NS	NS	NS
	Legume x Herb	NS	NS	NS
Functional Group Identity and Soil Moisture Interaction Effects	Grass x SM	NS	0.04082 *	NS
	Legume x SM	NS	NS	NS
	Herb x SM	NS	NS	NS
Functional Group Diversity and Soil Moisture Interaction Effects	Grass x Legume x SM	NS	NS	NS
	Grass x Herb x SM	NS	NS	NS
	Legume x Herb x SM	NS	NS	NS

SM = soil moisture. NS = not significant. *** < 0.001, ** < 0.01, * < 0.05. See Section ***2.9 Statistical analysis*** for description of effect types.

Figures

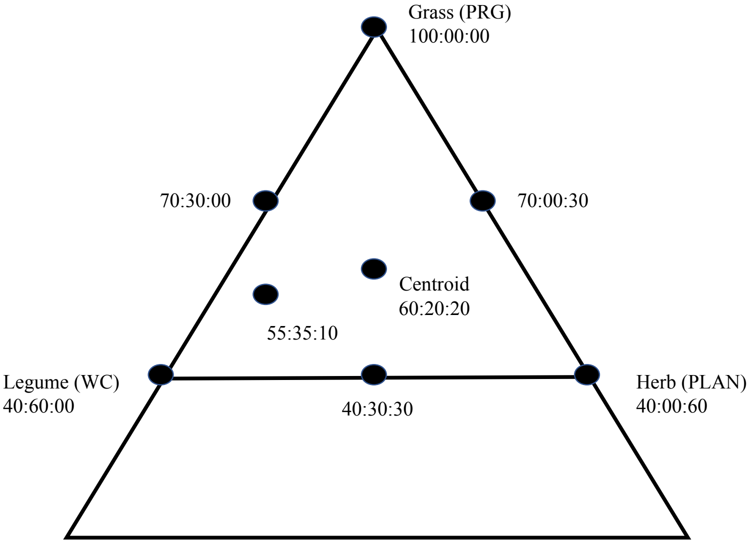

Figure 1: The simplex experimental design demonstrating the eight proportions of each of the functional groups (grass: legume: herb) with constraint imposed (minimum of 40 % grass inclusion in each mixture). Adapted from Grace et al., (2018).

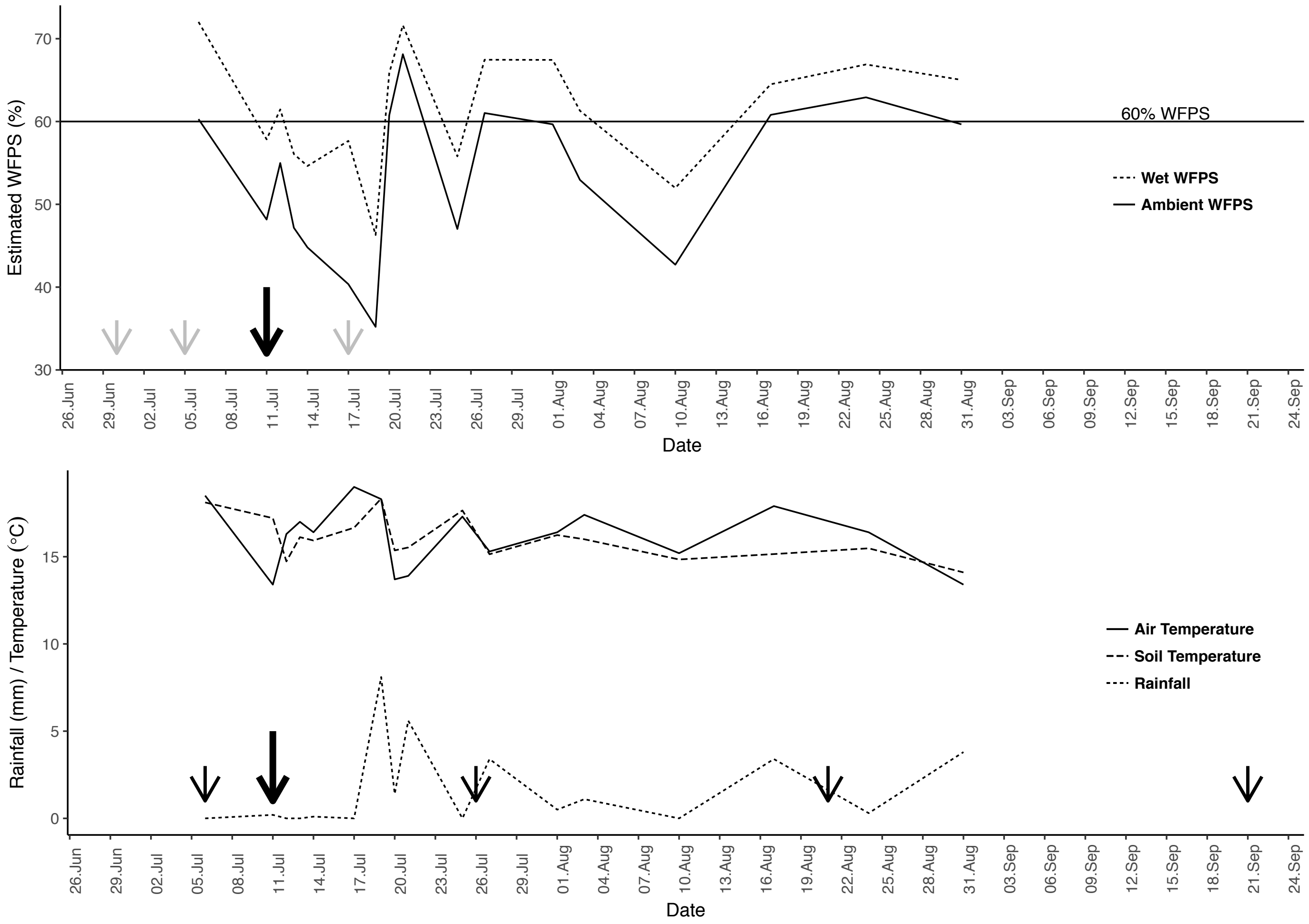

Figure 2: Estimated soil WFPS (%), rainfall (mm), air and soil temperature data as recorded on sampling dates. Large black arrows = fertiliser application. Upper plot: grey arrows = water applications to wet soil. Lower plot: small black arrows = herbage harvest dates.

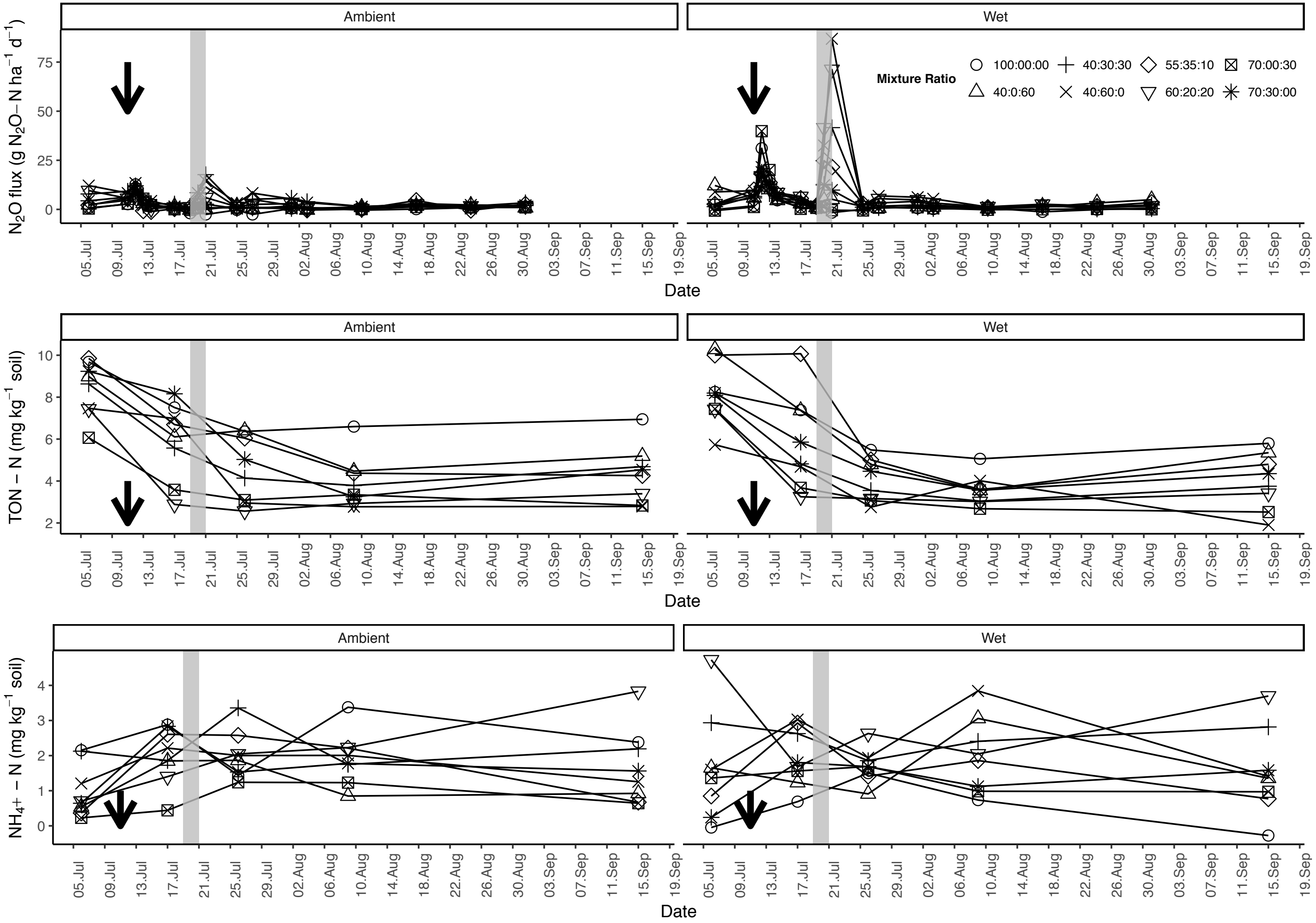

Figure 3: Daily N_2O emissions (g N_2O-N ha^{-1} day^{-1}); KCl-extractable soil TON-N and NH_4^+-N concentrations (mg kg^{-1} soil) from different mixtures; black arrow = fertiliser application date, shaded area = heavy rainfall period. (Mixture Ratio = proportions of PRG:WC:PLAN).

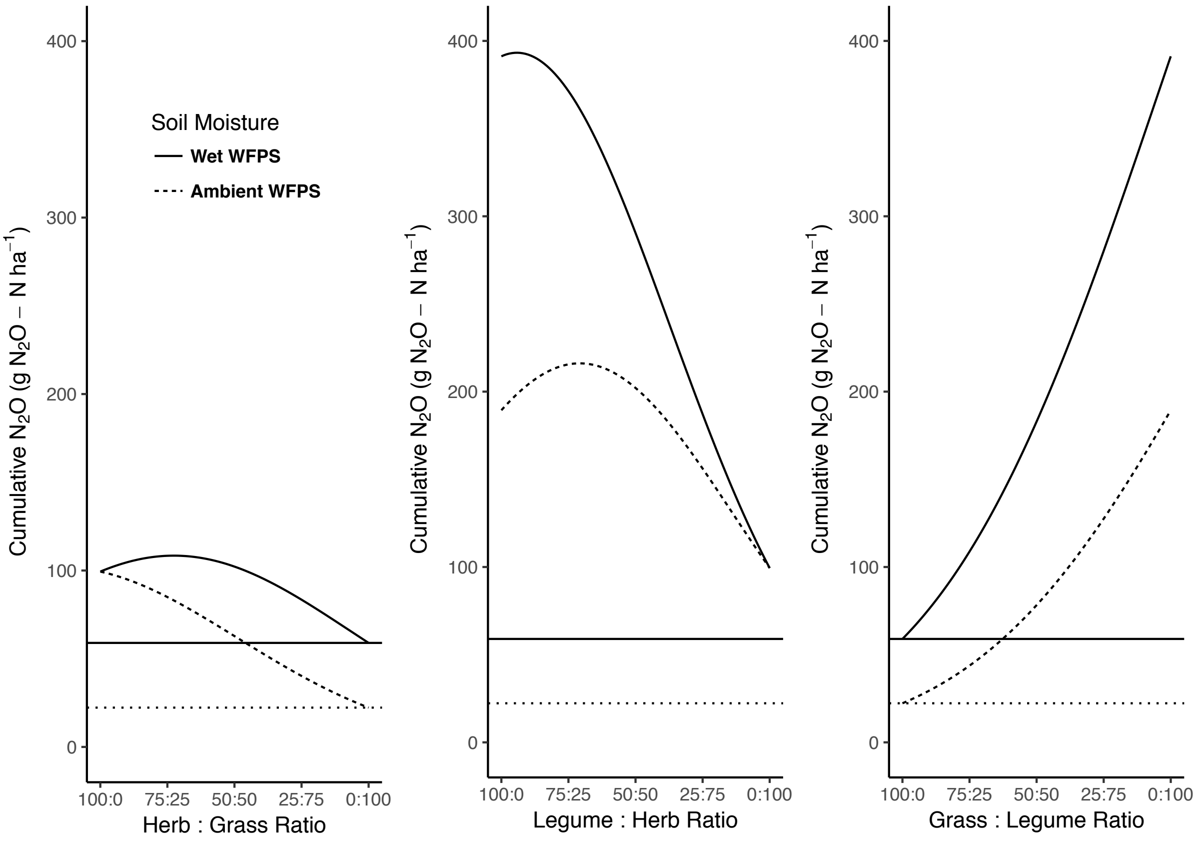

Figure 4: Effects plot of predicted cumulative N_2O emission (g N_2O-N ha^{-1}) with increasing proportions of individual plant functional groups under wet (solid line) and ambient (dotted line) soil moisture conditions. Horizontal lines: PRG monoculture (100:0:0) response. (Cumulative N_2O emissions were for the two-month post fertiliser sampling period).

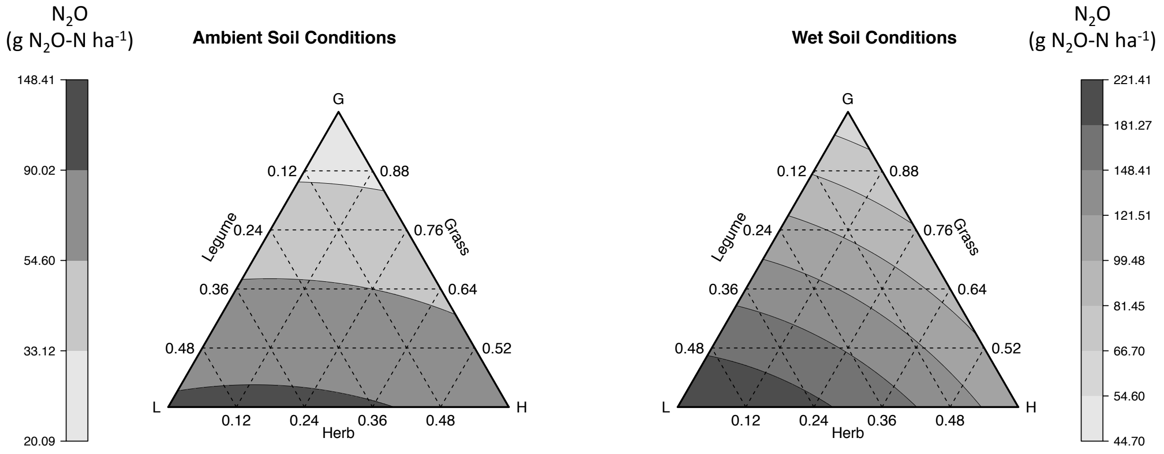

Figure 5: Contour plots of the two-month post fertiliser cumulative N_2O emissions (g N_2O-N ha^{-1}) post-fertiliser application under ambient and wet soil moisture conditions.

Supplementary Information

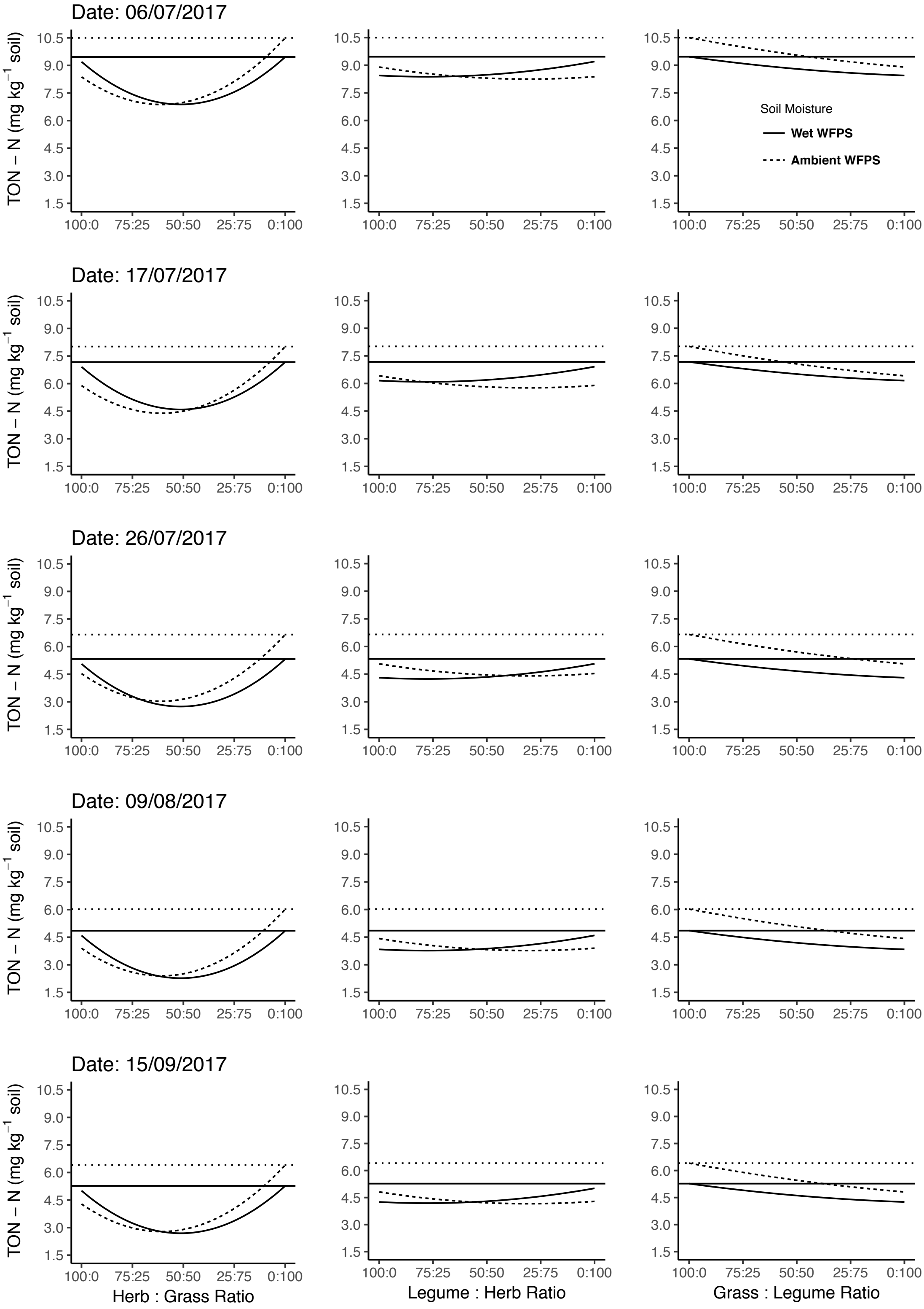

Figure S1: Effects plots of predicted TON (mg kg^{-1} soil) for each soil sampling date with increasing proportions of individual plant functional groups under wet and ambient soil moisture conditions. Horizontal lines: PRG monoculture (100:0:0) response.

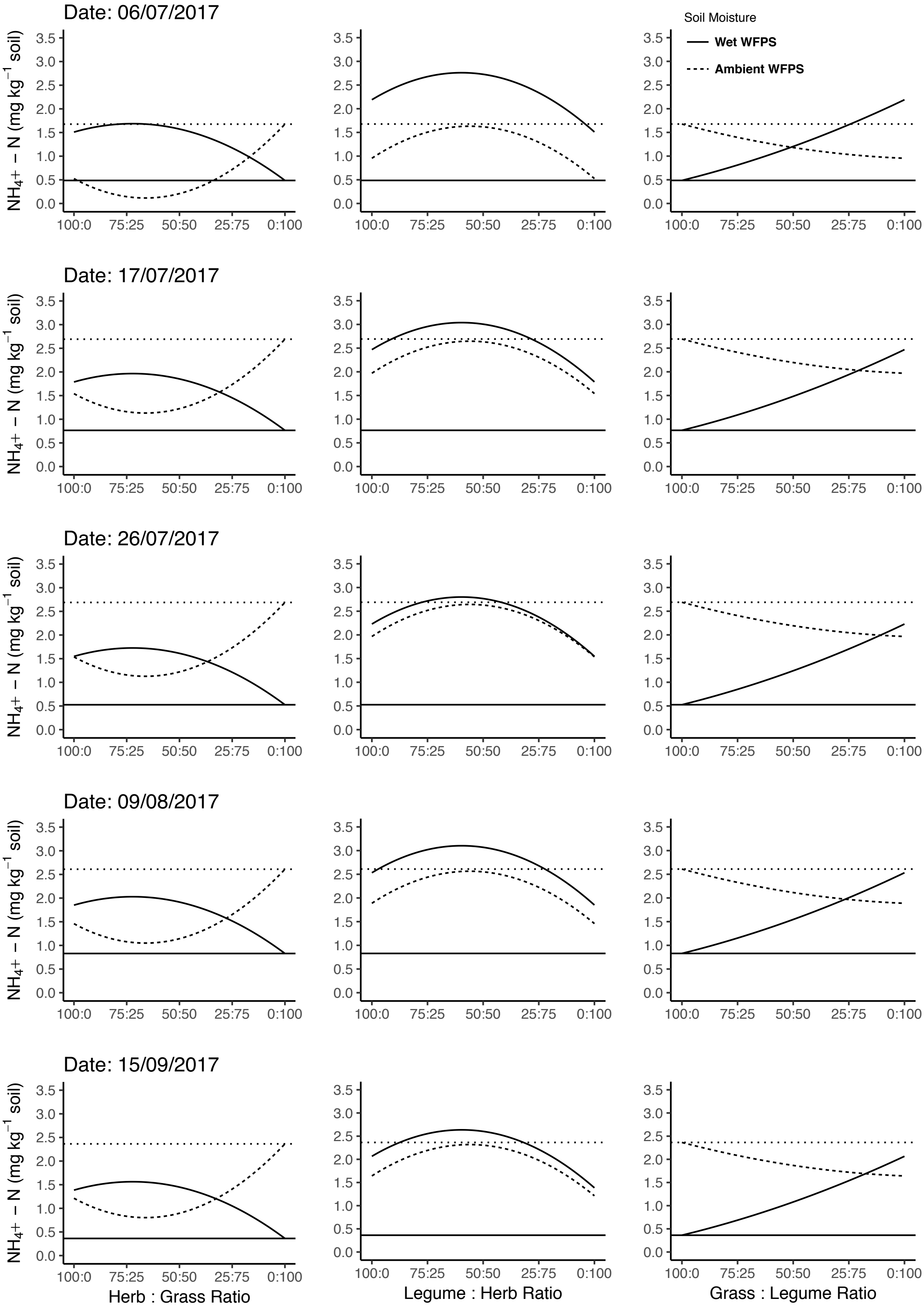

Figure S2: Effects plots of predicted NH_4^+ (mg kg^{-1} soil) for each soil sampling date with increasing proportions of individual plant functional groups under wet and ambient soil moisture conditions. Horizontal lines: PRG monoculture (100:0:0) response.

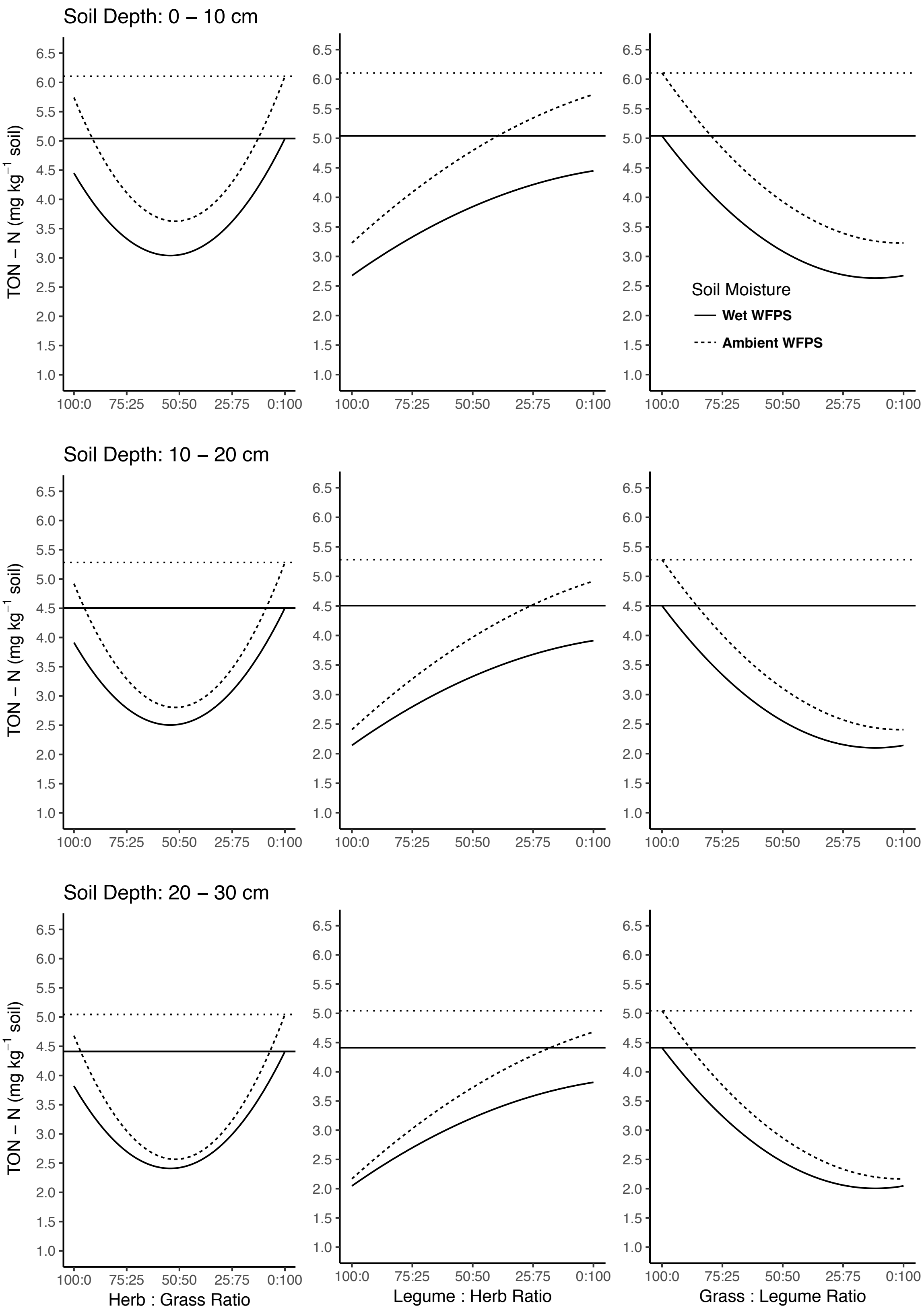

Figure S3: Effects plots of predicted TON (mg kg^{-1} soil) for each soil sampling depth with increasing proportions of individual plant functional groups under wet and ambient soil moisture conditions. Horizontal lines: PRG monoculture (100:0:0) response.

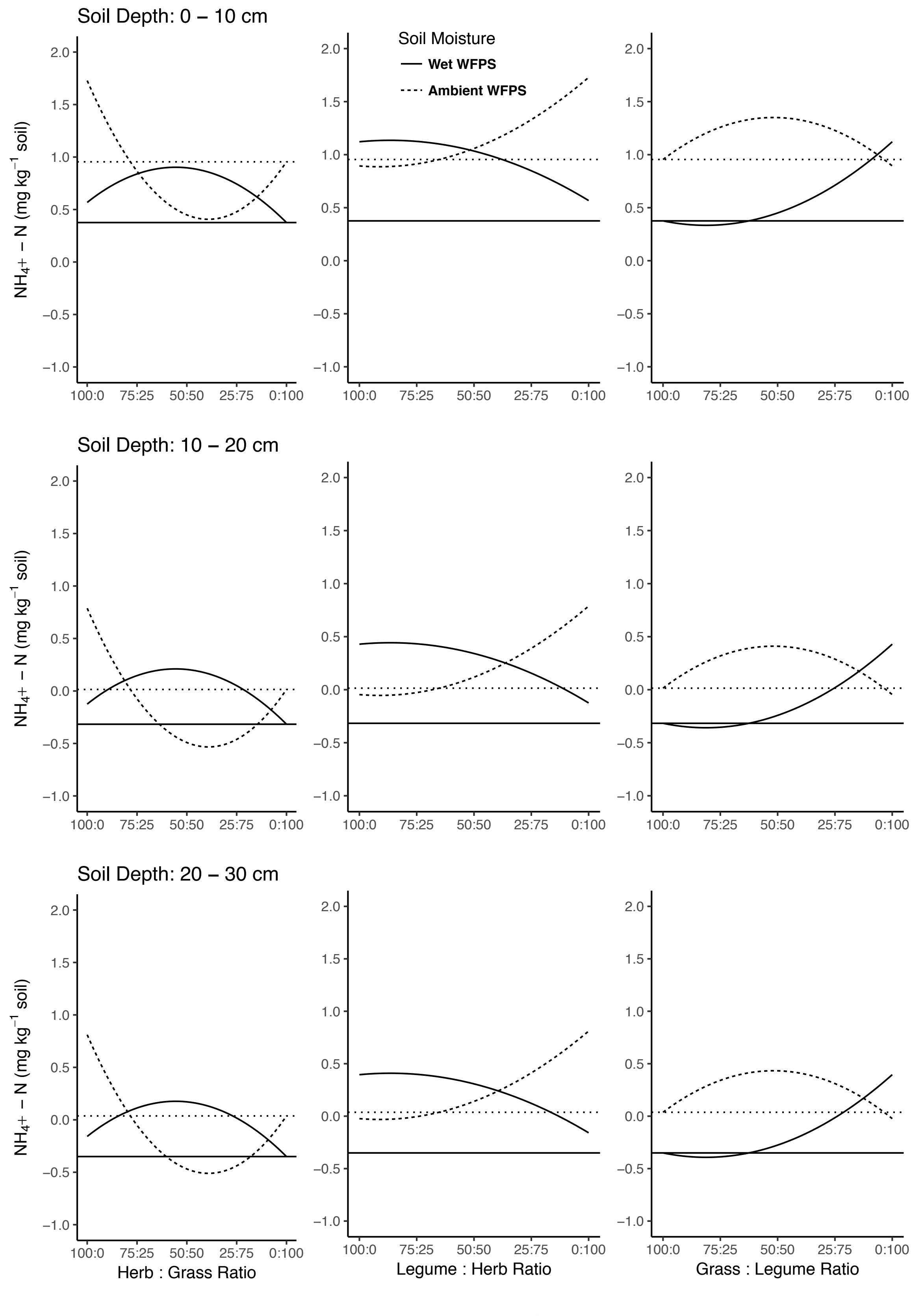

Figure S4: Effects plots of predicted NH_4^+ (mg kg^{-1} soil) for each soil sampling depth with increasing proportions of individual plant functional groups under wet and ambient soil moisture conditions. Horizontal lines: PRG monoculture (100:0:0) response.

Table S1: Original species ratios based on seeding rates (Grace et al. 2018) and 2017 species ratios as a percentage of total DM.

Original Species Ratios			**2017 Species Ratios**		
PRG	**WC**	**PLAN**	**PRG**	**WC**	**PLAN**
100	0	0	100	0	0
40	0	60	35	34	31
55	35	10	41	59	0
70	30	0	54	46	0
40	30	30	39	61	0
70	0	30	67	0	33
60	20	20	34	66	0
40	60	0	27	73	0