



RESEARCH ARTICLE

Manipulating plant community composition to steer efficient N-cycling in intensively managed grasslands

Diego Abalos^{1,2} | Gerlinde B. De Deyn¹ | Laurent Philippot³ | Natalie J. Oram¹ | Barbora Oudová^{1,4} | Ioannis Pantelis¹ | Callum Clark¹ | Andrea Fiorini⁵ | David Bru³ | Ignacio Mariscal-Sancho⁶ | Jan Willem van Groenigen¹

¹Soil Biology Group, Wageningen University, Wageningen, The Netherlands; ²Department of Agroecology, Aarhus University, Tjele, Denmark; ³Université Bourgogne Franche-Comté, INRA, AgroSup Dijon, Agroécologie, Dijon, France; ⁴School of Biological Sciences, University of East Anglia, Norwich, UK; ⁵Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, Italy and ⁶Departamento de Producción Agraria, ETS Ingeniería Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, Madrid, Spain

Correspondence

Diego Abalos

Email: d.abalos@agro.au.dk

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Handling Editor: Lei Cheng**Abstract**

1. Minimizing nitrogen (N) losses and increasing plant N uptake in agroecosystems is a major global challenge. Ecological concepts from (semi)natural grasslands suggest that manipulating plant community composition using plant species with different traits may represent a promising opportunity to face this challenge. Here, we translate these trait-based concepts to agricultural systems in a field experiment, aiming to reveal the main determinants of how plant community composition regulates N-cycling in intensively managed grasslands.
2. We focused on key N pools (plant N from soil and from biological N-fixation, soil mineral N and N₂O emissions) as well as on biological drivers of N-cycling in soil (abundance of N-cycling microbial communities, earthworm populations and arbuscular mycorrhizal fungi), using three common grass and one legume species in monoculture, two- and four-species mixtures. We hypothesized that: (a) plant species mixtures increase plant N uptake, reduce soil mineral N concentrations and N₂O emissions and promote the abundance of biological N-cyclers; (b) legume presence stimulates N pools, fluxes and biological N-cycling activity, (c) but in combination with a grass with acquisitive traits, more N is retained in the plant community, while N₂O emissions are reduced.
3. We found that mixtures increased plant N and lowered the soil mineral N pool compared to monocultures. However, plant species identity played an overarching role: Legume presence increased N₂O emissions, plant N pools, soil mineral N and the abundance of N-cycling microbes and earthworms. Combining the legume with a grass with low leaf dry matter content and high root length density (and with high root biomass) reduced the higher soil mineral N and N₂O emissions induced by the legume, while harnessing positive effects on plant N pools and biological N-fixation.

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4. *Synthesis and applications.* Our results show the potential of plant community composition to steer N-cycling in fertilized agroecosystems, paving the way for a more biologically based agriculture. Legumes will play a crucial role, but selecting an optimum companion species is key for the sustainability of the agroecosystem.

KEYWORDS

functional traits, grass legume mixtures, N₂O emissions, nitrogen cycling, nitrogen losses, plant and soil interactions, plant mixtures, plant species identity

1 | INTRODUCTION

The invention of the Haber–Bosch process, by which inert atmospheric nitrogen (N₂) is transformed into reactive forms, has marked a profound shift in the relationship between humans and the global N cycle. This reactive N enters the N cycle as an external input via mineral fertilizers applied to boost yields in intensive agroecosystems (Moreau et al., 2019). However, plants only take up and retain approximately 40% of fertilizer N in their biomass (Cassman et al., 2002). Most of the remaining N can be lost from the agroecosystem with dire environmental consequences. Nitrogen can be lost as nitrous oxide (N₂O) emissions from nitrification, denitrification and related processes, contributing to global warming and ozone depletion. Alternatively, soil mineral N may leach to water bodies, resulting in harmful algal blooms and human health problems (van Groenigen et al., 2015). One promising strategy to steer the biological processes that underpin soil N availability, N losses and plant N retention is by manipulating the plant community composition of the agroecosystem.

Translating concepts from ecology in natural grasslands, recent field studies in fertilized, intensive agroecosystems have shown that increasing plant species richness from monocultures up to four-species mixtures can increase yields (Finn et al., 2013) and plant N retention (Suter et al., 2015). In these studies, resource use complementarity was the most likely mechanism behind the biomass increases in mixtures. Therefore, plant species mixtures may mitigate N losses by reducing the availability of soil mineral N to micro-organisms due to high rates of plant N uptake (Moreau et al., 2015). Within these mixtures, the presence of specific plant functional groups may be a key determinant of plant community effects on N-cycling. Legumes, because of their ability to fix N₂, represent a keystone biological input of N in natural and managed ecosystems. Grown together with grasses, legumes can meet more than 80% of their own N-demand from fixation, promoting facilitation effects by transferring biologically fixed-N to grasses (Rasmussen et al., 2013), and enhancing exploitation of soil resources through spatial or temporal niche complementarity in resource uptake (Mueller et al., 2013). Since the identity of the companion grass exerts a strong effect on the overall distribution of N in plants and soil (Pirhofer-Walzl et al., 2012), we need to identify species combinations that increase the sustainability of our agroecosystems and understand the main determinants behind such positive plant–plant interactions.

A robust approach to obtain a mechanistic understanding of how plant communities regulate N-cycling is to study relationships between plant traits and ecosystem functioning (Pommier et al., 2018). In mesocosm experiments, grasses with leaf traits related to fast N acquisition (high specific leaf area, SLA, and low leaf dry matter content, LDMC) have been linked to reduced N losses in fertilized systems via decreases in N₂O emissions (Abalos et al., 2018) and N leaching (De Vries & Bardgett, 2016). Below-ground, grasses with root traits associated with fast N acquisition are expected to lead to the same results, and empirical evidence to validate these theoretical claims is starting to emerge (Abalos et al., 2014, 2018; Cantarel et al., 2015). It is therefore plausible that combining a legume with an acquisitive grass will result in higher plant N uptake and lower N losses. A key next step is to (a) test these findings under realistic field conditions and (b) unveil whether the functional traits of the dominant species in the community (Garnier et al., 2004) or the functional trait diversity among species (Laliberte & Legendre, 2010) determines the overall association between plant traits and N pools and fluxes.

Plant community composition can be strategically designed to regulate N losses and N retention not only via direct effects through plant nutrient acquisition but also indirectly by affecting below-ground biotic communities contributing to N-cycling. Conceptual frameworks illustrating these mechanistic links have been recently proposed (Abalos et al., 2019; Moreau et al., 2019). For example, previous research has shown positive relationships between plant species richness and earthworm species number and abundance, particularly when legumes are present (Milcu et al., 2008). In turn, higher earthworm abundance may enhance N mineralization and N₂O emissions (Lubbers et al., 2013). Legumes can also increase the abundance of soil diazotrophs (Xiao et al., 2020) rising N inputs into the soil. Leaf traits such as SLA have been linked to microbial N uptake and ecosystem N retention (De Vries & Bardgett, 2016). By producing higher root biomass, plant species richness can promote the abundance of arbuscular mycorrhizal fungi (AMF; Chung et al., 2007; Wolf et al., 2003); plants with low specific root length (SRL) generally have high levels of AMF colonization of their roots (Cortois et al., 2016). In turn, increased AMF colonization may favour plant N retention and reduce the emission of N₂O and N leaching (Bender et al., 2016; Martínez-García et al., 2017). Specific plant traits are also linked to the abundances and activities of microbial nitrifiers (e.g. SRL; Cantarel et al., 2015) and nitrate reducers

(e.g. root N uptake rate; Moreau et al., 2015), and these processes regulate in which form N is lost (Hallin et al., 2018). To date, most studies on plant community effects on N-cycling have overlooked these meaningful plant–soil interactions, despite their importance to transition into more biologically based agroecosystems (Abalos et al., 2019; Bowles et al., 2015).

The objective of this field study was to explore the potential of plant community composition (species mixtures, species identity and functional trait dominance/diversity) to regulate N-cycling in intensively managed grasslands. We hypothesized that: (a) plant species mixtures would increase plant N retention and decrease soil inorganic N concentrations and N₂O emissions; by providing more diverse litter inputs, plant mixtures would also promote the abundance and activity rates of below-ground biotic communities; (b) the presence of a legume would increase N pools, fluxes and biological N-cycling activity, but (c) in combination with an acquisitive grass more N can be retained in the plant community with lower N losses; and (d) the relationships between plant communities and N-cycling can be explained by the dominant traits or the trait divergence of the plant community.

2 | MATERIALS AND METHODS

2.1 | Experimental design

We conducted our field study at 'Droevendaal' experimental farm, Wageningen, the Netherlands (51°59'N, 5°39'E). The soil is classified as a *typic endoaquoll* (Soil Survey Staff, 1999) with 75% sand, 23% silt and 2% clay. Initial physico-chemical characteristics of the 0–15 cm soil layer were pH-CaCl₂, 5.1; soil bulk density, 1.42 Mg/m³; organic matter, 2.2%; total N, 1.30 g/kg. The climate is temperate maritime (Cfb according to Köppen–Geiger), with an average temperature of 9.4°C and mean annual precipitation of 780 mm. During the years before the establishment of our experiment, the field site had been used as an arable field involving annual crops (mainly wheat and maize), managed following standard farming practices (i.e. conventional tillage, mineral fertilizers according to crop requirements, use of herbicides and pesticides).

The experiment was set up using a randomized block design in March 2016, and consisted of 60 plots (3 × 3 m) laid out in five blocks, with grass strips (*Phleum pratense* L.) of 3 m between plots. The treatments covered a plant species richness gradient of one, two and four species, and two functional groups: grass (*Lolium perenne* L., Lp; *Festuca arundinacea* Schreb., Fa; *Poa trivialis* L., Pt) and legume (*Trifolium repens* L., Tr). In total, there were 12 treatments: no plants (control); monocultures of the four species; all possible combinations of two species; and a mixture with all four species ($n = 60$ plots). The levels of species richness from one to four species were selected based on the strong benefits for ecosystem functioning of this diversity gradient in intensive grasslands (Finn et al., 2013; Nyfeler et al., 2009), while maintaining a level of management complexity that is feasible to adopt by farmers. The selected plant species are

commonly found in N-rich soils and cover a relatively wide range of functional traits, both above- and below-ground (e.g. Abalos et al., 2014; Mason et al., 2016). All plots were weeded manually two to three times per year to maintain the target species composition.

Plowing took place in March 2016 and sowing in April 2016 (establishment year), at a final seeding density of 1,500 viable seeds per m² divided equally among the species of each mixture (i.e. 750 seeds m⁻² for each species in the two-species mixtures, and 375 seeds m⁻² for each species in the four-species mixtures). During 2016 and 2017, N fertilizer (calcium ammonium nitrate) was split into three applications at a total rate of 150 kg N/ha (50 kg N/ha in May, June and September). This total N rate is realistic from a farmer's perspective, but lower than the highest levels of N application to grass monocultures in intensive grassland management (200–300 kg N/ha), and it does not impair positive grass–legume interactions (Nyfeler et al., 2009). All plots also received superphosphate (50 kg P ha⁻¹ year⁻¹) and potassium sulphate (200 kg K ha⁻¹ year⁻¹). All measurements were conducted during the second productive year (2017), when the grassland communities were well established.

2.2 | Soil and plant N pools and fluxes

Plant N pools were quantified for above- and below-ground plant biomass. Above-ground biomass was cut in subplots (25 × 25 cm) three times over the growing season (May, July and October) and the vegetation was sorted per species. The remaining above-ground biomass was harvested with an experimental plot harvester immediately after sampling. Root biomass was sampled at the end of the growing season after the last harvest (October 2017) with a soil corer (8 cm diameter, 0–30 cm depth) and roots were separated from soil and cleaned with water using a root washing facility; a representative root subsample of the cleaned roots was collected and stored in 50% ethanol to be used for quantifying root traits (see below). Ball-milled dry plant samples were analysed for N concentration using a CN elemental analyser (LECO). For plant communities consisting of more than one species, we used species-specific above-ground N uptake (kg N/ha) per plot to quantify the net, complementarity and selection effects using the additive partitioning method (Loreau & Hector, 2001).

Flux measurements of N₂O were taken daily during the first 4 days after fertilizer applications, every other day in the second week, two consecutive days after rainfall events and two to three times per week for the remainder of the experiment. The flux measurement protocol largely followed that of previous studies (Abalos et al., 2014, 2018; Lubbers et al., 2015). Polypropylene flux chambers (20 cm diameter, 14 cm height) were placed on top of permanently installed collars (10 cm depth) in each plot for approximately 30 min. Gas measurements were taken from the headspace with an Innova 1312 photo-acoustic infrared gas analyser (LumaSense Technologies A/S). Emission rates of N₂O fluxes were consequently estimated as the slope of the linear regression between concentration and time. Cumulative emissions were calculated by linear interpolation.

To determine soil mineral N, five soil cores per plot were taken with a soil auger (25 cm deep \times 1.5 cm diameter) in May, June, July and October. Due to the exponential decline of root biomass with soil depth (Frank et al., 2010) and the mobility of NO_3^- , high NO_3^- concentrations in the top 25 cm soil layer are generally an indicator of high risk of N leaching (Klaus et al., 2018). Soil samples were extracted for mineral N analyses by shaking (1/10 w/v, dry weight basis) with 0.01 M CaCl_2 for 2 hr (Houba et al., 2000). Extracts were centrifuged (15 min), filtered (0.45 μm) and NH_4^+ and NO_3^- concentrations were determined by colorimetry (Brann en LuebbeTrAAcs 800 Autoanalyzer, Skalar Analytical B.V. Breda).

2.3 | Soil microbiological properties, biological N-fixation, arbuscular mycorrhizal fungi and earthworms

To quantify the abundances of microbial populations involved in N-cycling by real-time quantitative PCR (qPCR), five soil samples (25 cm depth \times 1.5 cm diameter) were collected in July 2017 and combined into a composite sample per plot ($n = 60$). Samples were frozen (-20°C) immediately after sampling until further analysis. DNA extraction (0.25 g of soil) was performed using the DNeasy PowerSoil HTP 96 Kit (QIAGEN) according to manufacturer instructions. DNA concentrations were measured using the Quant-iTTM dsDNA High-Sensitivity Assay Kit (Invitrogen); qPCR was performed in a 15- μl final reaction volume with the Takyon low ROX SYBR 2X MasterMix blue dTTP, Eurogentec. Reactions were carried out in a ViiA7 real-time PCR system. Potential inhibitory effects of co-extracted humic compounds in soil extracts were checked by mixing a known amount of plasmid DNA with soil DNA extract or water prior to qPCR. Primers and amplification conditions of qPCR assays are shown in Table S1.

To determine the influence of carbon availability on N_2O production and reduction by denitrification, we determined denitrification capacity as described in Abalos et al. (2018). Briefly, we supplied 10 ml of a 1 mM KNO_3 solution to airtight 1 L flasks containing two subsamples per plot (20 g each) collected in July. For one of the duplicate flasks, 10% C_2H_2 was added to inhibit N_2O -reductase activity and the flasks were incubated at 20°C . Nitrous oxide was quantified after 24 and 48 hr, and the ratio of N_2O to total $\text{N}_2\text{O} + \text{N}_2$ production was derived from the ratio of N_2O production in flasks without and with C_2H_2 .

To sample earthworm populations, a soil monolith of 20 \times 20 \times 20 cm was dug out and hand-sorted from every plot in October. Earthworms were counted and their intestines voided for 48 hr following the wet paper method of Dalby et al. (1996) before being weighed.

To estimate arbuscular mycorrhizal fungi (AMF) colonization in the root samples collected in October 2017, the fungal structures in roots were stained following Brundett et al. (1996). The AMF colonization percentage of the roots was estimated according to the grid line intersection method (McGonigle et al., 1990).

Biological N-fixation in the plants was assessed using the natural ^{15}N abundance method as percentage of N derived from the atmosphere (%Ndfa). The shoot biomass of five legume leaves per plot was sampled in October, dried, ball-milled and analysed for total N and atom% ^{15}N at the UC Davis Stable Isotope Facility, on an ANCA-SL Elemental Analyser coupled to a 20–20 mass spectrometer using the Dumas dry combustion method. The %Ndfa was calculated following Shearer and Kohl (1986).

2.4 | Plant traits

Plant functional traits were measured following standard procedures described in Perez-Harguindeguy et al. (2013). Above-ground traits were measured on the youngest fully expanded leaf of five individuals per species per plot, sampled in October. SLA was quantified using an LI-3100C Area Meter (Li-Cor Inc.). Leaf dry matter content (LDMC) was estimated as the ratio of dry to saturated weight. The root subsamples that were stored in alcohol were stained with neutral red (Bouma et al., 2000) and scanned (EPSON Perfection V700/V750 3.92). Root length and diameter were estimated using WINRHIZO (Regent Instruments Inc.). Specific root length (SRL) and root length density (RLD) were calculated.

To understand whether the functional leaf traits of the dominant species in the community or the functional leaf trait dissimilarity among species were the main regulators of N-cycling, we used the measured trait values of each plant species (in monocultures and in mixtures) to calculate the community-weighted means (CWM; Garnier et al., 2004), and the functional trait diversity (FD; Laliberté & Legendre, 2010). Root traits are expressed on the community level.

2.5 | Calculations and statistical analysis

All statistical analysis was performed in R version 3.5.0. (R Core Team, 2018). The effects of plant species mixtures (1 vs. 2 or 4 species), species identity and functional trait dominance (CWM) and diversity (FD) on N pools, fluxes and biological drivers of N transformations were tested with linear mixed effects models, lme from package NLME (Pinheiro et al., 2016), fitted with a type III SS ANOVA. Plant species mixtures, species identity and functional trait measures were considered as explanatory variables, and block was included as a random factor. When residual variances were heterogeneous, a variance structure (varIdent, package NLME) was included in the model. Models with and without the variance structure were compared based on Akaike information criterion (AIC) and the model with significantly lower AIC was retained. The same models with monoculture data were used to test differences in functional traits at the plant species level. Since the abundances of N-cycling microbial communities were highly correlated ($r = 0.72\text{--}0.99$; $p < 0.001$), we performed a principal component

analysis (PCA) on gene abundances using the package VEGAN (Oksanen et al., 2013), and PC1 was used in subsequent analysis. Spearman correlations were used to assess the relationships between biotic N drivers and N pools.

3 | RESULTS

3.1 | Plant monocultures versus species mixtures

Plant N pools increased in the mixtures compared to the monocultures ($p = 0.04$; $R^2 = 0.11$; $F = 3.34$), with the four-species mixture having a 29% larger plant N pool (N uptake in above- and below-ground biomass) than the average of the monocultures (Figure 1c). This effect was mainly due to root N uptake rather than above-ground plant N uptake. Root biomass in the mixtures was higher than in the monocultures ($p < 0.001$; $R^2 = 0.34$; $F = 16.57$; Figure S1). The additive partitioning analysis of above-ground N uptake (Figure S2)

showed a positive net effect of mixtures (i.e. enhanced performance of plant mixtures relative to that expected from monocultures). These results also revealed that the complementarity effect contributed more to the net effect (61%–75%) than the selection effect (i.e. the presence of a specific species). Soil NO_3^- availability ($p = 0.01$; $R^2 = 0.14$; $F = 4.32$) and earthworm biomass ($p = 0.007$; $R^2 = 0.07$; $F = 5.51$) were lower in mixtures than monocultures (Figure 1). All the other N variables were not different between plant mixtures and monocultures.

3.2 | Plant species identity and community composition

Trifolium repens presence increased N_2O emissions by 58% ($p < 0.001$; $R^2 = 0.12$; $F = 10.61$), total plant N uptake by 49% ($p < 0.001$; $R^2 = 0.45$; $F = 45.40$), soil NO_3^- by 60% ($p \leq 0.05$; $R^2 = 0.06$; $F = 3.42$), NH_4^+ by 17% ($p = 0.09$; $R^2 = 0.05$; $F = 2.96$), earthworm biomass by

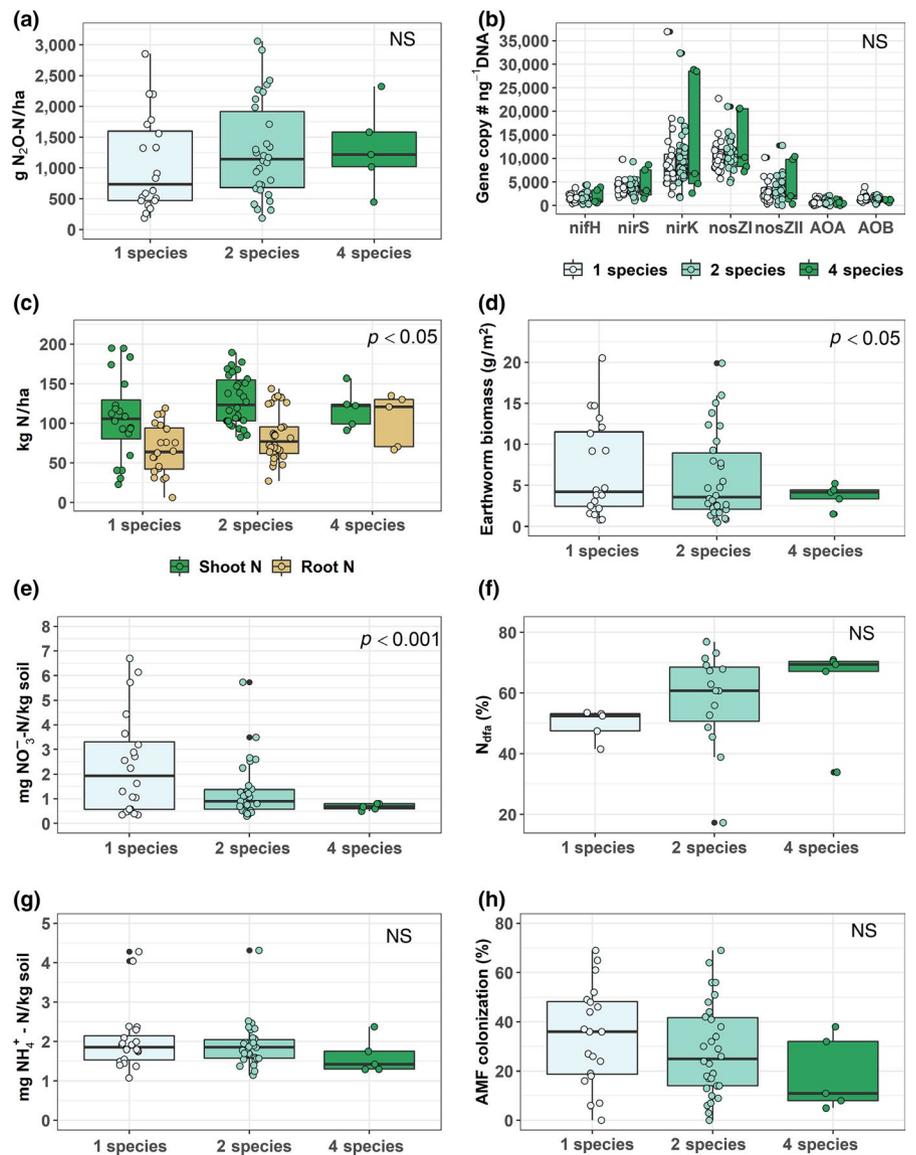


FIGURE 1 Plant monocultures and species mixtures (two and four species) impacts on (a) cumulative N_2O emissions; (b) the abundance of N-cycling microbial communities; (c) plant N uptake, depicted separately for above- and below-ground components; (d) earthworm biomass; (e) soil NO_3^- concentration; (f) N-fixation, expressed as N derived from the atmosphere (Ndfa); (g) soil NH_4^+ concentration; and (h) arbuscular mycorrhizal fungi (AMF) colonization in roots. Significant differences are tested using linear models (NS: non-significant)

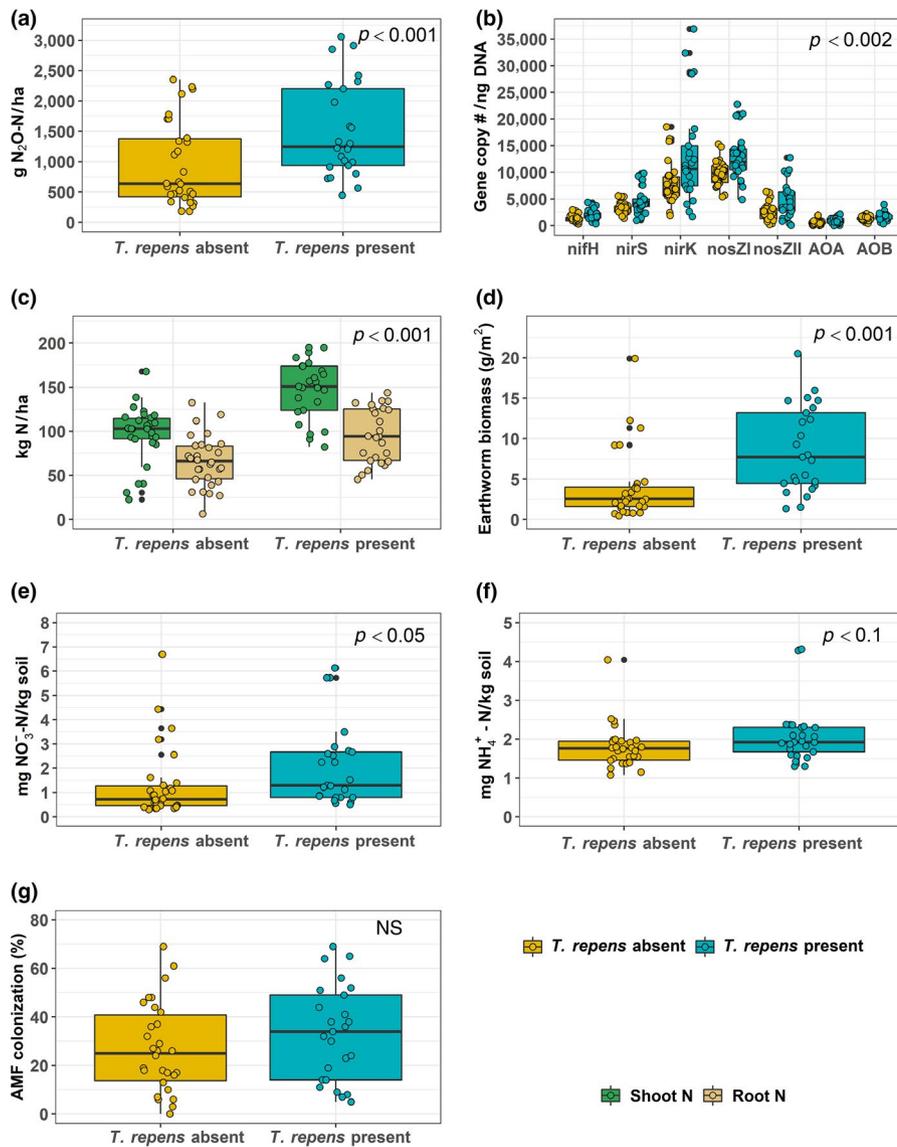


FIGURE 2 Effect of *Trifolium repens* presence/absence on (a) cumulative N_2O emissions; (b) the abundance of N-cycling microbial communities; (c) plant N uptake, depicted separately for above- and below-ground components; (d) earthworm biomass; (e) soil NO_3^- concentration; (f) soil NH_4^+ concentration; (g) arbuscular mycorrhizal fungi (AMF) colonization in roots. Significant differences are tested using linear models (NS: non-significant)

116% ($p < 0.001$; $R^2 = 0.19$; $F = 15.29$) and on average N-cycling microbial abundance by 53% ($p = 0.002$; $R^2 = 0.11$ – 0.22 ; $F = 10.45$ – 21.03 ; Figure 2). The presence of *T. repens* also increased the harvested yield ($p = 0.02$; $R^2 = 0.05$; $F = 5.38$; Figure S3), and had a marginally positive effect ($p = 0.08$; $R^2 = 0.05$; $F = 3.15$) on the ratio of N_2O -producers (*nirK* + *nirS*) to N_2O -reducers (*nosZI* + *nosZII*; Figure S4). The strength of the *T. repens* effects depended on the identity of the companion grass species in the species mixtures (Figure 3). Compared to the other mixtures with *T. repens*, combining the legume with the grass *L. perenne* led to 50% lower N_2O emissions ($p = 0.02$; $R^2 = 0.26$; $F = 3.47$), low soil mineral N concentrations (similar to the combination with *F. arundinacea* and lower than with *P. trivialis*; $p < 0.001$; $R^2 = 0.57$; $F = 14.95$) and without differences for AMF colonization levels. Denitrification capacity was not affected by species identity or plant community composition (Figure S5). Nitrogen fixation (%Ndfa; Figure 4) was higher when the legume *T. repens* was grown in combination with the grasses *L. perenne* and *F. arundinacea* than when grown in monoculture or in combination with *P. trivialis* ($p = 0.005$; $R^2 = 0.47$; $F = 6.96$).

3.3 | Relationships between biotic N drivers and N pools

Earthworm biomass was related to all the measured variables, whereas AMF colonization was only related to earthworm biomass (Table 1). The collective abundance of genes of the soil microbes involved in N-cycling (PCA axis projection 1) had a positive relationship with N_2O emissions and with N-fixation. Plant N pools were related to all N variables except AMF, and the strongest association was with N-fixation.

3.4 | Plant functional traits in monocultures

Above-ground, *L. perenne* and *T. repens* had the lowest LDMC, *T. repens* had the highest LNC and lowest C:N ratio and *P. trivialis* had the lowest SLA values (Table 2). Belowground, *P. trivialis* had the highest SRL and lowest root diameter; *P. trivialis* and *L. perenne* showed significantly larger RLD values than *F. arundinacea* and *T. repens*. The legume *T. repens* had lowest root C:N ratio.

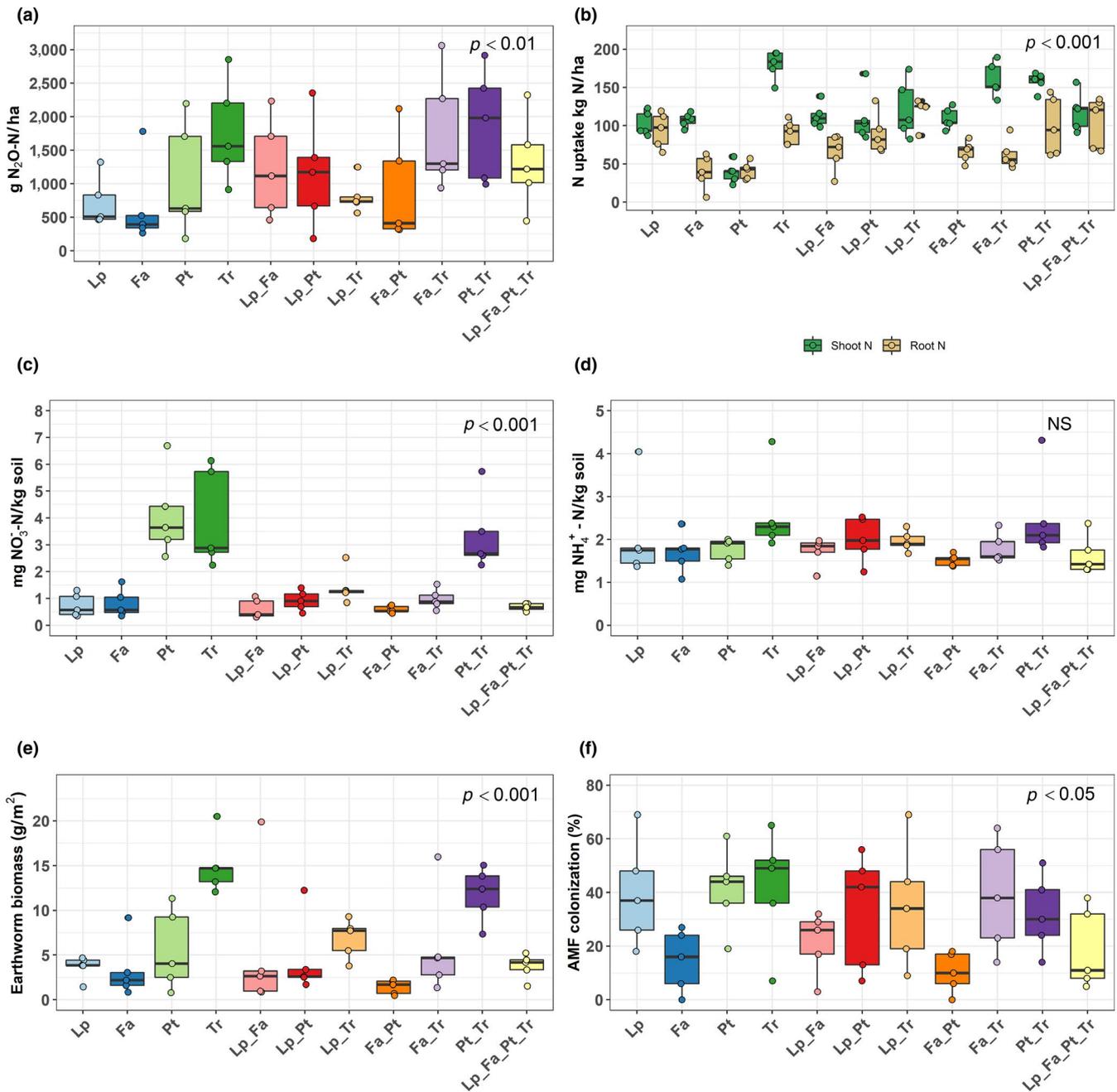


FIGURE 3 Plant community composition impacts on (a) cumulative N₂O emissions; (b) plant N uptake, depicted separately for above- and below-ground components; (c) soil NO₃⁻ concentration; (d) soil NH₄⁺ concentration; (e) earthworm biomass; and (f) arbuscular mycorrhizal fungi (AMF) colonization in roots. Significant differences are tested using linear models (NS: non-significant). *Lolium perenne* (Lp); *Festuca arundinacea* (Fa); *Poa trivialis* (Pt); *Trifolium repens* (Tr)

3.5 | Trait dominance and trait diversity effects on N-cycling

Community-weighted means (CWM) of leaf traits showed more associations and stronger effects on N pools and biotic drivers of N-cycling than functional diversity of leaf traits (Table 3). Higher CWM values for LNC and lower CWM for LDMC were linked to N pool increases (plant N pools, N₂O emissions, N-fixation and soil mineral N) and enhanced abundance of biological N drivers (N-cycling microbial communities, earthworm biomass and AMF). Higher CWM values

for SLA were linked to higher plant N retention and abundance of N-cycling microbial communities. Plant communities with high functional diversity in LNC had lower soil NO₃⁻ concentrations, and higher N₂O emissions, plant N retention and N-fixation. Belowground, SRL was negatively related with plant N retention (communities with finer roots had lower plant N uptake), whereas root diameter was positively related with plant N retention, earthworm biomass and N-fixation. Higher RLD was negatively related to soil NO₃⁻ concentration, the abundance of N-cycling microbial communities and earthworm biomass.

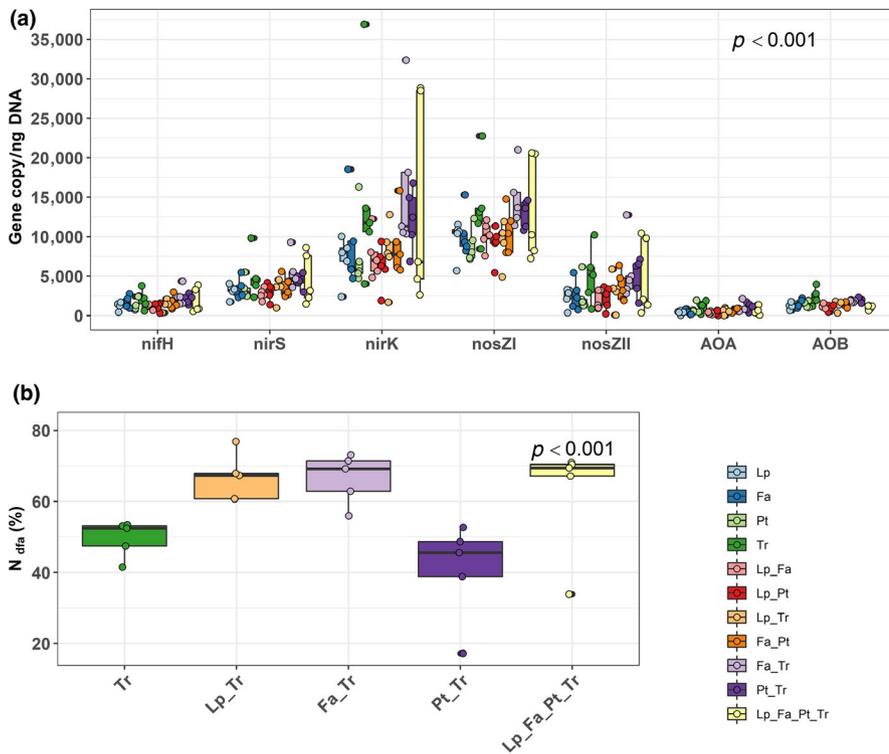


FIGURE 4 Plant community composition impacts on (a) the abundance of N-cycling microbial communities; and (b) N-fixation, expressed as N derived from the atmosphere (N_{dfa}). Significant differences are tested using linear models (NS: non-significant). *Lolium perenne* (Lp); *Festuca arundinacea* (Fa); *Poa trivialis* (Pt); *Trifolium repens* (Tr)

TABLE 1 Spearman's rank correlations of N pools and biotic drivers of N-cycling. N₂O, cumulative N₂O emissions over the experimental period; Plant N retention, above- (4 harvests) and belowground plant N uptake; NO₃⁻ and NH₄⁺, mean concentrations over the experimental period; N-cycling microbial communities, axis 1 of PCA with microbial gene abundances; EW biomass, earthworm biomass; N-fixation, biological N-fixation by legume plants (%N_{dfa}); AMF, arbuscular mycorrhizal fungi colonization in roots

	N ₂ O	Plant N retention	NO ₃ ⁻	NH ₄ ⁺	N-cycling microbial communities	EW biomass	N-fixation	AMF
N ₂ O		0.24	0.18	0.21	0.31*	0.39**	0.28*	—
Plant N retention			0.29*	0.38**	0.35**	0.38**	0.64**	—
NO ₃ ⁻				0.48**	0.22	0.40**	0.24	0.25
NH ₄ ⁺					0.12	0.52**	0.19	0.11
N-cycling microbial communities						0.38**	0.36**	0.18
EW biomass							0.37**	0.34*
N-fixation								0.13
AMF								

Note: Numbers indicate ρ values; values in bold are $p < 0.05$ (*, **significant at the 0.05 and 0.01 probability level respectively).

TABLE 2 Results of linear mixed effects models for functional traits of plant monocultures (mean values based on $n = 5$). SLA, specific leaf area; LDMC, leaf dry matter content; LNC, leaf nitrogen concentration; SRL, specific root length; RLD, root length density

Plant species identity	SLA (cm ² /g)	LDMC (g/g)	LNC (mg N/g)	Leaf C:N	SRL (m/g)	Root diameter (mm)	RLD (cm/cm ³)	Root C:N
<i>Lolium perenne</i>	178.2	0.16	27.01	16.85	153.46	0.21	101.94	33.75
<i>Festuca arundinacea</i>	214.6	0.20	22.78	19.22	112.85	0.24	44.53	39.58
<i>Poa trivialis</i>	101.6	0.25	29.01	15.01	449.02	0.14	100.24	27.78
<i>Trifolium repens</i>	209.5	0.13	42.28	10.41	91.46	0.28	23.46	16.44
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001
R^2	0.66	0.81	0.84	0.81	0.65	0.68	0.47	0.85
F	16.46	29.54	36.50	34.10	13.47	13.75	10.63	42.59

4 | DISCUSSION

4.1 | Plant community composition and N-cycling

We found that an agriculturally feasible level of plant species mixing (from one to two or four species) increased plant N retention by 25% and reduced soil NO_3^- availability and earthworm biomass compared to monocultures (Figure 1). The positive effect of species mixtures on plant N retention is consistent with results previously found in natural grasslands (Scherer-Lorenzen et al., 2003; Wang et al., 2018), yet our study is one of the few reporting this finding for an intensively fertilized grassland (Suter et al., 2015). This effect could be due to enhanced complementarity (De Deyn et al., 2009), and/or due to the presence of a certain influential species or functional groups (i.e. selection effect; Hooper, 1998). Our results suggest that both mechanisms may have taken place during the experiment. Above-ground, complementarity was the main mechanism causing enhanced N uptake in plant mixtures relative to that expected from monocultures (Figure S2). The importance of complementarity is also supported by our finding that increased functional diversity of LNC (higher for the four-species mixtures) was associated with higher plant N pools (Table 3). The higher resource complementarity and plant N capture probably led to the lower NO_3^- availability for the four-species mixtures (Figure 1). Despite this evidence for the occurrence of resource use complementarity, the dominance of CWM over FD trait measures in explaining biotic and abiotic N processes (Table 3) points to the overarching role of specific plant species and their functional traits as regulators of N-cycling during our experiment.

Indeed, we found that the presence of the legume *T. repens* increased N_2O emissions, plant N pools, soil NO_3^- and NH_4^+ concentrations, the abundance of N-cycling microbial communities and earthworm biomass at plant community level (Figure 2). Legume presence also played a minor but significant role for higher yield (Figure S3). By establishing a symbiotic association with N-fixing bacteria (and by increasing N-fixers abundance, i.e. *nifH*), legumes add an internal input of biological N resulting from the mineralization of their N-rich litter (*T. repens* had the lowest leaf and root C:N ratios; Table 2) and root exudates. In semi-natural grasslands with low soil fertility levels, positive effects of legumes on N_2O (Niklaus et al., 2006), soil mineral N (and associated N-leaching; Scherer-Lorenzen et al., 2003), plant N pools (De Deyn et al., 2009), earthworm biomass (Milcu et al., 2008) and some N-cycling microbes (e.g. AOB; Malchair et al., 2010) have been previously reported. Here, we show for the first-time similar effects for an intensively managed grassland receiving external inputs of N via fertilizer. Moreover, our study shows that legumes can also increase the abundance of *nirS*, *nirK*, *nosZI* and *nosZII* microbial guilds, further stimulating soil N-cycling.

Among the combinations of *T. repens* with grasses, *L. perenne* was the species leading to lower N_2O emissions and a high plant N pool, without a negative influence on the other N-cycling drivers or pools. This was probably because this fast-growing grass had higher N uptake rates than the other grass species (Figure 3), allowing

L. perenne to use the available sources of N from soil, fertilization and N-fixation more efficiently. Indeed, *L. perenne* was the grass species with the lowest LDMC (Table 2), indicative of an acquisitive strategy for N capture. It was also the species with the highest RLD and with high SRL, architectural root traits that determine the configuration of the root system and thereby the plant's ability to acquire N (Bardgett et al., 2014) and potentially reduce N_2O emissions (Abalos et al., 2019). As such, higher RLD led to lower soil NO_3^- concentrations (Table 3), and because *L. perenne* had the highest root biomass production of all the species (Figure S1), its combination with *T. repens* had the highest RLD values among the combinations with the legume.

Although grasses with acquisitive leaf traits and high RLD appear to be of pivotal importance for efficient N-cycling in combination with legumes, these traits alone did not explain a significant fraction of the variation in N pools and soil N-cycling processes (Table 1; Table 3). *P. trivialis*, for example, also presented these set of traits, but did not result in the same positive effects when mixed with *T. repens*. This was probably because high root biomass production may be a critical factor as well, since *L. perenne* in monoculture and two-species mixtures had high biomass production, whereas *P. trivialis* had very low biomass (also aboveground), contributing little to the overall traits of the plant communities. The negative correlation between root biomass and the abundance of AOB ($r = -0.45$; $p < 0.05$) and AOA ($r = -0.37$; $p < 0.05$) supports the relevance of root biomass for soil mineral N capture. Hence, the lower abundance of both AOA and AOB suggests a lower soil N availability and that ammonia oxidizers are out-competed when the root biomass production is higher. Root biomass could also stimulate denitrification and N_2O emissions, as the quantity of root exudation, which may provide a carbon source for heterotrophic microorganisms as denitrifiers, is thought to be positively correlated with root biomass (Bengtson et al., 2012; Orwin et al., 2010). However, denitrification capacity was not affected by plant community composition and N_2O emissions were not correlated with root biomass, suggesting that root biomass alone was not a good indicator of carbon availability in our study. These results also indicate that future studies should consider other traits or factors not measured in our study to unravel the full potential of plant community composition to improve N-cycling.

4.2 | Relationships between N pools and soil N-cycling processes

Our results shed light on the relative importance of N-cycling processes as regulators of N pools in intensive agroecosystems. Earthworm biomass was a particularly pivotal player for N-cycling, showing positive associations with all the evaluated variables (Table 1). By stimulating mineralization from plant residues and soil organic matter, earthworms can increase soil mineral N and N leaching (Dominguez et al., 2004), N_2O emissions (Lubbers et al., 2013) and plant N uptake (van Groenigen et al., 2015). As opposed to the

prominent role of earthworms, AMF colonization showed a general absence of direct relationships with biological drivers of N-cycling or with the measured N pools and fluxes. By improving plant nutrient (mainly phosphorus (P) but also N and other plant growth limiting nutrients) and water acquisition, AMF can reduce soil mineral N and N leaching, N₂O emissions (Storer et al., 2017) and increase plant N retention (Bender et al., 2016). However, since P and N availability were not limiting plant growth strongly in our experiment due to fertilizer application, the chance of a large mycorrhizal benefit through improved plant nutrition may have disappeared (Ryan & Graham, 2018).

The abundance of N-cycling microorganisms was related to the emission of N₂O (Table 1), which was probably mediated by the presence of *T. repens*. As explained above, legume presence increased the availability of NH₄⁺ in the soil (Figure 2), resulting in higher abundance of AOA (91%) and AOB (38%), and accordingly the potential N₂O emissions from nitrification (Prosser et al., 2019). *Trifolium repens* also had positive effects on the abundance of all the denitrification genes, and marginally increased the ratio of N₂O-producers (*nirK* + *nirS*) to N₂O-reducers (*nosZI* + *nosZII*; Figure S4), and as expected this ratio was positively correlated with N₂O emissions ($r = 0.25$; $p < 0.05$). Therefore, *T. repens* probably also increased N₂O emissions from denitrification. Some authors argue that the N₂O emissions induced by the growth of legumes may be estimated solely as a function of the above- and below-ground N inputs from their residue during pasture renewal (Rochette & Janzen, 2005). Here, we show that, to some extent, the positive feedback between legumes and the abundance of N-cycling microbial communities may be a hitherto overlooked mechanism contributing to the higher emissions of N₂O when legumes are grown.

4.3 | Implications of our study and future research directions

Intensively managed grasslands are commonly renewed after 2–5 years (Nevens & Reheul, 2003; Schils et al., 2002), and therefore, the effects of any intervention must become apparent within a relatively short timeframe. Our results show that manipulating plant community composition has a profound effect on multiple facets of N-cycling already in the second productive year. However, the extent and direction of such effects and the ecological processes involved should be confirmed in longer term studies. For example, it remains unknown whether the legume effects persist when soil fertility levels increase after several years of legume use. Such long-term studies can also clarify whether legumes increase (Fornara et al., 2009) or decrease (Prommer et al., 2019) soil carbon sequestration in fertilized systems. Legume effects on P-cycling should also be addressed, since legumes have high P requirements compared to grasses (Haling et al., 2016), but also an advantage in P acquisition in P-limited soils (Houlton et al., 2008).

In productive temperate grasslands, grass monocultures are often used to simplify management options (e.g. easier to estimate the harvesting time, more stable forage quality). Here, we show that using plant species mixtures of two or four species instead of monocultures in these systems may increase plant N pools due to complementary resource acquisition, without increasing N losses. Our experiment also indicates that grass–legume mixtures have strong potential to optimize N-cycling in agroecosystems: they reduced soil mineral N and the potential N losses associated with this pool compared to legume monocultures, and increased plant N retention and the abundance of N-cycling microbial communities compared to grass monocultures. By generating high forage yields of high nutritive value, these mixtures can also lead to improved livestock performance (Lüscher et al., 2014). Combining legumes with grasses with low LDMC and high RLD may provide a promising opportunity to further promote rhizosphere biological interactions that regulate internal soil N-cycling, while ensuring productive and sustainable agroecosystems that retain and use N more efficiently.

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AUTHORS' CONTRIBUTIONS

D.A., G.B.D.D., L.P. and J.W.v.G. conceived the ideas and designed the methodology; D.A., N.J.O., B.O., I.P., C.C., A.F., D.B. and I.M.-S. collected the data; D.A. and N.J.O. analysed the data; D.A., G.B.D.D., L.P. and J.W.v.G. led the writing. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available via the Dryad Digital Repository <https://doi.org/10.5061/dryad.9p8cz8wf0> (Abalos et al., 2020).

ORCID

Diego Abalos  <https://orcid.org/0000-0002-4189-5563>

Laurent Philippot  <https://orcid.org/0000-0003-3461-4492>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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