REGULAR ARTICLE

Effect of four plant species on soil ^{15}N-access and herbage yield in temporary agricultural grasslands

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Abstract

Background and aims We carried out field experiments to investigate if an agricultural grassland mixture comprising shallow- (perennial ryegrass: *Lolium perenne* L.; white clover: *Trifolium repens* L.) and deep- (chicory: *Cichorium intybus* L.; Lucerne: *Medicago sativa* L.) rooting grassland species has greater herbage yields than a shallow-rooting two-species mixture and pure stands, if deep-rooting grassland species are superior in accessing soil ^{15}N from 1.2 m soil depth compared with shallow-rooting plant species and vice versa, if a mixture of deep- and shallow-rooting plant species has access to greater amounts of soil ^{15}N compared with a shallow-rooting binary mixture, and if leguminous plants affect herbage yield and soil ^{15}N-access.

Methods ^{15}N-enriched ammonium-sulphate was placed at three different soil depths (0.4, 0.8 and 1.2 m) to determine the depth dependent soil ^{15}N-access of pure stands, two-species and four-species grassland communities.

Results Herbage yield and soil ^{15}N-access of the mixture including deep- and shallow-rooting grassland species were generally greater than the pure stands and the two-species mixture, except for herbage yield in pure stand lucerne. This positive plant diversity effect could not be explained by complementary soil ^{15}N-access of the different plant species from 0.4, 0.8 and 1.2 m soil depths, even though deep-

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rooting chicory acquired relatively large amounts of deep soil ^{15}N and shallow-rooting perennial ryegrass when grown in a mixture relatively large amounts of shallow soil ^{15}N. Legumes fixed large amounts of N_2, added and spared N for non-leguminous plants, which especially stimulated the growth of perennial ryegrass.

Conclusions Our study showed that increased plant diversity in agricultural grasslands can have positive effects on the environment (improved N use may lead to reduced N leaching) and agricultural production (increased herbage yield). A complementary effect between legumes and non-leguminous plants and increasing plant diversity had a greater positive impact on herbage yield compared with complementary vertical soil ^{15}N-access.

Introduction

Positive plant diversity-productivity relationships have been reported for experimental semi-natural grasslands (Cardinale et al. 2006; Hector et al. 1999; Tilman et al. 1996) as well as temporary agricultural grasslands (Frankow-Lindberg et al. 2009; Kirwan et al. 2007; Nyfeler et al. 2009; Picasso et al. 2008). Generally, these relationships are explained, on the one hand, by niche differentiation and facilitation (Hector et al. 2002; Tilman et al. 2002) and, on the other hand, by greater probability of including a highly productive plant species in high diversity plots (Huston 1997). Both explanations accept that diversity is significant because species differ in characteristics, such as root architecture, nutrient acquisition and water use efficiency, to name a few, resulting in composition and diversity being important for improved productivity and resource use (Naeem et al. 1994; Tilman et al. 2002). Plant diversity is generally low in temporary agricultural grasslands grown for ruminant fodder production. Grass in pure stands is common, but requires high nitrogen (N) inputs. In terms of N input, two-species grass-legume mixtures are more sustainable than grass in pure stands and consequently dominate low N

input grasslands (Crews and Peoples 2004; Nyfeler et al. 2009; Nyfeler et al. 2011).

In temperate grasslands, N is often the limiting factor for productivity (Whitehead 1995). Plant available soil N is generally concentrated in the upper soil layers, but may leach to deeper layers, especially in grasslands that include legumes (Scherer-Lorenzen et al. 2003) and under conditions with surplus precipitation (Thorup-Kristensen 2006). To improve soil N use efficiency in temporary grasslands, we propose the addition of deep-rooting plant species to a mixture of perennial ryegrass and white clover, which are the most widespread forage plant species in temporary grasslands in a temperate climate (Moore 2003). Perennial ryegrass and white clover possess relatively shallow root systems (Kutschera and Lichtenegger 1982; Kutschera and Lichtenegger 1992) with effective rooting depths of <0.7 m on a silt loamy site (Pollock and Mead 2008). Grassland species, such as lucerne and chicory, grow their tap-roots into deep soil layers and exploit soil nutrients and water in soil layers that the commonly grown shallow-rooting grassland species cannot reach (Braun et al. 2010; Skinner 2008). Chicory grown as a catch crop after barley reduced the inorganic soil N down to 2.5 m depth during the growing season, while perennial ryegrass affected the inorganic soil N only down to 1 m depth (Thorup-Kristensen 2006). Further, on a Wakanui silt loam in New Zealand chicory extracted water down to 1.9 m and lucerne down to 2.3 m soil depth, which resulted in greater herbage yields compared with a perennial ryegrass-white clover mixture, especially for dry-land plots (Brown et al. 2005).

There is little information on both the ability of deep- and shallow-rooting grassland species to access soil N from different vertical soil layers and the relation of soil N-access and herbage yield in temporary agricultural grasslands. Therefore, the objective of the present work was to test the hypotheses 1) that a mixture comprising both shallow- and deep-rooting plant species has greater herbage yields than a shallow-rooting binary mixture and pure stands, 2) that deep-rooting plant species (chicory and lucerne) are superior in accessing soil N from 1.2 m soil depth compared with shallow-rooting plant species, 3) that shallow-

rooting plant species (perennial ryegrass and white clover) are superior in accessing soil N from 0.4 m soil depth compared with deep-rooting plant species, 4) that a mixture of deep- and shallow-rooting plant species has greater access to soil N from three soil layers compared with a shallow-rooting two-species mixture and that 5) the leguminous grassland plants, lucerne and white clover, have a strong impact on grassland N acquisition, because of their ability to derive N from the soil and the atmosphere.

Materials and methods

Site and experimental grassland communities

The following five plant communities were sown at the research farm of Copenhagen University, Denmark (55°40′N. 12°18′E; 28 m above s.l.), on 6 September 2007 after a spring barley harvest. Pure stands of perennial ryegrass (*Lolium perenne* L. cv. Calibra 50 % and L. cv. Tivoli 50 %), lucerne (*Medicago sativa* L. cv. Pondus) and chicory (*Cichorium intybus* L. cv. Puna) were sown with seeding rates of 20 kg ha^{-1}, 15 kg ha^{-1} and 10 kg ha^{-1}, respectively; a two-species mixture of white clover and perennial ryegrass was sown with a seeding rate of 25 kg ha^{-1} (*Trifolium repens* L. cv. Sonja 11 % and L. cv. Rivendel 4 %, *Lolium perenne* L.cv. Calibra 30 %, L.cv. Sameba 27 %, L.cv. Tivoli 28 %); and a four-species mixture of white clover, perennial ryegrass, lucerne and chicory was sown with a seeding rate of 35 kg ha^{-1} (72 % of the two-species mixture: white clover and perennial ryegrass, 14 % of *Cichorium intybus* L. cv. Puna and 14 % of *Medicago sativa* L. cv. Pondus). Plant communities were chosen because of their relevance for agricultural grasslands. Pure stand chicory is not used for agricultural grasslands, but was investigated because of the plant's deep-rooting characteristic.

The sandy-loamy site, classified as a typical Hapludalf, contained 15 % clay, 18 % silt, 42 % fine sand and 25 % coarse sand and had a ploughing layer with a depth of approximately 0.25 m. The total N content in the ploughing layer was approximately 1.3 % with a C:N ratio of 9. The experimental area was mainly cropped with cereals before sowing the grassland communities. In 2008, all grassland communities were fertilized twice (13 May and 28 July) with ammonium-sulphate (NH_4SO_4; 49 kg N ha^{-1}). No fertilizer was applied in 2009. There were twenty plots (plot size: 8×1.5 m) comprised of five experimental plant communities and four replicates (Fig. 1).

^{15}N placement

The subplots for the ^{15}N placement experiment were 0.8×0.8 m with one subplot representing one depth, one experimental plant community and one replicate. One plot contained three subplots (Fig. 1). The distance between subplots was 2 m in order to avoid ^{15}N cross contamination. On 8 June, 2009, we placed a bullet-shaped frozen ammonium sulphate ($^{15}NH_4SO_4$) solution enriched with ^{15}N (99.5 atom%) at three soil depths: 0.4, 0.8 and 1.2 m. In order to see a clear difference in soil ^{15}N-access between shallow- and deep-rooting grassland species our study focused on soil depths under the ploughing layer.

A drilling machine with a rod of 25 mm diameter was used to make holes. Afterwards a hollow plastic tube with the length of the respective depths was inserted to avoid ^{15}N contamination on the sides of the hole during the ^{15}N placement. With a thin plastic rod we made sure that the frozen ^{15}N solution was placed at the right depth. After removing the plastic tube, we filled the hole with sand and covered it with a plastic lid (4 cm in diameter) to prevent rainwater from entering. Each hole received 3 ml of $^{15}NH_4SO_4$ solution (20 mg ^{15}N), resulting in a total N application of 100 mg ^{15}N per subplot (equivalent to 1.56 kg ^{15}N ha^{-1}). The subsequent ^{15}N enrichment in the above-ground plant biomass was thus an indicator of the ability of the plants to access soil ^{15}N from a certain depth.

Soil sampling

The soil columns taken up when drilling the holes were sampled according to their respective lengths. The soil column with 1.2 m in length was split into three parts (0–0.4, 0.4–0.8 and 0.8–1.2 m), the soil column with 0.8 m in length was split into two parts (0–0.4 and 0.4–0.8 m) and the soil column with 0.4 m in length was not split. The soil was frozen and later the inorganic

Fig. 1 Illustration of the plots and ^{15}N placement subplots. Each dot indicates a hole which was vertically drilled into the ground for one depth and one plant community. The numbers are the distances in meters
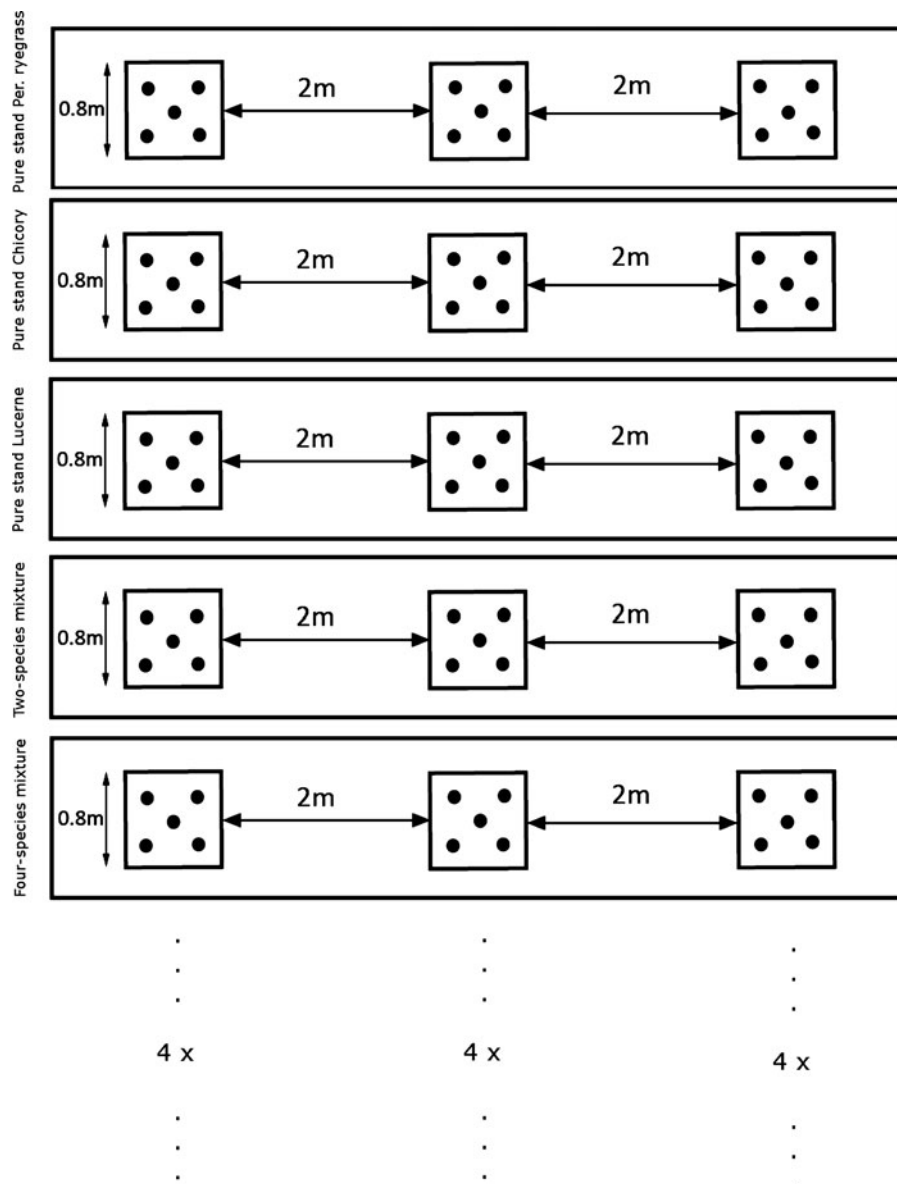
nitrogen content (NO_3^-, NH_4^+), the soil dry matter and the gravimetric soil water content were determined. The inorganic N content was measured spectrophotometrically after extraction with 1 M KCl (1:2 w/v).

Plant sampling, ^{15}N analyses

Herbage yield was determined four times in 2008 (4 June, 26 July, 30 August and 24 October) and in 2009 (14 May, 29 June, 3 August and 12 October) with a Haldrup plot harvester (J Haldrup A/S, Loegstoer, Denmark). The botanical compositions of the two-

and four-species mixtures were determined by separating a representative herbage subsample in single plant species.

To measure the ^{15}N-access from different soil depths, plants from subplots were harvested 10 days after ^{15}N placement. We timed the sampling on the basis of a preliminary study and to avoid great ^{15}N movements in the soil matrix. We harvested 0.8×0.8 m of above-ground plant tissue with an electric hand shear as close to the soil surface as possible. The mixtures were separated according to their respective plant species. Additionally, in order to determine the

natural ^{15}N abundance we sampled the above-ground plant tissue of each of the four plant species grown in the five plant communities adjacent to the treated plots. After drying the plant material to constant dry weight, it was ground to fine powder (using a Cyclotec 1093 sample mill, FOSS, Höganäs, Sweden). Then it was analyzed for ^{15}N enrichment and percentage total N in the different plant species (ANCA-SL Elemental Analyzer coupled to a 20–20 Mass Spectrometer, SerCon Ltd., Crewe, UK) using the Dumas dry-combustion method.

The stable isotope ^{15}N was placed into holes in the soil, and thus inhomogeneously over the experimental area. Therefore, we used the "negative discard method" for sampling (Powlson and Barraclough 1993). This method requires 1) that a larger plot than where ^{15}N was applied should be harvested to collect as much of the labelled ^{15}N as possible and 2) that the subsample was mixed very thoroughly before the ^{15}N analysis. Both requirements were fulfilled in our study.

Calculations and statistical analyses

The plant access of soil ^{15}N was defined as the ability of the plants to accumulate soil applied ^{15}N in the above-ground plant tissue. It was calculated as the ^{15}N enrichment in the foliar tissue of each plant species minus the natural ^{15}N enrichment. After taking the total N content of the above-ground plant tissue into account we could determine the amount of ^{15}N accessed by the plants per m^2 (mg [15]N m^{-2}). To prevent the abundance of plant species from affecting the soil ^{15}N-access, mg [15]N m^{-2} was divided by the dry plant weight (DW) of the respective plant species (mg [15]N g^{-1}DW).

The volumetric soil water content was calculated on the basis of measured gravimetric soil water content and soil bulk densities from an earlier study at the same site (Hansen et al. 1986).

Analysis of variance (ANOVA) was carried out to test plant community, cut and year impacts on herbage yield, and plant community impacts on inorganic soil N concentration and soil water content.

Linear regression analysis was used to test the decline rate of plant access to soil applied ^{15}N by increasing application depth. Linear regression was carried out on ln-transformed data for both the plant access to soil [15]N (response variable) and the soil application depth (covariate). The data was transformed

to normalize the distribution of residuals. Regression models were tested against analysis of variance models to test for the lack-of-fit, and the residuals of the regression models were inspected to check the models. Mixed models were used with plant community as fixed class variables and block replications as random. Hence, ^{15}N-access was described by

$$^{15}Naccess = {}^{15}N_0 \times \exp(-d \times (\ln(depth))) \qquad (1)$$

where ^{15}N-access (mg [15]N m^{-2} or mg [15]N g^{-1}DW) is access to soil ^{15}N, the $^{15}N_0$ parameter is the estimated ^{15}N-access when depth approaches 0 cm, and the d parameter is a decline rate parameter that describes the decline in ^{15}N-access in relation to the natural logarithm of depth. In linear regression (ln(^{15}N-access)) against (ln(depth)), the slope parameter, d, is identical to the decline rate parameter in Eq. (1) but the linear regression intercept is ln(N_0) due to the transformation. $^{15}N_0$ was not used for comparison, as the depth of 0 m was outside our measurements, which were between 0.4 and 1.2 m. The decline rate parameter (d) was used to compare the decline in ^{15}N-access by increasing soil depth of plant communities and plant species. The selection of Eq. (1) to describe data was based on empirical evidence: it fitted data well.

The effect of plant community and single plant species on the total ^{15}N-access was tested using analysis of variance. To determine the role of the different legumes in adding N to the grassland communities, the atmospheric N_2 fixation was calculated using the ^{15}N enrichment of legume plants from plots with ^{15}N placement at 0.4 m depth. Perennial ryegrass in mixture and in pure stand was chosen as a reference plant for mixtures and pure stands, respectively. The proportion of N derived from the atmosphere (%Ndfa) was calculated according to Peoples et al. (1989):

$$\%Ndfa = \left(1 - \frac{\delta^{15}N_{legume}}{\delta^{15}N_{reference\ grass}}\right) \times 100 \qquad (2)$$

where $\delta^{15}N$ is the ^{15}N excess (atom%), which describes the measured ^{15}N enrichment in the legume and reference grass, respectively, minus the natural ^{15}N enrichment measured on plants growing in the same plant communities adjacent to the ^{15}N placement study.

All statistical analyses were done with the open source program R (R Development Core Team 2010).

Results

Herbage yield

Herbage yield in the five plant communities over 2 years varied between 103 ± 30 kg ha^{-1} in pure stand perennial ryegrass at the fourth cut in 2009 and 4000 ± 263 kg ha^{-1} in pure stand lucerne at the first cut in 2009. The analysis of variance showed a significant 'plant community x cut x year' interaction ($p<0.0001$, Fig. 2, Table 1), which meant that herbage yield in the different experimental plant communities was influenced differently during various cuts and years. Herbage yield decreased from the first to the fourth cut in both years, with exception of the second cut in 2009. Even though plant communities were fertilized in the first year and not in the second year, herbage yield was greater in the second year compared to the first year, except pure stand perennial ryegrass and the two-species mixture. On average, pure stand perennial ryegrass had the lowest herbage yield while pure stand lucerne and the four-species mixture had the highest herbage yields (Fig. 2). Plant communities with deep-rooting plant species (pure stand chicory, pure stand lucerne and four-species mixture) showed greater yield compared with pure stand perennial ryegrass, besides pure stand chicory at the first and fourth cut in the first year. Further, plant communities with legumes (pure stand lucerne, two-species and four-species mixture)
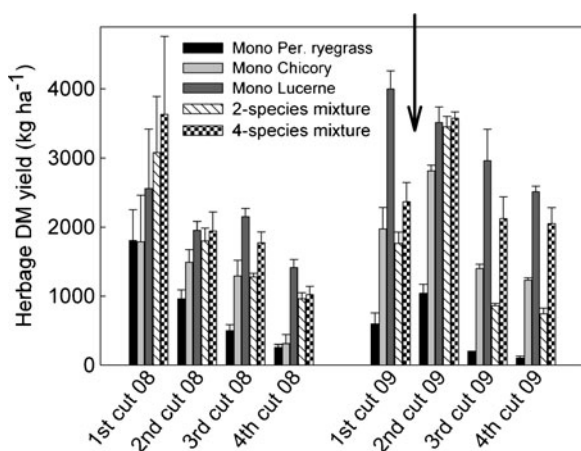
Table 1 Analysis of variance with main effects and interaction effects on herbage yield. Mean values ± standard error ($n=4$). Plant comm. = Plant community, d.f. =degrees of freedom

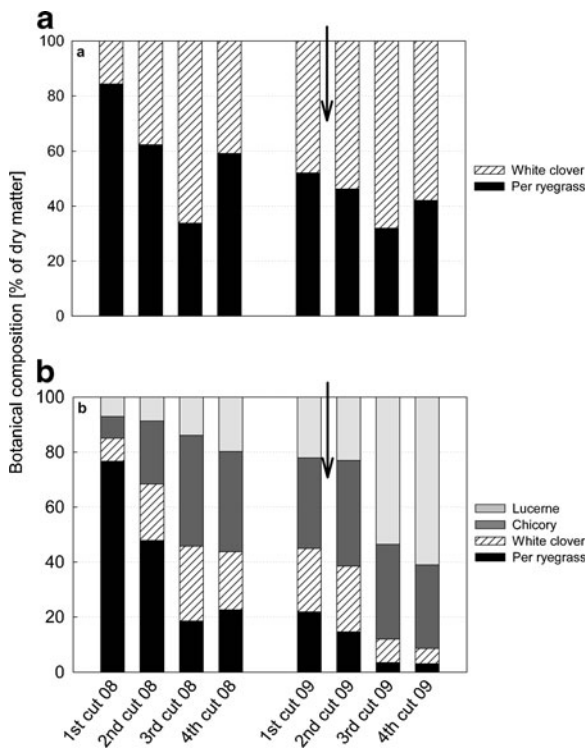| Factors	Herbage yield (kg ha^{-1})	
	d.f.	*p*-value
Plant comm.	4	<0.0001
Cut	3	<0.0001
Year	1	0.0032
Plant comm. x Cut	12	<0.0001
Plant comm. x Year	4	<0.0001
Cut x Year	3	<0.0001
Plant comm. x Cut x Year	12	<0.0001
Residuals	117	

yielded on average 73, 65 and 72 % more, respectively, than perennial ryegrass in pure stand.

Botanical composition in two- and four-species mixtures

The proportions of plant species in the perennial ryegrass-white clover mixture showed a similar pattern from the first to the fourth cut in 2008 and 2009 (Fig. 3a). In 2008, when some N fertilizer was applied, perennial ryegrass contributed with a greater proportion to the two-species mixture compared with 2009. The proportion of perennial ryegrass in the four-species mixture decreased by approximately 75 % from the first cut in 2008 to the last cut in 2009 (Fig. 3b). White clover proportion decreased by 12 % at the third and fourth cut in 2009 and was only 8 % at the first cut in 2008 after N fertilization. The proportion of chicory was stable in all cuts, apart from the first cut in 2008. The proportion of lucerne increased from 8 % at the first cut in 2008 to 40 % at the last cut in 2009, which thus may have displaced white clover and perennial ryegrass at the end of the growing season in 2009. At the second cut in 2009, shortly after the ^{15}N placement experiment, the components of the two-species and four-species mixtures were relatively evenly distributed (Fig. 3a and b).
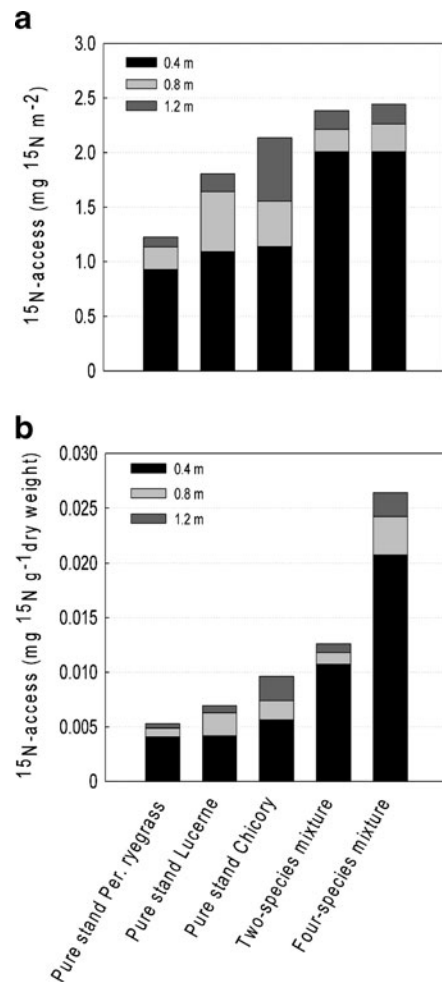
Soil ^{15}N-access

Total ^{15}N-access measured m^{-2} between 0.4 and 1.2 m soil depth did not differ among the different plant communities ($p=0.09$), yet perennial ryegrass in pure

Fig. 2 Herbage DM (dry matter) yields of five plant communities at four cuts in 2008 and 2009. Mean values ± standard error ($n=4$). The arrow (↓) indicates the time of the ^{15}N placement experiment. Figure 3. Botanical composition of the two-species **a**) and the four-species **b**) mixtures at four cuts in 2008 and 2009. The arrow (↓) indicates the time of the ^{15}N placement experiment

Fig. 3 Botanical composition of the two-species **a**) and the four-species **b**) mixtures at four cuts in 2008 and 2009. The arrow (↓) indicates the time of the ^{15}N placement experiment

Fig. 4 Soil ^{15}N-access at three different soil depths in the five different plant communities presented as **a**) mg ^{15}N per m^{-2} and **b**) mg ^{15}N per gram dry plant weight

stand tended to have lower total ^{15}N-access values than the other communities, and the two- and four-species mixtures tended to have a greater ^{15}N-access than the pure stands (Fig. 4a). When total ^{15}N-access was calculated per gram dry plant weight, access to soil ^{15}N increased significantly from pure stands perennial ryegrass, lucerne, chicory, the two-species mixture to the four-species mixture ($p < 0.0001$; Fig. 4b). Plant ^{15}N-access declined significantly ($p < 0.0001$) from 0.4 to 1.2 m soil depth according to Eq. 1. The d parameter describes the plant's ability to access ^{15}N with increasing depth. The larger/smaller the parameter the quicker/slower the plants ^{15}N-access decreases with increasing depth. Plants with different rooting depths are expected to show varying d parameters. Contrary to our expectation, there were no significant differences in parameter d between experimental plant communities. The common d parameter (Eq. 1) was 1.917±0.203 and 1.915±0.181 for ^{15}N-access m^{-2} and g^{-1} dry plant weight, respectively ($p > 0.05$). This corresponds to approximately 80 % decline of ^{15}N-access from 0.4 to 0.8 m and approximately 90 % decline from 0.4 to 1.2 m independently of the five plant

communities (Fig. 4a and b). Chicory in pure stand, however, tended to have the smallest slope parameter (Table 2), indicating a relatively lower decline rate of ^{15}N-access by increasing depth compared with the other plant communities.

Furthermore, we compared individual plant species and their soil ^{15}N-access in different soil depths. In the two-species mixture, perennial ryegrass had a larger d-parameter than white clover (Table 2). This means that perennial ryegrass derived a greater relative proportion of its N from shallow soil layers than white clover (Fig. 5a and c). Total ^{15}N-access was approximately five times greater in perennial ryegrass than in white clover. In the four-species mixture the d-parameter declined in the order perennial ryegrass > lucerne >

Table 2 Parameter estimates and standard errors of the *d*-parameter in Eq. 1 and p-values for individual plant species, showing whether the parameters are statistically different. DW = dry weight

Plant community	Plant species	Parameter estimates (*d*)			
		^{15}N-access per plant species			
		mg^{15}N m^{-2}		mg^{15}N g^{-1}DW	
Pure stand	Per. ryegrass	1.823±0.537	*p*>0.05	1.909±0.446	*p*>0.05
Pure stand	Lucerne	1.733±0.537		1.700±0.446	
Pure stand	Chicory	1.450±0.537		1.531±0.446	
Two-species mixture	Per. ryegrass	2.545±0.407	*p*=0.02	2.654±0.388	*p*=0.008
	White clover	1.010±0.407		1.017±0.388	
Four-species mixture	Per. ryegrass	3.775±0.569	*p*=0.019	3.481±0.467	*p*=0.006
	White clover	1.561±0.569		1.644±0.467	
	Lucerne	2.946±0.569		2.701±0.467	
	Chicory	1.539±0.569		1.194±0.467	

white clover > chicory, demonstrating that chicory had the greatest proportions of ^{15}N-access in the deeper soil layer (Table 2). Yet perennial ryegrass had access to the most ^{15}N between 0.4 and 1.2 m soil depth, chicory less than perennial ryegrass and the two legumes, lucerne and white clover, had only small total

Fig. 5 Soil ^{15}N-access at three different soil depths in single plant species of the two-species plant community presented as **a**) mg ^{15}N per m^{-2} and **b**) mg ^{15}N per gram dry plant weight and the four-species plant community presented as **c**) mg ^{15}N per m^{-2} and **d**) mg ^{15}N per gram dry plant weight
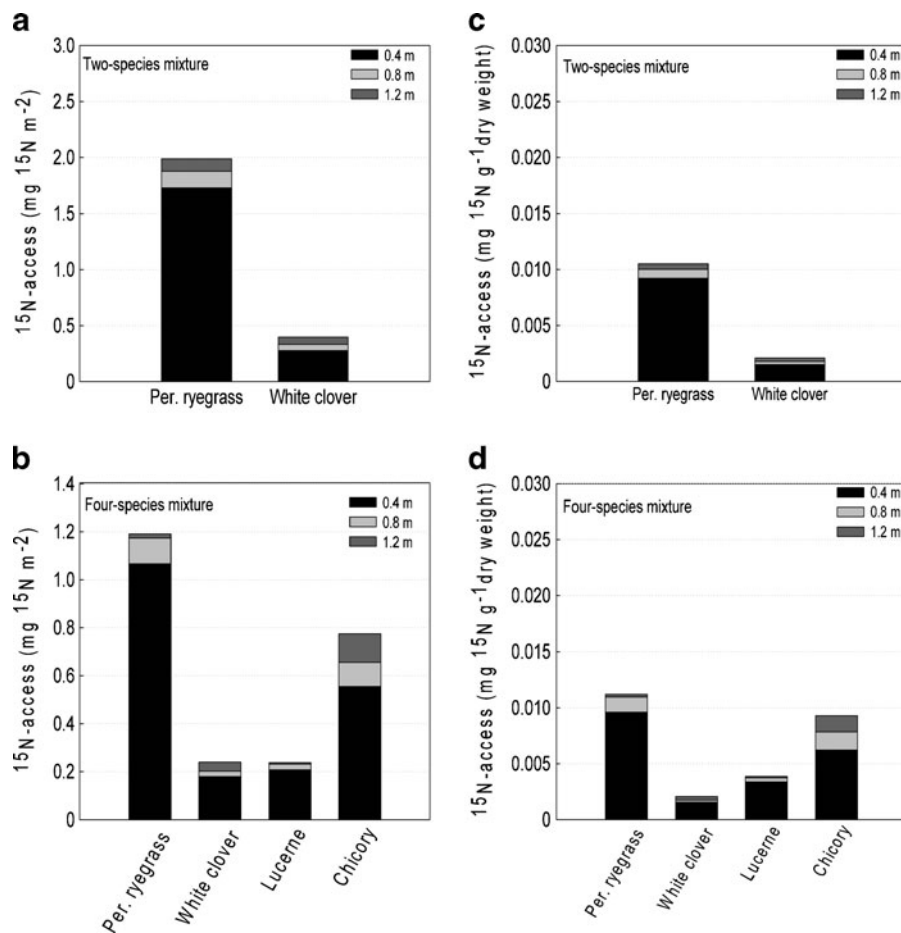
[15]N-access values (Fig. 5b and d; $p < 0.0001$). This difference in total ^{15}N-access among the plant species was also strong for the two-species mixture ($p = 0.0001$). Even though there was no difference in total ^{15}N-access from the combined three soil layers between the two- and four-species mixtures, the individual plant species in the mixtures had access to different amounts of ^{15}N in various soil depths. In both mixtures perennial ryegrass absorbed great amounts of ^{15}N from the 0.4 m soil depth in comparison with its neighbouring plants and access to soil ^{15}N in 0.8 and 1.2 m soil depths was much lower than at 0.4 m. This resulted in very similar total ^{15}N-access levels for both the two- and four-species mixtures.

Soil inorganic N and volumetric soil water content

At the time of ^{15}N placement soil ammonium content decreased significantly ($p < 0.0001$) with depth, but was unaffected by the experimental plant community ($p > 0.05$). At 0.4 m soil depth the ammonium content was 1.7 ± 0.13 mg (NH_4^+) kg^{-1} dry soil, at 0.8 m 0.4 ± 0.06 and at 1.2 m 0.2 ± 0.09. Nitrate content was generally small (on average 0.5 ± 0.08 mg $NO_3^- kg^{-1}$ dry soil) and only above the measurement limit (0.1 mg/L) at 0.4 m soil depth. In the 0 to 0.4 m soil column, experimental plant communities with legumes (pure stand lucerne, two- and four-species mixtures) showed significantly ($p = 0.04$) higher nitrate values (0.6 ± 0.02 mg $NO_3^- kg^{-1}$ dry soil) compared with pure stand perennial ryegrass and pure stand chicory (0.3 ± 0.03 mg $NO_3^- kg^{-1}$ dry soil).

Volumetric soil water contents were between 0.12 and 0.24 v/v, which indicate that the soils were rather dry, as the water content of a sandy-loamy soil would have values between 0.25 and 0.35 at field capacity (Certified Crop Advisor 2012) and the water content at field capacity is considered to be ideal for crop growth. Volumetric soil water content varied with soil depth and plant community ($p < 0.0001$). Generally it increased with increasing depth and was greatest for pure stand perennial ryegrass and smallest for the two-species mixture. The fact that the soils of all five plant communities were relatively dry presumably meant that there was no N leaching from the soils and that soil water content had no impact on plants' access to soil [15]N.

Symbiotic N_2 fixation in legumes

White clover and lucerne grown in a grassland mixture derived between 86 and 92 %, respectively, of total plant N from atmospheric N_2 fixation. Lucerne in pure stand derived significantly less N from atmospheric N_2 fixation than when grown in a four-species mixture ($p < 0.05$; 70 %).

Discussion

Positive plant diversity effect on herbage yield and total ^{15}N-access

Greater herbage yields in the four-species mixture compared with the two-species mixture and perennial ryegrass and chicory in pure stand confirmed our first hypothesis. Increasing herbage yields with increasing plant species richness (positive plant diversity effects) were also shown in other studies on agricultural grassland mixtures (Frankow-Lindberg et al. 2009; Kirwan et al. 2007; Nyfeler et al. 2009; Picasso et al. 2008). The same was not the case for lucerne in pure stand, but growth conditions must have been optimal for lucerne, as herbage yields were high in both years and the proportion of lucerne in the mixture increased from the first to the second year. A parallel study (Rasmussen et al. 2012) also showed good growth conditions for lucerne in those years. The combination of atmospheric N_2 fixation and a deep-rooting characteristic make lucerne robust towards low soil N levels as well as low soil water content (Brown et al. 2005). It was the opposite for perennial ryegrass where low yield in pure stand was a reflection of low plant-available soil N content. Total ^{15}N-access of the four-species mixture was greater than in the two-species mixture and the pure stands and thus correlates with greater herbage yields for mixtures compared to pure stands. This positive relationship of plant diversity, soil ^{15}N-access and herbage yield suggests that increased plant diversity in agricultural grasslands improves N use and enhances yield, which may reduce water pollution by N leaching. From the positive plant diversity effect on herbage yield and total ^{15}N-access we hypothesized that plant species differ in their ability to access soil ^{15}N from different soil depths and thus affect the soil ^{15}N-access of the whole plant community.

Weak vertical complementarity in soil ^{15}N-access

Our field study did not show that the positive plant diversity effect for total soil ^{15}N-access and herbage yield derives from complementary soil ^{15}N acquisition from different depths, thus rejecting our fourth hypothesis. Deep- and shallow-rooting plant species did not complement each other in ^{15}N-access from different vertical soil layers, but may have horizontal root complementarity (Mommer et al. 2010; von Felten and Schmid 2008). The fact that we only looked at above-ground plant tissue may have biased our result, as N acquisition by roots is fast and the transfer from the roots to above-ground plant tissue is slow and may vary among plant species. Further, next to root N uptake, microbial N uptake, especially by arbuscular mycorrhizal fungi (AMF), was shown to be more important in species-rich low N input grasslands compared with species-poor high N input grasslands (De Vries et al. 2012). Hence, differences in microbial and root N retention between different plant communities and plant species may have affected the N acquisition of plants. In spite of this, von Felten et al. (2012) drew similar conclusions from a mesocosm experiment analysing below- and above-ground plant tissues.

^{15}N-access of individual plant species

All species in the different experimental plant communities were able to access ^{15}N from all depths, even from 1.2 m. This surprised us, as the effective rooting depth of perennial ryegrass and white clover has been reported to be <0.7 m (Kutschera and Lichtenegger 1982; Kutschera and Lichtenegger 1992; Pollock and Mead 2008) and down to 1 m under drought stress conditions (Høgh-Jensen and Schjoerring 1997; Skinner 2008). In our study, relatively dry soil layers down to 0.8 m could possibly have stimulated deeper root growth and can therefore explain ^{15}N-access from deep soil layers for shallow-rooting plant species. Nevertheless, perennial ryegrass in mixture was superior in acquiring ^{15}N from the shallow soil layer and white clover generally accessed only small amounts of ^{15}N. Lucerne, one of the two deep-rooting plant species, was shown to derive more N from the symbiotic N_2 fixation process than from N foraging by roots when grown in mixtures (Carlsson and Huss-Danell 2003;

Hardarson et al. 1988; Nyfeler et al. 2011). This may have spared soil N for the non-leguminous plants of the mixture (Chalk 1998) and resulted in interspecific competition for soil N mainly between chicory and perennial ryegrass, as also white clover fixed large amounts of N from the atmosphere when grown in the four-species mixture. The deep-rooting characteristic of chicory (Skinner 2008; Thorup-Kristensen 2006) was confirmed by greater deep soil ^{15}N-access compared with the other three plant species in the four-species mixture. Yet, chicory had less access to below-ground ^{15}N from three soil depths altogether than perennial ryegrass, which is in agreement with Høgh-Jensen et al. (2006). Thus, our second and third hypotheses on the superiority of certain plant species in accessing ^{15}N from deep and shallow soil layers, respectively, were only partly confirmed. Chicory was superior in accessing deep soil ^{15}N (1.2 m) and perennial ryegrass when grown in mixture was superior in accessing shallow soil ^{15}N (0.4 m), but lucerne and white clover, the two leguminous grassland plants, generally acquired only small amounts of ^{15}N from the soil.

Legume effect on ^{15}N-access and herbage yield

The legumes in this study derived large amounts of N from atmospheric N^2 fixation, which affected the N acquisition of grassland plant communities and thus confirmed our fifth hypothesis. Plant available soil N was limited in all soil layers (0 to 0.7 mg (NO_3) kg^{-1} dry soil and 0.1 to 1.9 mg (NH_4^+) kg^{-1} dry soil). Because of dynamic nutrient processes in the soil (e.g. mineralization, nitrification) ammonium and nitrate concentrations are very short lived. Yet nitrate contents in 0.4 m depth were significantly greater in plant communities with legumes than without legumes, because N-rich legumes release N into the soil (Palmborg et al. 2005; Paynel et al. 2008; Wichern et al. 2008). This deposition of nutrients by plant roots may stimulate soil microorganisms, which consequently makes even more nutrients available to the plants (Wichern et al. 2008). Probably because of this, we observed high herbage yields for plant communities including legumes in the second year even without fertilization. Furthermore, positive effects of legumes on neighbouring plants by below-ground N transfer (Pirhofer-Walzl et al. 2012) can have resulted in greater herbage yields in the two- and four-species mixture

as compared with perennial ryegrass and chicory in pure stand. Perennial ryegrass grown in pure stand was not very productive and accessed only little ^{15}N, but shifted from small and relatively deep soil ^{15}N-access when grown in pure stand to high and relatively shallow soil ^{15}N-access in the 0.4 m soil layer when grown in mixture. This shows that grasses grown in mixture with legumes are strongly stimulated and increase their competitiveness for soil N. Lucerne in pure stand accessed relatively deep soil ^{15}N, indicating that the deep-rooting lucerne may forage for deep soil resources other than N, such as water (Brown et al. 2005; De Boeck et al. 2006).

Increased N availability in plant communities with legumes stimulated grass root growth (Paynel et al. 2008) as indicated by the high grass ^{15}N-access in grass-legume mixtures of our study. However, plant diversity effects on herbage yield have also been found in mixtures comprising only grasses and non-leguminous dicotyledonous plant species (Roscher et al. 2008; Van Ruijven and Berendse 2003), suggesting that legume N_2 fixation is not the only process that improves productivity of mixtures. A recent study shows that soil microbes play a major role in the positive plant diversity-productivity relationship (Schnitzer et al. 2011).

Reflections on methodology

We are aware of presenting a ^{15}N-study, which is only a snapshot of the growing season. Different grassland plant species change growth patterns during the growing season, for instance when soil temperature affects root activity (Fitter 1986). For that reason, more ^{15}N placement campaigns would have given more insight into temporal differences. Further, the import of ^{14}N into the plant-soil system by N_2-fixation of white clover and lucerne can have diluted above-ground plant ^{15}N enrichment and thus legume ^{15}N-access could have been underestimated. The ^{15}N-study, however, was carried out within a relatively short time frame, meaning that the ^{14}N-input in the system was kept at a low level. Moreover, ^{15}N was placed at depths lower than where we would expect the main body of ^{14}N from N_2-fixation to be deposited. Low root abundances in deep soil layers can have caused lower ^{15}N-access values than reported in other studies (e.g. Jumpponen et al. 2002; Kristensen

and Thorup-Kristensen 2004; von Felten et al. 2009) and fairly high variations among replicates. More ^{15}N placements per subplot would have improved the accuracy. Additionally, the ^{15}N-dilution method to estimate symbiotic N_2-fixation (Fried and Middleboe 1977) is based on the assumption that the legume and its reference plant have a spatially similar nutrient access pattern. It was hypothesized that different grassland plant species differ in their vertical ^{15}N-access pattern and therefore would contradict this assumption. Nonetheless, Hardarson and Danso (1990) showed that for high N_2-fixation levels, as found in our study, the selection of the reference plant is not a critical factor. For low N_2-fixation levels (<30 %), a good reference plant is still crucial for the accuracy of the method.

Conclusions

In a two-year field experiment, a four-species mixture comprising deep- and shallow-rooting plant species had greater herbage yields than the two-species mixture comprising only shallow-rooting plant species and pure stands, besides lucerne in pure stand. Access to soil ^{15}N was also greater for the four-species mixture compared with the two-species mixture and pure stands. This stresses the positive effect of grassland plant diversity on the environment (improved N use may lead to reduced N leaching) and agricultural production (increased herbage yield). Lucerne and white clover, deep- and shallow-rooting legume, respectively, fixed large proportions of their N symbiotically, only acquired small amounts of soil ^{15}N and thus, spared soil N for non-leguminous plants. Chicory confirmed its deep-rooting characteristic in terms of soil ^{15}N-access, and perennial ryegrass grown in mixture its shallow-rooting characteristic, acquiring large amounts of ^{15}N from the shallow soil layer. Nevertheless, including deep-rooting plant species to a shallow-rooting grassland mixture did not improve soil ^{15}N-access in deep soil layers. This suggests that complementarities between legumes and non-leguminous plants and increasing plant diversity have a greater affect on herbage yield compared with complementary vertical soil ^{15}N-access.

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