



# Adding forbs and legumes to a grass-clover mixture suppressed weeds and maintained herbage yield and crude protein content across slurry application rates

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## ABSTRACT

Grassland leys are valuable for improving the performance of agricultural production systems. Multispecies mixtures have been proposed as a means of improving yields and reducing weed pressure in temporary grasslands, but the effect is influenced by both the length of the grass phase and fertilization. Based on a long-term organic dairy crop rotation experiment with four years of grass-clover in a six-course rotation, we tested the benefits of multispecies grassland leys (3- or 12-species) on herbage yield, crude protein content and weed suppression in one- to four-year-old leys with different cattle slurry inputs (0–300 kg total-N ha<sup>-1</sup>) over two years. Herbage yield and crude protein content were maintained as species diversity was increased, but the proportion of weeds was reduced. Weed suppression was most pronounced in the four-year-old swards, where a reduction from 18.5% to 11.0% was observed. Irrespective of the mixture, increasing the slurry application rate increased herbage yield, reduced the proportion of legumes and increased the proportion of grasses. The proportion of forbs in the 12-species mixture remained stable across N rates. The most abundant additional species in the 12-species mixture were plantain, chicory, caraway, and for longer leys, lucerne. Nevertheless, ryegrass and white clover accounted for 54–84% of the herbage yield in the 12-species mixtures across the combinations of year, N rate and sward age. This indicates that establishing very diverse mixtures in productive grassland leys for cutting may be challenging and highlights the need for studies focusing on increasing the evenness of diverse mixtures.

## 1. Introduction

The simplification of agricultural production systems has been identified as a key driver of e.g. losses in soil organic carbon or a low nutrient use efficiency, both of which are threats to the systems sustainability (Lemaire et al., 2015). Crop rotations in general, and especially grassland leys using multispecies grasslands have been identified as an avenue to enhance the environmental performance of agricultural production (Malisch et al., 2024). The enhanced plant species diversity in the grassland community can cause large increases in herbage yield due to a combination of niche complementarity and facilitation effects (Cong et al., 2018; Nyfeler et al., 2009). Complementarity occurs when species split resource allocation in time or space (e.g. development phases are different during the vegetation period or different rooting or canopy systems), whereas facilitation describes the provision of benefits from one species to the other (e.g. transfer of nitrogen fixed by legumes

via biological nitrogen fixation to non-legumes) (Tilman, 1999; Kirwan et al., 2009). Among the interspecific interactions, the interactions between grasses and N<sub>2</sub> fixing legumes have been identified to be strongest in a large pan-European experiment (Finn et al., 2013), and in that experiment, the minimum legume proportion to attain 95% of the maximum total nitrogen yield was 35, 24 and 25% in year 1, 2 and 3, respectively, across all sites and environmental conditions (Suter et al., 2015). At the same time, mixtures with around 1/3 of legumes that were fertilized with 150 kg N ha<sup>-1</sup> achieved similar yields to grass monocultures fertilized with 450 kg N ha<sup>-1</sup>, thus illustrating the potential to reduce N fertilizer application rates by approximately two thirds (Nyfeler et al., 2009). Therefore, the benefits of grass-legume mixtures have been utilized for a long time in organic agriculture, where mineral-N fertilizer is not available (Pirhofer-Walzl et al., 2012), but also to a large extent in conventional farming (Kristensen et al., 2022). However, while there are clear indications that increasing species

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diversity from monocultures improves yields and show substantial benefits with regards to N use efficiency, it is unclear how high species diversity can be increased, as the number of ecological niches becomes saturated at some point, thereby reducing the benefits of each added species. Accordingly, in [Hearn et al. \(2024\)](#), increasing the species richness from monocultures to binary mixtures and then to mixtures consisting of three species improved yields significantly, yet successive increments to five species mixtures had no additional yield benefits. Furthermore, the observed yield benefits observed by [Nyfeler et al. \(2009\)](#) were reduced at higher N application rates - due to grasses becoming more dominant and reduced mixture evenness - and generally decreased over time, due to a generally low legume persistence. However, mixtures with higher species richness might have benefits with regards to ecosystem services, such as habitat and feed provision for pollinators ([Cong et al., 2020](#), [Beye et al., 2022](#)), weed suppression ([Connolly et al., 2018](#)) and soil carbon sequestration ([Cong et al., 2014](#)). Species-rich grasslands will therefore be an important tool for organic dairy crop rotations in particular, where they have the greatest potential to provide high quality forage at low nitrogen inputs, close nutrient cycles and reduce food-feed competition, while also providing the ecosystem services mentioned above. In addition, higher species richness in semi-natural grasslands was shown to be positively associated with yield stability across a gradient of environmental conditions and time points, and of 147 species tested, 84% contributed at least once to yield improvements under drought ([Isbell et al., 2015](#)). Few multi-site experiments exist that have tested the effects of drought on sown intensive grassland mixtures with substantially lower species richness, but effects have been confirmed in several individual drought experiments. Increasing plant species diversity of grasslands up to four species was shown to significantly support forage yield stability under simulated drought events ([Haughey et al., 2018](#)), and this was linked to a combination of legumes resistance to drought and non-legumes resilience to drought, and their mixtures therefore resulted in the highest overyielding potential compared to monocultures ([Hofer et al., 2016](#)). Therefore, multispecies grasslands will become even more important for European agroecosystems as climate change increases the frequency and severity of droughts and floods.

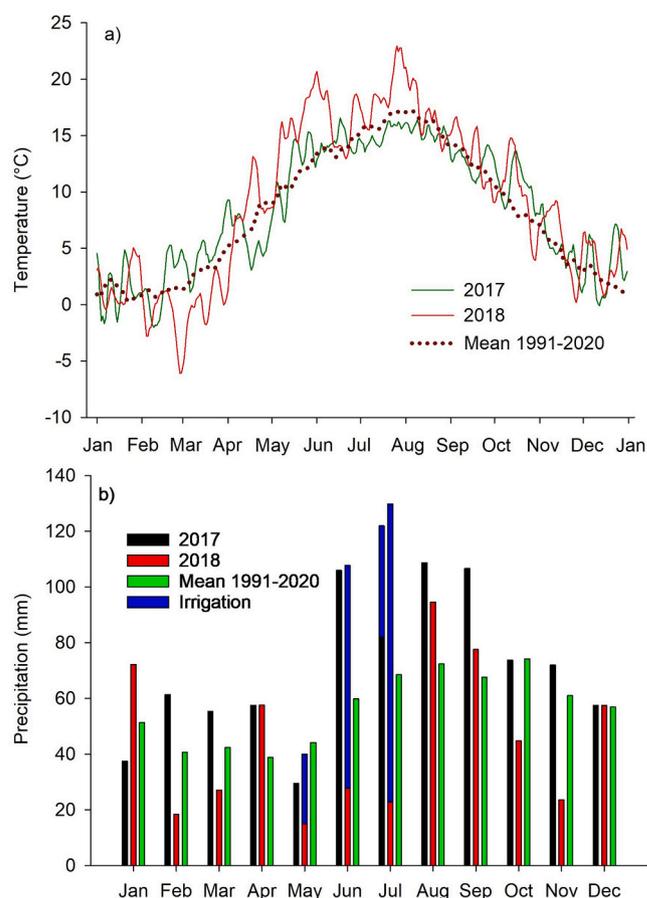
Maintaining forage species diversity in multispecies swards over time can be difficult, depending on management and environmental factors ([Haughey et al., 2018](#); [Jing et al., 2017](#)). This is problematic as longer ley durations are directly linked to higher C stocks ([Jensen et al., 2022](#)). The ability of the swards to maintain their composition is highly affected by management (i.e., grazing or cutting, cutting frequency) and especially N fertilization intensity ([Cong et al., 2018](#); [Ergon et al., 2016](#); [Eriksen et al., 2014](#); [Jing et al., 2017](#)). Organic manures are an integral part of dairy farming and therefore sward response to fertilization is important. Some knowledge for two- and three-species exist, e.g., in mixtures of ryegrass, white and red clover a yield benefit of applying up to 200 kg total-N ha<sup>-1</sup> was found, but with decreasing clover proportion, crude protein content and N use efficiency ([Thers et al., 2022](#)), but similar investigations for mixtures with higher species numbers are lacking. To our knowledge, no experiment has previously been conducted to date using a factorial design to test the effects of species richness, ley duration and nitrogen application rate on herbage yield and species composition. Therefore, the aim of this study was to identify effects of nitrogen application rates on the persistence of diverse grassland swards over a four-year ley period. We hypothesize that increasing species richness in grasslands will maintain or improve herbage yield while maximizing the potential for ecosystem services such as weed suppression, especially when maintained over longer ley durations. Thus, this study explored the herbage yield, weed suppression and crude protein content in 3- and 12-species mixtures on highly productive organically managed grass-clover leys used for cutting across different nitrogen application rates and sward ages.

## 2. Materials and methods

Herbage yield, botanical composition and crude protein content in four annual cuts of one- to four-year-old multispecies grassland mixtures (3- or 12-species) either unfertilized or fertilized with 50, 100, 200 or 300 kg total-N ha<sup>-1</sup> in cattle slurry were investigated in 2017 and 2018.

### 2.1. Study site and experimental design

The organic dairy crop rotation experiment was initiated in 1987 at Foulumgaard Experimental Farm, Aarhus University, Denmark (56°29'N, 09°34'E). The soil is a loamy sand (8% clay, 10% silt, 48% fine sand and 34% coarse sand) ([Heidmann, 1989](#)) and classified as a Typic Hapludult ([Soil Survey Staff, 2014](#)). Soil C and N concentrations were 18.6 and 1.65 g kg<sup>-1</sup> ([Jensen et al., 2022](#)), soil pH is 5.9 (0.01 M CaCl<sub>2</sub>, 1:2.5 w/w) and exchangeable K is 64 mg kg<sup>-1</sup> DW soil (extraction of 5 g soil with 50 mL of 0.5 M NH<sub>4</sub>OAc for 0.5 h) ([Thers et al., 2022](#)). Average annual temperature and precipitation are 8.2 °C and 673 mm, respectively (means of 1991–2020). [Fig. 1](#) shows air temperature and precipitation for the two experimental years and the average during 1991–2020. The weather conditions varied significantly between years. The year 2017 was close to the average during 1991–2020, whereas 2018 was characterized by high temperatures and low precipitation during the growing season. The latter was being compensated for by irrigation.



**Fig. 1.** Temperature and precipitation for the two experimental years measured at the meteorological station at Foulumgaard Experimental Station. a) Daily air temperature at 2 m height and the mean daily temperature during 1991–2020 are both calculated as the running average of five days. b) Monthly precipitation at 1.5 m height and mean monthly precipitation during 1991–2020. Blue bar extensions indicate irrigation, which was mainly performed in the dry summer of 2018.

A six-year rotation with two years of grassland was established in 1987. From 2006, the six-year rotation was split into two crop rotations differing in grassland proportions, being two years of grass-clover and four years of arable crops in rotation 1 and four years of grass-clover and two years of arable crops in rotation 2. For the current study, we selected grassland plots in rotation 2 only. Rotation 2 consisted of spring barley (*Hordeum vulgare* L.) with grassland mixtures undersown, four years of grassland and after grassland cultivation, spring barley for wholecrop silage with Italian ryegrass (*Lolium multiflorum* L.) undersown. Each of the six fields in the rotation was divided into two blocks, in which five treatments with varying cattle slurry input to grasslands (unfertilized, 50, 100, 200 and 300 kg total-N ha<sup>-1</sup>) were randomly allocated (Fig. 2). Cattle slurry was injected into the swards by placing it in a vertical 8 cm deep slot cut into the soil by a disc and applied in spring before first cut for the 50 and 100 kg total-N treatments, while the slurry application was split into 100 and 200 kg N ha<sup>-1</sup> in spring and 100 kg N ha<sup>-1</sup> after the first cut for the 200 and 300 kg total-N treatments. In addition, the slurry added a total of 9, 17, 35 and 52 kg P ha<sup>-1</sup> and a total of 39, 78, 156 and 234 kg K ha<sup>-1</sup> for the 50, 100, 200 and 300 kg total-N treatments, respectively (average of 2017 and 2018). The total N content of the slurry was 3.3 and 4.2 kg Mg<sup>-1</sup> FW and the NH<sub>4</sub>-N content 57 and 56 % of the total-N in 2017 and 2018, respectively. All treatments received an annual application of 200 kg K ha<sup>-1</sup> in potassium sulfate. The potassium sulfate application was split into 100 kg K ha<sup>-1</sup> in spring and 100 kg K ha<sup>-1</sup> after the first cut.

In 2013, each grassland plot was divided into two subplots with two different seed mixtures: The existing grass-clover seed mixture (3-mix) and a mixture including nine additional species (12-mix) (Table 1). The 3-mix was the reference mixture commonly used in the long-term experiment (Eriksen et al., 2014) and the 12-mix composition was based on previous experiences during 2006–2010 at the site with alternative legumes and forbs with regard to productivity, persistence and quality (Jing et al., 2017) and N transfer and dynamics between

legumes and non-legumes (Pirhofer-Walzl et al., 2012). The grass-clover seed mixture (25 kg ha<sup>-1</sup>) was a Danish commercial mixture of 14 % large- and -medium-leaved white clover (*Trifolium repens* L.) varieties and 82 % medium tetraploid and late diploid and tetraploid perennial ryegrass (*Lolium perenne* L.) varieties, supplemented with 4 % red clover (*Trifolium pratense* L.) (Table 1). The 12-mix consisted of 61.2 % perennial ryegrass, 24.4 % legumes (six species) and 14.4 % forbs (five species). The legume species were white clover, red clover, lucerne (*Medicago sativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.), melilot (*Melilotus officinalis* L.) and sainfoin (*Onobrychis viciifolia* Scop.), and the forb species were plantain (*Plantago lanceolata* L.), chicory (*Cichorium intybus* L.), caraway (*Carum carvi* L.), yarrow (*Achillea millefolium* L.) and salad burnet (*Sanguisorba minor* Scop.). The seeding rate for each species is given in Table 1. Plots measured 18 × 15 m (0, 100 and 300 kg total-N treatments) and 9 × 15 m (50 and 200 kg total-N treatments). Each plot included one 6 m wide strip of the 3-species mixture (3-mix) in the middle and two 4.5 m wide strips of 12-species mixture (12-mix) on the sides (Fig. 2). Both mixtures were undersown in spring barley shortly after sowing of barley (within a week). The mixtures were undersown by machine with 0.12 m row distance. A seed rate of 160 seeds m<sup>-2</sup> was used for spring barley. A total seed quantity of 25 kg ha<sup>-1</sup> was used for both mixtures. Hence, the current study includes four sward ages (1, 2, 3 and 4 years), five nitrogen application rates (unfertilized, 50, 100, 200 and 300 kg total-N ha<sup>-1</sup> in cattle slurry), two mixtures (3- and 12-species mixtures) replicated twice and investigated in two years (2017 and 2018) providing a total of 160 records.

### 2.2. Herbage yield and botanical composition

A plot harvester (J. Haldrup a/s, Løgstør, Denmark) was used to cut the herbage (8 cm stubble height) and record the fresh weight of herbage from each plot. A total area of 20–24 m<sup>2</sup> located in the center of the plot was used as harvesting area for both mixtures. Cutting dates were 2

The organic dairy crop rotations experiment:

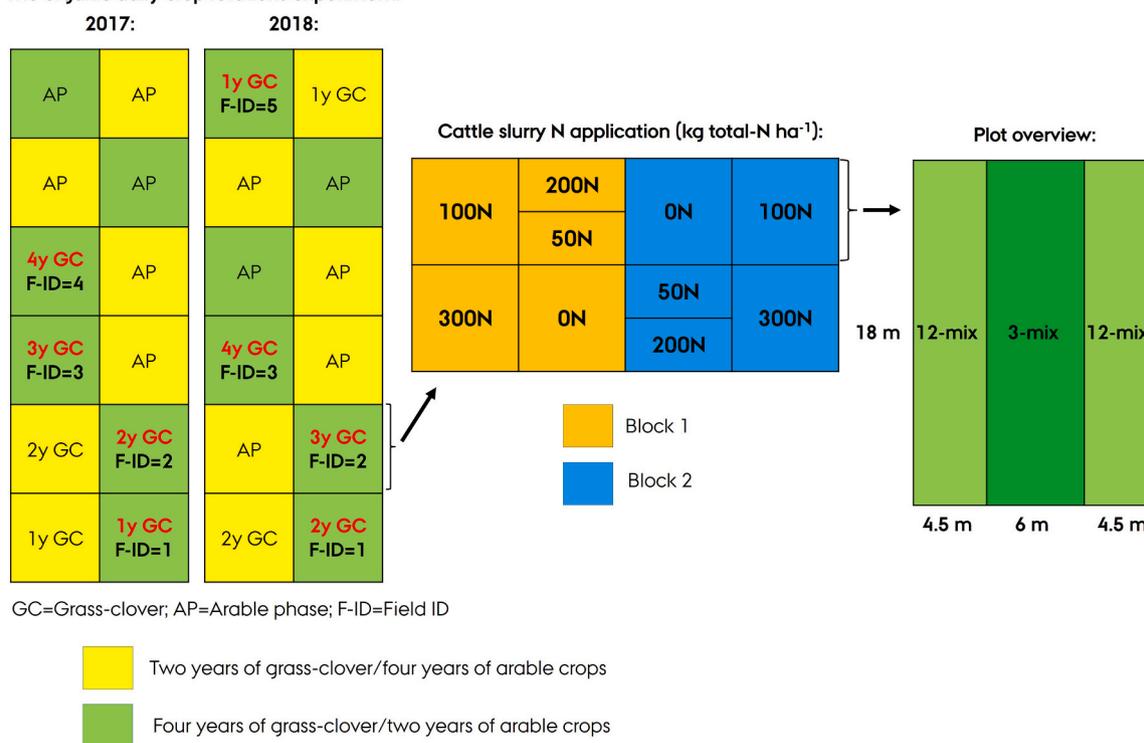
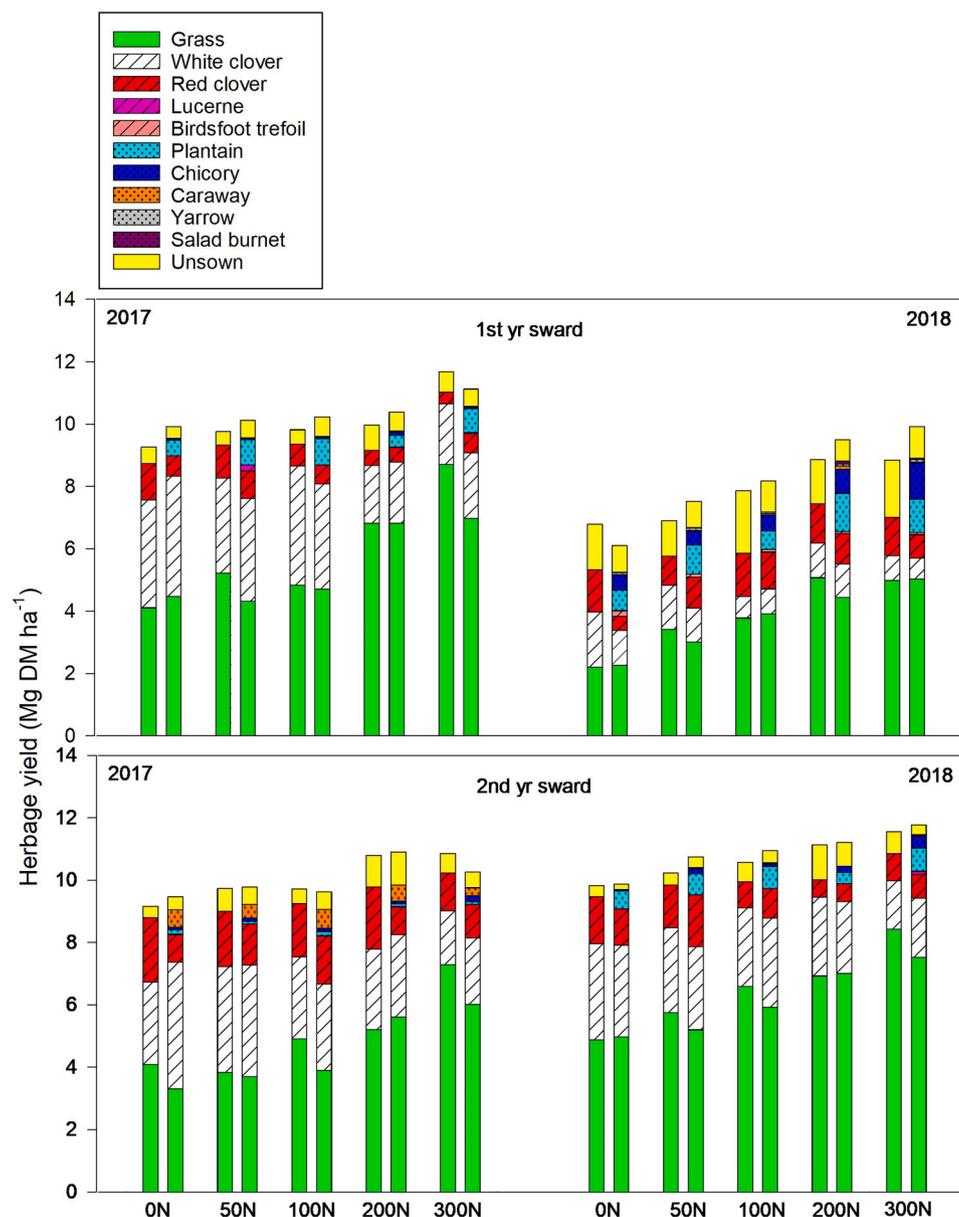


Fig. 2. Overview of the experimental design of the organic dairy crop rotations experiment showing the selected fields in 2017 and 2018 (marked with red bold text), the division of the fields into two blocks with five nitrogen application rates (0, 50, 100, 200 and 300 kg total-N ha<sup>-1</sup>) and finally the division of each plot into subplots with different seed mixtures (3- and 12-mix). Field ID is indicated.

**Table 1**  
Species included in the experiment, their functional group classification, the variety sown, and seeding rates for the two mixtures.

Functional group	Species	Latin name	Variety	1000 seed weight (g)	3-mix (kg ha <sup>-1</sup> )	12-mix (kg ha <sup>-1</sup> )
Grass	Perennial ryegrass	<i>Lolium perenne</i> L.		2.7	20.0	15.3
Legume	White clover	<i>Trifolium repens</i> L.		0.7	4.0	2.7
	Red clover	<i>Trifolium pratense</i> L.	Rajah	1.8	1.0	0.75
Forb	Lucerne	<i>Medicago sativa</i> L.	Creno	2.1		0.6
	Birdsfoot trefoil	<i>Lotus corniculatus</i> L.	Lotanova	1.1		0.6
	Melilot	<i>Melilotus officinalis</i> L.	Wild type	2.2		0.6
	Sainfoin	<i>Onobrychis viciifolia</i> Scop.	Wild type	17.3		0.85
	Plantain	<i>Plantago lanceolata</i> L.	Velhouden	1.6		0.6
	Chicory	<i>Cichorium intybus</i> L.	Wild type	1.5		0.6
	Caraway	<i>Carum carvi</i> L.	Wild type	2.9		0.8
	Yarrow	<i>Achillea millefolium</i> L.	Wild type	0.2		0.6
	Salad burnet	<i>Sanguisorba minor</i> Scop.	Wild type	5.3		1.0



**Fig. 3a.** Herbage yield and species composition for the 3- (always on the left of each N rate) and 12-species (always on the right of each N rate) mixture as a function of N rate (0–300 kg total N ha<sup>-1</sup> y<sup>-1</sup> in cattle slurry) for the 1st (top) and 2nd year (bottom) swards in 2017 and 2018 (n = 2). Colors are indicated in the legend, the dashed species are legumes, the dotted species are forbs.

June, 10 July, 17 August, and 9 October in 2017, and 23 May, 2 July, 15 August, and 9 October in 2018. A 500 g subsample was dried at 60 °C for 48 h to determine herbage dry matter (DM) content. Furthermore, the botanical composition of the herbage was determined by collecting and separating a 2–4 kg subsample into individual plant species. The sample for DM from each plot was milled and N content was determined by Elemental Vario EL C/N Analyzer (Hanau, Germany). N content was converted to crude protein by a factor of 6.25.

### 2.3. Statistics

For the statistical analysis, the R-project software package Version 4.3.0 (R Foundation for Statistical Computing) was used. Linear mixed effect models were used to test the effect of N rate (N: 0, 50, 100, 200 or 300 kg total-N ha<sup>-1</sup> in cattle slurry), sward age (A: 1st, 2nd, 3rd or 4th year), mixture (M: 3- or 12-species mixture) and year (Y: 2017 or 2018) on herbage parameters using the *lmer* function of the *lme4* package (Bates et al., 2015). Sward age, mixture and year were considered as

categorical fixed effects, N rate as continuous fixed effect, while Field ID was set as random effect (Fig. 2). Interactions between the fixed effects were assessed except for the interaction between sward age and year. The significance of management parameters (fixed effects) was assessed by using a Type III analysis of variance (ANOVA). The criterion used for statistical significance was  $P < 0.05$ . The assumptions of normality and homoscedasticity were checked with the Shapiro-Wilk test and visual examination of the residuals against fitted values.

### 3. Results

#### 3.1. Herbage yield

Herbage yields ranged from 7.6 to 11.7 Mg DM ha<sup>-1</sup> in 2017 and from 6.1 to 11.8 Mg DM ha<sup>-1</sup> in 2018 (Figs. 3a and 3b). A significant interaction between mixture, N rate and year on herbage yield was found (Table 2, Fig. 4). This implies that the N rate was affecting herbage yield differently depending on the combination of mixture and year.

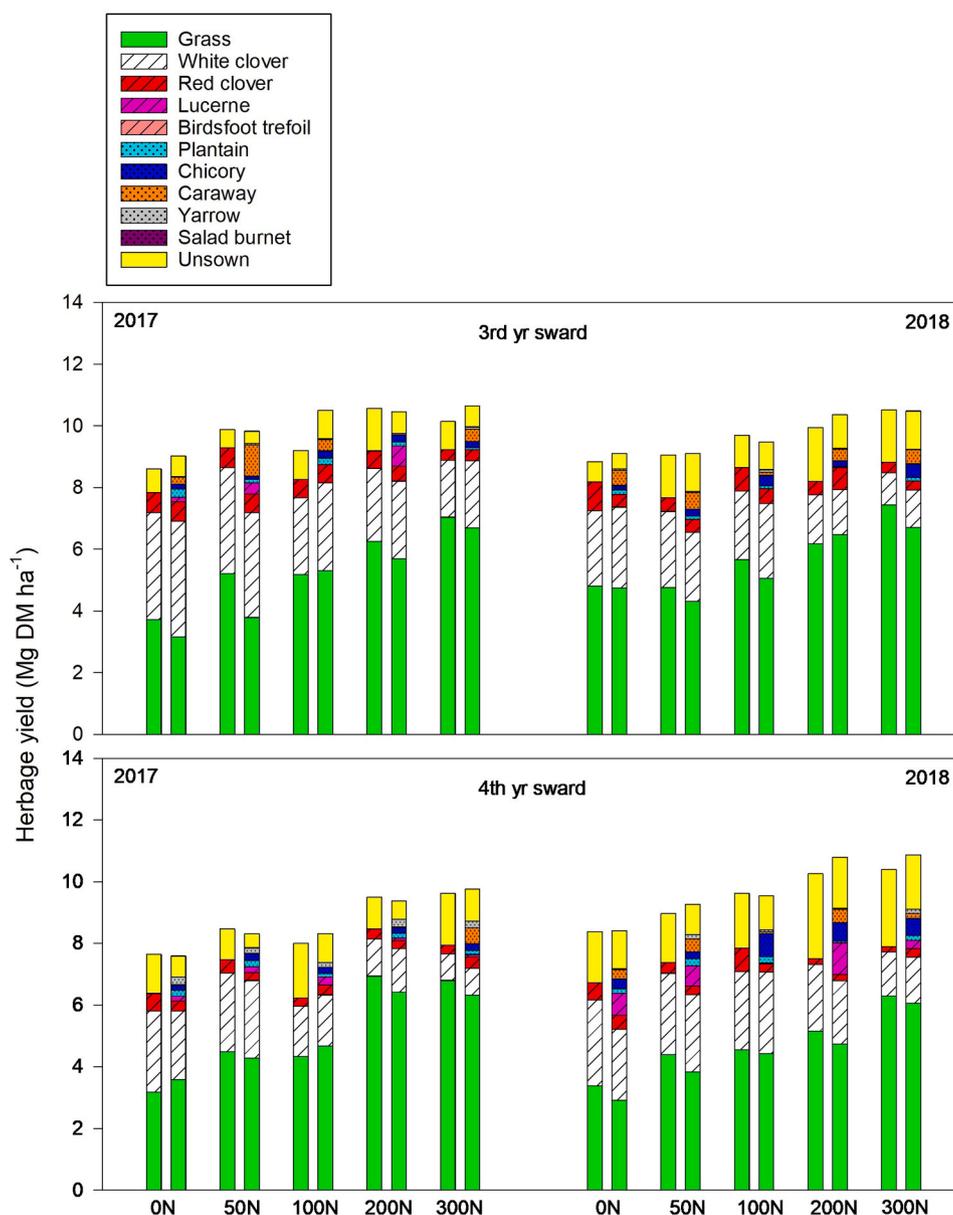


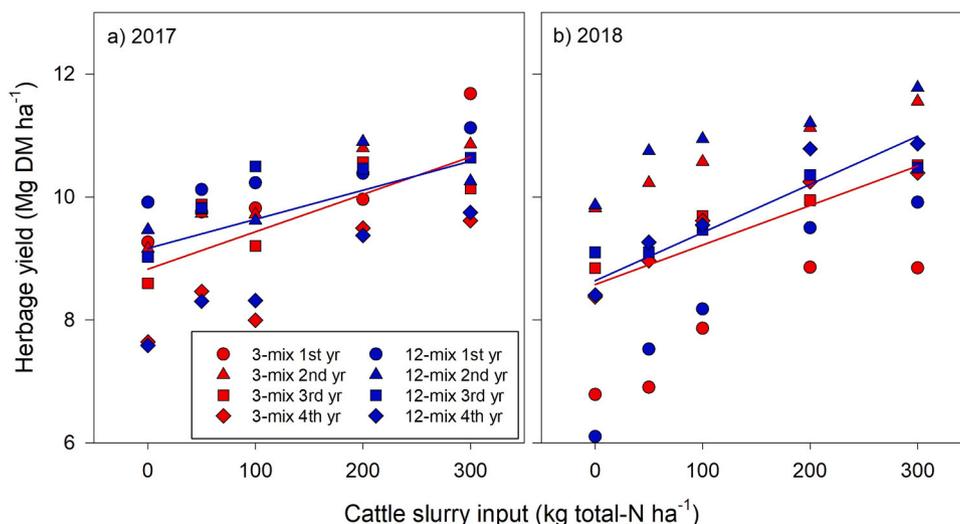
Fig. 3b. Herbage yield and species composition for the 3- (always on the left of each N rate) and 12-species (always on the right of each N rate) mixture as a function of N rate (0–300 kg total N ha<sup>-1</sup> y<sup>-1</sup> in cattle slurry) for the 3rd (top) and 4th year (bottom) swards in 2017 and 2018 (n = 2). Colors are indicated in the legend, the dashed species are legumes, the dotted species are forbs.

**Table 2**

Analyses of variance (Type III) for annual herbage yield, species group composition (grass, legume, forb and unsown), crude protein content and N removal for the two mixtures (M: 3-mix or 12-mix), four sward ages (A: 1st, 2nd, 3rd or 4th year), five N rates (N: Unfertilized, 50, 100, 200 or 300 kg total-N ha<sup>-1</sup> in cattle slurry) and two years (Y: 2017 and 2018). Field ID was included as random effect. Three-way interactions were excluded if they were non-significant.

Fixed effects	df	Annual yield	Grass	Legume	Forb*	Unsown	Crude protein	N removal
Mixture (M)	1	< 0.05	0.052	< 0.01	-	0.111	0.272	0.134
Nrate (N)	1	< 0.001	< 0.001	< 0.001	0.901	0.734	< 0.001	< 0.001
Year (Y)	1	0.635	0.106	< 0.001	< 0.05	< 0.01	< 0.001	< 0.01
Age (A)	3	< 0.001	< 0.001	< 0.001	0.245	< 0.001	< 0.001	< 0.001
M×A	3	0.700	0.670	< 0.05	-	< 0.01	0.240	0.274
M×N	1	0.170	0.241	< 0.05	-	0.261	0.083	< 0.05
M×Y	1	0.244	0.804	0.240	-	< 0.05	0.407	0.098
N × A	3	< 0.01	0.988	0.865	-	0.254	< 0.05	0.440
N × Y	1	0.726	< 0.001	< 0.01	-	0.329	< 0.001	< 0.05
M×N × A	3	-	-	-	-	-	-	-
M×N × Y	1	< 0.05	-	-	-	-	-	< 0.05

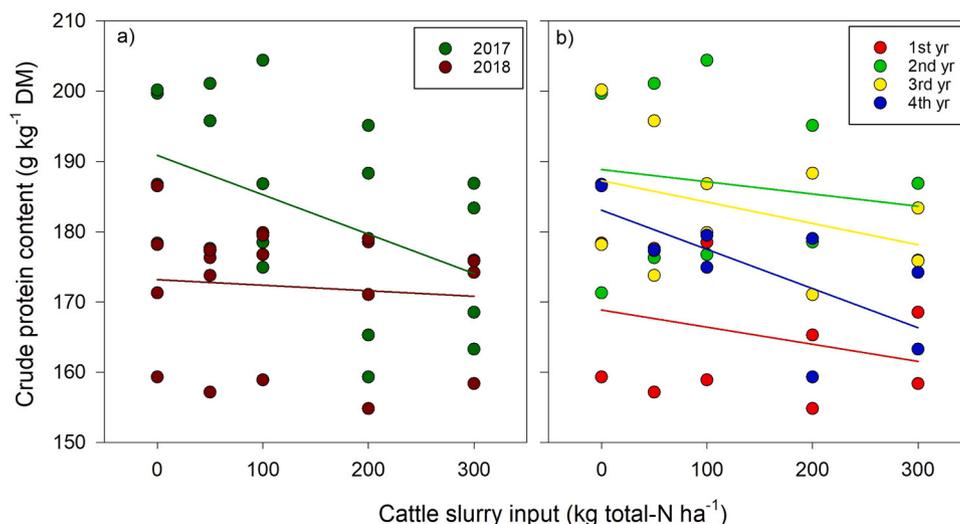
\* Only the 12-species mixture.



**Fig. 4.** Annual herbage dry matter yield as a function of cattle slurry input (0–300 kg total-N ha<sup>-1</sup>) for the two mixtures (3- and 12-species mixtures) in (a) 2017 and (b) 2018. The linear regressions are averaged over sward age.

Hence, the slope of the linear regression for the herbage yield across fertilizer levels was significantly lower in 2017 (0.0047 Mg DM per kg total-N) than in 2018 (0.0078 Mg DM per kg total-N) for the 12-species

mixture, while no significant difference in the slopes between years for the 3-species mixture were found (2017: 0.0061; 2018: 0.0064). When comparing the 3-species mixture to the 12-species mixture, the yield



**Fig. 5.** Annual crude protein content as a function of N rate (0–300 kg total-N ha<sup>-1</sup> in cattle slurry) for (a) the two years (2017 and 2018) and (b) the four sward ages (1st, 2nd, 3rd and 4th year). The data points are averaged over the two species mixture treatments.

difference was largest under unfertilized conditions in 2017, where the diverse mixture had on average  $0.33 \text{ Mg DM ha}^{-1}$  higher yields, while in 2018, the largest yield difference was found at the highest N application, where the diverse mixture had  $0.48 \text{ Mg DM ha}^{-1}$  higher yields. No interaction between mixture and sward age was observed (Table 2). A significant interaction between sward age and N rate on annual DM yield was found (Table 2). The herbage yields generally increased with increasing fertilizer rate (Fig. 4). However, the response was more pronounced in the one- and four-year-old swards than in the two- and three-year-old swards with the slope being significantly larger for the one- as compared to the three-year-old sward. The yield level was in general lowest in the one- and four-year-old swards, and highest in the two-year-old swards.

### 3.2. Crude protein content and total N removal

The 3- and 12-species treatments did not significantly influence the annual crude protein content and no interactions involving the species mixtures were significant (Table 2). Significant interactions between year and N rate as well as between sward age and N rate were found. In 2017, the crude protein content was in general larger and decreased with N rate, while it was lower and unaffected by N rate in 2018 (Fig. 5a). The significant interaction between N rate and sward age revealed lower crude protein content in the one-year-old sward than in the two- and three-year-old swards and that crude protein decreased more with increasing N rate in the four-year-old sward as compared to the two-year-old sward (Fig. 5b). The crude protein content was linearly and positively related to the legume percentage of total herbage yield, and the response and coefficient of determination was larger in 2017 than 2018 (Fig. 6).

Total N removal ranged from 195 to  $328 \text{ kg N ha}^{-1}$  in 2017 and from 138 to  $300 \text{ kg N ha}^{-1}$  in 2018. A significant interaction between mixture, N rate and year on N removal was found (Table 2, Fig. 7). This implies that the N rate was affecting N removal differently depending on the combination of mixture and year. Hence, the slope of the linear regression for N removal across N applicates rate was significantly lower in 2017 ( $0.007 \text{ kg N per kg total-N}$ ) than in 2018 ( $0.180 \text{ kg N per kg total-N}$ ) for the 12-species mixture, while no significant difference in the slopes between years for the 3-species mixture were found (2017: 0.090; 2018: 0.157). The total N removal was in general lowest in the one- and

four-year-old swards, and highest in the two-year-old swards.

### 3.3. Botanical composition of sown species

Figs. 3a and 3b shows the differences in the sward composition for the 3- and 12-species mixture depending on sward age, N rate and year. The sown mixture diversity had a close to significant effect on the percentage of ryegrass ( $P = 0.052$ ) (Table 2). The percentage of grass for the 3- and 12-species mixture was 55.5 and 50.8 % (averaged over year, N rate and sward age). No interactions between mixture combined with other effects on percentage of grass were found. However, the grass proportion in the swards was affected by the interaction between year and N rate (Table 2). Thus, the slope of the linear regression describing grass proportion as a function of N rate across sward age and mixture was larger in 2017 ( $0.085 \text{ % per kg total-N}$ ) than in 2018 ( $0.057 \text{ % per kg total-N}$ ).

A significant interaction between mixture and N rate on the percentage of legumes was found (Table 2) revealing a more pronounced negative effect of N rate on legume percentage in the 3- than in the 12-species mixture ( $-0.086$  vs.  $-0.071 \text{ % per kg total-N}$ ). In addition, a significant interaction between mixture and sward age was found with the 12-species mixture having a significantly lower legume proportion than the 3-species mixture (31 vs. 35 %) in the one-year-old sward. The most prevalent legume species was white clover, and it was not affected by mixture (Table 3). However, a significant interaction between year and N rate was found revealing an in general larger white clover proportion in 2017 than 2018, namely 26.7 and 20.5 % (averaged over sward age, N rate and mixture). A significant interaction between mixture and N rate was found for red clover (Table 3) revealing that the difference in red clover percentage was largest under unfertilized conditions with 12.9 and 7.1 % in the 3- and 12-species mixtures, respectively. The red clover percentage was also affected by sward age with the largest percentage being present in the one- and two-year-old swards (10.7 and 13.7 %, respectively) irrespective of mixture.

A significant effect of year on the total forb percentage was found, while no effect of sward age and N rate was detected (Table 2). The forb percentage was 8.5 and 12.0 % in 2017 and 2018, respectively (averaged over sward age and N rate). However, the one-year-old sward in 2018 stands out by having a larger forb percentage of 20.5 % (Figs. 3a and 3b). Sainfoin and melilot was not present in both years, while birdsfoot trefoil and salad burnet only were present in 2018 in the one-year-old sward in negligible proportions. The forb species present in the largest proportions were plantain, chicory and caraway (Figs. 3a and 3b). The plantain proportion was largest in the one-year-old sward with 6.4 and 10.8 % in 2017 and 2018, respectively (averaged over N rate). A large proportion was also found in the two-year-old sward in 2018 (5.6 %). A significant interaction between N rate and year was found for chicory (Table 4). A larger prevalence was in general found in 2018 and the percentage was increasing with N rate in 2018, while it was unaffected by N rate in 2017. In 2017, the largest proportion was found in the four-year-old sward (2.2 %), while in 2018, the largest proportions were found in the one- and four-year-old swards with 7.9 and 5.0 %, respectively. No effect of N rate, sward age and year on caraway percentage was found (Table 4). In 2017, the largest percentages were observed in the two- and three-year old swards with 4.8 and 4.0 %, respectively, while the proportion was largest in the three- and four-year-old swards in 2018 with 4.0 and 2.9 %, respectively. Yarrow was significantly affected by sward age. The largest proportions were found in the four-year-old swards, especially in 2017 (2.4 %).

The legume, lucerne, being present in the 12-species mixture only was significantly affected by sward age (Table 3), and mainly present in the three- and four-year-old swards in 2017 with 2.3 and 1.8 %, and in the four-year-old sward in 2018 with 5.5 % (averaged over N rates).

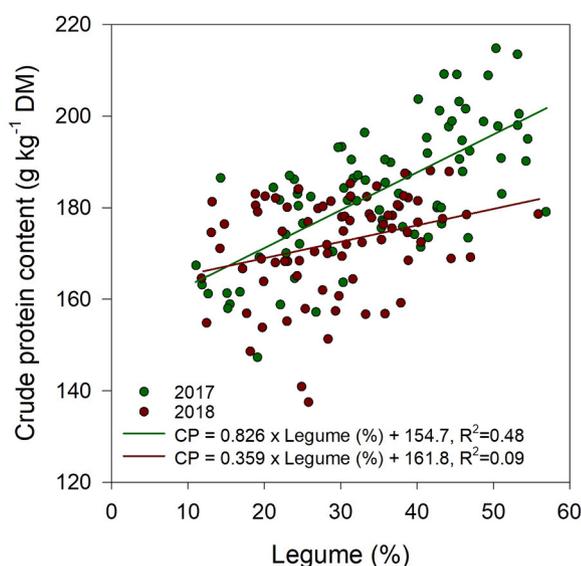


Fig. 6. Annual crude protein content as a function of legume percentage of total herbage dry matter yield across both species mixture treatments, N application rates and sward ages at plot level for 2017 and 2018 ( $n = 80$  for each year). The linear regression and coefficient of determination is indicated.

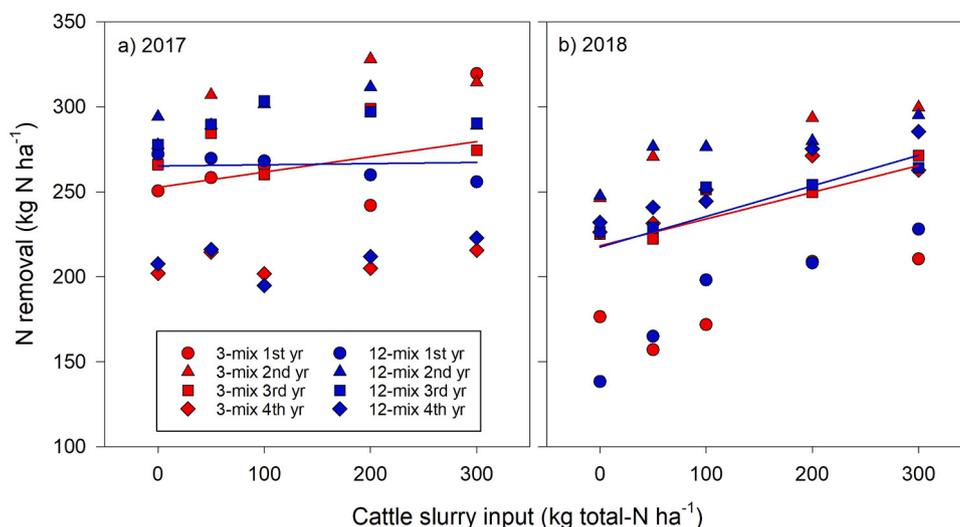


Fig. 7. Total N removal as a function of cattle slurry input (0–300 kg total-N ha<sup>-1</sup>) for the two mixtures (3- and 12-species mixtures) in (a) 2017 and (b) 2018. The linear regressions are averaged over sward age.

Table 3

Analyses of variance (Type III) for legume, white clover, red clover, lucerne and birdsfoot trefoil for the two mixtures (M: 3-mix or 12-mix), four sward ages (A: 1st, 2nd, 3rd or 4th year), five N rates (N: Unfertilized, 50, 100, 200 or 300 kg total-N ha<sup>-1</sup> in cattle slurry) and two years (Y: 2017 and 2018). Field ID was included as random effect. Three-way interactions were excluded if they were non-significant.

Fixed effects	df	Legume	White clover	Red clover	Lucerne*	Birdsfoot trefoil**
Mixture (M)	1	< 0.01	0.466	< 0.001	-	-
Nrate (N)	1	< 0.001	< 0.001	< 0.001	0.337	< 0.05
Year (Y)	1	< 0.001	< 0.001	0.893	0.922	-
Age (A)	3	< 0.001	0.671	< 0.001	< 0.001	-
M×A	3	< 0.05	0.123	0.205	-	-
M×N	1	< 0.05	0.315	< 0.05	-	-
M×Y	1	0.240	0.059	0.896	-	-
N × A	3	0.865	0.279	0.186	-	-
N × Y	1	< 0.01	< 0.001	0.759	-	-
M×N × A	3	-	-	-	-	-
M×N × Y	1	-	-	-	-	-

\* Only the 12-species mixture.

\*\* Only observed in 1st year sward in 2018.

### 3.4. Unsovn species

A significant interaction between mixture and year was observed with regard to the proportion of unsovn species (Table 2). The percentage of unsovn biomass (w/w DM) for the 3- and 12-species mixture was 9.8 and 7.1 % in 2017 and 15.1 and 9.5 % in 2018 (Fig. 8a). Hence, the difference between the mixtures was in general larger in 2018 than in 2017. In addition, a significant interaction between mixture and sward age was found (Table 2). The 12-species mixture consistently had a lower percentage of unsovn than the 3-species mixture, but the magnitude of this varied between sward ages (Fig. 8b). The largest difference in unsovn percentage between the two mixtures was found in the four-year-old sward and the lowest in the two-year-old sward. The

percentage of unsovn biomass for the 3- and 12-species mixture was 18.5 and 11.0 % in the four-year-old swards and 6.1 and 4.8 % in the two-year-old swards.

## 4. Discussion

### 4.1. Diverse grassland mixtures can be maintained for up to four years

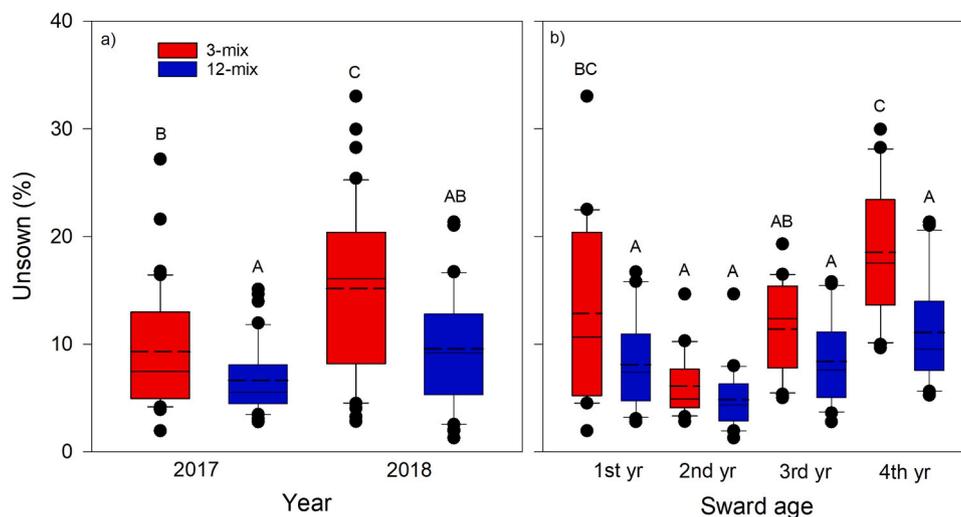
A primary goal in designing grass-arable crop rotations is to optimize the grass phase, specifically the balance between the number of grassland cultivations and the duration of the grass phase (Eriksen et al., 2008). Typically, grassland cultivation is motivated by yield loss resulting from sward deterioration due to factors like compaction from

Table 4

Analyses of variance (Type III) for forb, plantain, chicory, caraway, yarrow and salad burnet for the four sward ages (A: 1st, 2nd, 3rd or 4th year), five N rates (N: Unfertilized, 50, 100, 200 or 300 kg total-N ha<sup>-1</sup> in cattle slurry) and two years (Y: 2017 and 2018). Field ID was included as random effect. Two-way interactions were excluded if they were non-significant.

Fixed effects	df	Forb	Plantain	Chicory	Caraway	Yarrow	Salad burnet*
Nrate (N)	1	0.901	0.065	0.265	0.498	0.801	< 0.05
Year (Y)	1	< 0.05	< 0.01	0.097	0.271	0.646	-
Age (A)	3	0.245	< 0.01	0.447	0.581	< 0.05	-
N × A	3	-	-	0.704	-	-	-
N × Y	1	-	-	< 0.05	-	-	-

\* Only observed in 1st year sward in 2018.



**Fig. 8.** The unsown percentage of annual herbage dry matter yields for (a) the interaction between mixture (3- and 12-species mixture) and year (2017 and 2018) (averaged over N rate and sward age), and (b) the interaction between mixture and sward age (1st, 2nd, 3rd, and 4th year) (averaged over N rate and year). Dashed lines indicate mean values. Lines within the boxes represent median values, box boundaries include the 25th and 75th percentiles, and the whiskers extend from the box boundary to the 10th and 90th percentiles. Data points that lie outside the 10th and 90th percentiles are shown as symbols. Different letters indicate significant differences ( $P < 0.05$ ).

wheel traffic and invasion of less productive natural grasses. Additionally, maintaining or enhancing soil fertility and nutrient utilization is also of significant consideration. The fact that the grassland mixtures did not differ significantly in productivity even in the third or fourth year was very promising, as the duration of a grassland ley is an important factor that determines the extent of its benefits. According to Lemaire et al. (2015), the grassland ley duration in a crop rotation reduced the risk of leaching exponentially. Accordingly, an 8-year rotation with 4 years of grassland ley reduced the concentration of nitrate in the leachate by approx. 50 % compared to arable cropping. Similarly, the ley duration has a substantial impact on the soil organic carbon stocks. Jensen et al. (2022), e.g., found a linear increase in SOC stock by  $2.7 \text{ Mg C ha}^{-1}$  from 2006 to 2018 when the grassland proportion was increased from 2 to 4 years of grass-clover in a six-course rotation. However, especially, the persistence of red clover is normally limited, and in an international experiment across 31 sites, it was completely outcompeted in most sites by the third experimental year (Brophy et al., 2017). Therefore, there is a trade-off between maintaining agronomic potential and species composition, while maximizing ecosystem services. The issue with maintaining red clover was also apparent in this study, where especially in the fourth year, red clover barely contributed to the yield anymore. This issue may, however, be partly overcome by selecting more persistent red clover cultivars such as the Swiss Matenkle cultivars (Hoekstra et al., 2017). While legume proportion was generally reduced with higher amounts of slurry being added, legumes managed to persist even at  $300 \text{ kg total-N ha}^{-1}$ , especially until the third year. While this appears surprising, as legumes downregulate their biological nitrogen fixation under increased fertilization (Burchill et al., 2014) thereby losing their competitive advantage over grasses, a previous multisite experiment has identified climatic conditions to be a stronger determining factor for legume proportion than fertilizer addition (Suter et al., 2015). Furthermore, due to the application of organic fertilizer, both N, P and K are being added, thereby potentially increasing the legumes' ability to persist in mixtures, as P increases the nodulation of legumes and P limitations are critical in preventing symbiotic interactions with rhizobia (Mendoza et al., 2016). However, in our experiment, the grass proportion was surprisingly dominant already from the first year onwards. While sufficient grass proportions are normally required to reduce weeds effectively, they also are in competition with the forbs. This trade-off cannot be overcome by merely increasing sowing densities of the forbs, but rather requires an optimization of the

niches that can be utilized (Jiang and Hitchmough, 2022). A better solution than increasing the sowing density of forbs or legumes has accordingly been, to change the sowing time, whereby legumes and forbs can be sown first to obtain a head start, thereby offering a competitive advantage, and improving evenness in the established species composition (von Gillhausen et al., 2014). This is in line with findings from Husse et al. (2016), who also found that asynchrony in shoot growth has the largest effect on yield performance, whereas the ability of species to intercept light was of minor importance.

Regarding the annual herbage yield, the fact that the yield generally was sustained in the 12- as compared to the 3-species mixture may be explained by a greater variety of functional traits and hence more efficient resource use in the more diverse mixture, and aligns with Grange et al. (2021), who found that forbs can contribute to overyielding by interacting synergistically with grasses and legumes. The relatively small yield increments are most likely explained by the already high diversity of the reference mixture, and the presence of the functional groups of legumes. Most of the studies revealing significant positive yield effects by increased plant diversity of the grassland community chose pure stands (i.e. monocultures) or two-species mixtures as reference (Baker et al., 2023; Cong et al., 2018; Eriksen et al., 2014; Grange et al., 2021). The reference 3-species mixture of our experiment included already two different species of clover (white and red clover) and ryegrass, which is virtually 'multispecies', but less diverse than the 12-species mixture. This may hence explain why only a marginal yield effect was observed between the reference and multispecies mixture. From semi-natural grasslands two general mechanisms are known that limit the benefits of increasing species numbers: i) the saturation curve, which assumes that with each added species the availability of vacant ecological niches is reduced, thereby resulting in lower yield gains with increasing addition of species (Tilman et al., 1997); and ii) the yield reduction theory indicating that each added species increases the likelihood of adding a species with lower agronomic potential at a given site (Roscher et al., 2005). Experiments from sown grasslands suggest that similar mechanisms exist under intensive management, thus, to design seed mixtures for highest yields, species identities are important and only species with high yield potential should be selected; and more balanced mixtures should be targeted, thus the species should be selected for contrasting and complementary functional traits. Nevertheless, mixtures in intensively used grasslands reach saturation in terms of yield at lower total species numbers. Accordingly, mixtures of up to

four species, consisting of two legumes and two grasses, generally gave comparable yields to mixtures of up to six species (Finn et al., 2013; Grange et al., 2021; Hearn et al., 2024). Also, at the site of this experiment, the inclusion of red clover in a ryegrass and white clover mixture has previously been shown to increase forage yields significantly (Eriksen et al., 2014), thereby already exploiting many mixture effects successfully. However, it should be noted that many studies have included comparably short ley durations of up to two years, and higher species richness might be more beneficial in older swards and additionally might still improve ecosystem services other than yield, such as weed suppression. The present study, with its long-term design, provides insight into species persistence and productivity for ley durations of up to four years over two experimental years. We encourage similar studies in other geographical areas with different climates and soil types. Although the study is unique, a longer experimental period is required to fully assess the ecological benefits of multispecies mixtures, such as soil carbon sequestration.

#### 4.2. Diverse mixtures were more effective at suppressing weeds in older grassland swards

The suppression of unsown species in the 12- compared to the 3-species mixture was more pronounced in the one- and four-year-old swards. The fact that multispecies mixtures are more effective at suppressing weeds compared to monocultures or less diverse mixtures has been found in several studies (Baker et al., 2023; Cong et al., 2018; Connolly et al., 2018; Jing et al., 2017; Suter et al., 2017). Baker et al. (2023) e.g., demonstrated a lower proportion of weeds in a 6-species mixture as compared to a ryegrass monoculture or a binary mixture of ryegrass and white clover with absolute weed proportions in the 6-species mixture being 3–6 %-points lower. Even stronger effects were found by Connolly et al. (2018), who reported that across 31 different research sites and three experimental years, mixtures containing between 2 and 6 species had on average 3 %-point lower weed biomass of even the best monocultures, and 26 %-point lower weed biomass of the average monocultures. However, these weed reductions were usually compared to monocultures, whereas in the present study, the less diverse mixture already contained 3-species and was therefore closer to the most diverse mixtures in Connolly et al. (2018) than to the monocultures. Thus, the already comparably diverse reference mixture in this experiment may have led to generally lower reductions in weed proportions than in previous studies. While unsown species were not botanically classified in our study, weeds can be problematic as they potentially reduce the forage quality or might even devalue it completely (e.g. if weeds are poisonous). However, most of the above-mentioned studies had limited ley durations of a maximum of three years, and the weed content of ley swards usually increases with sward age, as was especially apparent in the monocultures of the multisite analyses of Connolly et al. (2018) already within the three-year duration. Therefore, using multispecies mixtures in ley-arable rotations with longer grassland phases appears to be a reasonable approach to lowering weed pressure, especially in the four-year-old sward, where the absolute unsown percentage was reduced by as much as 7.5 %-points corresponding to a relative reduction of 40 %.

#### 4.3. Crude protein content was mostly affected by legume proportion

In the present study, crude protein (CP) content did not differ between the two mixtures. This can probably be explained by the small differences in legume content between mixtures. The CP content of forage mainly depends on the content of legumes, at least at nitrogen application rates below  $300 \text{ kg N ha}^{-1} \text{ y}^{-1}$ ; a higher proportion of N-fixing legumes is correlated with increased CP contents (Søgaard, 2009). In this context, a critical factor is the N fertilization rate: Lower rates support the presence of legumes in the sward and thereby result in higher CP contents in the forage material, but at rates above  $300 \text{ kg N ha}^{-1} \text{ y}^{-1}$  the CP content of grasses is increased and therefore total CP content is also increased. As N

application rates in this study were limited to a maximum of  $300 \text{ kg N ha}^{-1} \text{ y}^{-1}$ , the legume proportion was the driving factor for the CP concentration of each mixture, and the legume proportion was, as expected, negatively correlated with N application rate. The general lack of effect of species richness is in line with Jing et al. (2017), who showed that the increased CP content in a grass-clover mixture compared to a multispecies mixture was explained by species-specific differences within the legumes, as mainly the CP concentrations of lucerne differed compared to white clover, while forbs generally had comparable CP contents to grasses. The marginal difference in the botanical composition of the forage between the mixtures in this experiment did not seem to be sufficient to have an effect on the CP content.

Overall, the annual CP concentrations in this experiment ranged from  $138 \text{ g CP kg DM}^{-1}$  to  $215 \text{ g CP kg DM}^{-1}$  across both years, sward ages, mixtures and N rates (Fig. 6). Katongole and Yan (2020) have previously shown that milk yields continue to increase up to  $176 \text{ g CP kg DM}^{-1}$ , whereas higher concentrations only increased urinary N excretion. Similar findings were observed by Colmenero and Broderick (2006), however here the most efficient diet with the highest milk yields was achieved with  $165 \text{ g CP kg DM}^{-1}$ . Accordingly, while the exact number of optimal CP concentration also depends on other dietary factors, as well as the animal breed and lactation stage, overall, it can be stated that very high CP concentrations only result in reduced efficiencies. While therefore generally in a suitable range, especially the completely unfertilized swards are at risk of increasing urinary N losses.

## 5. Conclusions

Adding forbs and legumes to a grass-clover ley maintained herbage yield and suppressed weeds. However, when designing mixtures, the focus should be on species with the best agronomic potential. Based on this study, for temperate grasslands this could entail red clover, chicory, plantain and caraway, and for longer ley durations, lucerne. Due to the potential of species-rich long-term leys to suppress weeds, they could be an option, particularly in organic dairy crop rotations, to reduce weed pressure in the follow-on crop. This is promising as longer leys may enhance ecosystem services in crop rotations. Minor yield increments were observed under unfertilized conditions in 2017 and at the highest N application rate in 2018, but as a general trend fertilization increased yield. However, it seems promising for the inclusion of forbs that even in intensively managed grasslands the proportion of forbs was generally stable across N application rates. In contrast, legumes were generally strongly suppressed at high N rates. In general, the minor differences in legume proportions between the 3- and 12-species mixtures indicate that in intensively managed systems with high cutting frequencies, 3-species mixtures already show a level of species richness close to saturation effects under optimal environmental conditions. Future studies should focus on the yield stability of species-rich mixtures, and on how to increase the evenness of diverse mixtures to ensure that less competitive species establish in greater proportions.

### CRedit authorship contribution statement

**Johannes Lund Jensen:** Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Carsten Stefan Malisch:** Writing – review & editing, Supervision, Funding acquisition. **Henrik Thers:** Writing – review & editing. **Jørgen Eriksen:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

### Declaration of Competing Interest

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

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## Data availability

Data will be made available on request.

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