



Deep N acquisition, overyielding and vertical niche differentiation in hemiboreal cultivated grass-clover mixtures

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Abstract

Aims To explore the contribution of vertical root niche differentiation to recovery of deep NH_4^+ by species in cultivated grass-clover mixtures, and to mixtures' overyielding.

Methods The uptake of a deep-placed (42 cm) slow-release $^{15}\text{NH}_4^+$ label was tracked in the herbage of two grass-clover mixtures during one growing season and the subsequent spring (3 + 1 harvests) and compared with uptake in monocultures.

Results Mixtures overyielded in biomass (+11%), total N (+24%), and deep $^{15}\text{NH}_4^+$ recovery (+17%). The latter was most pronounced in the autumn (+47% to +62%). Diversity effects on deep ^{15}N recovery were a combined result of changes in growth vigor and ^{15}N recovery per biomass. We

found evidence of vertical niche differentiation: *Schedonorus arundinaceus*, *Schedonorus pratensis* and *Lolium perenne* displayed positive diversity effects on deep ^{15}N uptake per biomass combined with increased total N concentration; not so in *Phleum pratense* and *Poa pratensis*. Though the diversity effect on deep ^{15}N per biomass was small in N-fixing *Trifolium pratense*, due to its high yields this translated into a large negative diversity effect on deep N recovery. Enhanced deep N uptake was contingent on enhanced growth vigor.

Conclusions Improved N nutrition in grasses due to clovers did not prevent overall positive diversity effects on deep N recovery. This is potentially beneficial for reducing N leaching. The grass-clover mixtures better combined recovery of deep-placed $^{15}\text{NH}_4^+$ with yield performance and high protein content than any single species. Diversity effects on deep N uptake were species-specific and could not be predicted by performance in monoculture.

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Introduction

There is an increasing demand for forage of high quality and protein content, produced with high nitrogen use efficiency and reduced N losses.

Combining species with different growth habits can increase capture and use efficiency of resources such as light and nutrients and confer tolerance and resilience to adverse physical and biological factors, prompting *diversity effects* on the biomass and quality of a species in a mixture compared to monoculture (Weisser et al. 2017). A classical case is growing grasses with N-fixing legumes, which can give positive diversity effects on grass N nutrition (Hooper 1998; Jing et al. 2017; Lüscher et al. 2014; Nyfeler et al. 2011) and thus reduce fertilizer needs. A mixture is said to be *overyielding* when its overall performance is superior to the performance of its components in monoculture and their proportional presence in the mixture (Lüscher et al. 2008; Nyfeler et al. 2011). Thus, mixture overyielding is the sum of the components' diversity effects. A mixture yield surpassing that of the highest-yielding species grown in monoculture is defined as *transgressive overyielding* (Schmid et al. 2008).

Mineral N fertilizer is easily leached below the upper and densest root zone. Combining species with varied rooting depths can enhance N utilization throughout the soil profile (Hoekstra et al. 2015; Mamolos et al. 1995; Mommer et al. 2010; Pirhofer-Walzl et al. 2013), and may contribute to overyielding. It has been theorized that competition between species in the topsoil can stimulate species capable of producing deeper roots to increase N uptake from deeper soil (*vertical niche differentiation*, Hoekstra et al. 2015; Mommer et al. 2010), and thus enhance recovery of N which has leached below the topsoil.

Vertical niche differentiation is difficult to study as it is related to many simultaneous diversity effects which are difficult to disentangle (Hoekstra et al. 2015; Mamolos and Veresoglou 2000; Pirhofer-Walzl et al. 2013; von Felten et al. 2012; von Felten and Schmid 2008) and may vary over time (Ravenek et al. 2014). While many studies have confirmed that deep-rooting grassland species can access N from deep soil layers (Hoekstra et al. 2015; Kristensen and Thorup-Kristensen 2004), mixtures including deep-rooting species do not always obtain more N from deeper soil layers than mixtures without them (Hoekstra et al. 2015). Mommer et al. (2010) observed an early increase in root mass but shallower root distributions in mixtures relative to monocultures, which they attributed to inter- and intraspecific root recognition. Though plants modify root distribution and

allocation in response to other species and competition for limiting nutrients (Cahill and McNickle 2011; Hodge 2004; Robinson et al. 1999), root position is not necessarily indicative of nutrient uptake, as roots can vary in affinity for NH_4^+ or NO_3^- (Maire et al. 2009). Further, it has been suggested that species-rich communities invest less in belowground biomass but use it more efficiently (Bessler et al. 2009).

It is also unclear how the enhanced N nutrition of grasses when grown with N-fixing clovers may affect the grasses' exploitation of N at different soil depths. For example, upon surface N application in a nutrient-poor permanent grassland, N-competitive non-legumes increased shallow root activity (5 cm, traced by alkali earth metals) while legumes increased deeper (15 cm) root activity and less N-competitive species showed little change (Mamolos et al. 1995). Pirhofer-Walzl et al. (2013) found that N-competitive perennial ryegrass increased growth vigor and N uptake at 40 cm, and more so than deeper-rooting chicory, when they were grown together with legumes in a moderately-fertilized ley.

Our study aimed at exploring diversity effects on deep N acquisition in two well-established grass-clover mixtures grown in a hemiboreal climate typical of southern Norway, where due to soil conditions and the short growing season roots of grassland species are mainly confined within the shallow plough layer (upper 20–25 cm, Bleken et al. 2022). To do this we developed a novel slow-release $^{15}\text{NH}_4^+$ label (Byers et al. 2024) and followed its uptake by the mixtures' components through the third production year.

We chose five common forage grasses and two N-fixing clovers covering a variety of expected deep rooting patterns, N acquisition strategies, forage qualities, and persistence in hemiboreal conditions. The five grasses were: timothy (*Phleum pratense* L.) and meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv.), which are the most commonly-grown forage species in Norway as they combine good forage quality with winter hardiness (Jørgensen et al. 2023); tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort), which is known to be deep-rooting (e.g. Hernandez and Picon-Cochard 2016; Malcolm et al. 2015) as well as drought and winter resistant in Nordic countries; perennial ryegrass (*Lolium perenne* L.), which is fast establishing and reputed for its high yields and nutritional value, but overwinters poorly in Norway (Østrem et al. 2015) and is considered to

be shallow rooting (Finn et al. 2013), and Kentucky bluegrass (*Poa pratensis* L.), which is known for lower yields but high N concentration and a shallow rhizomatous root system which makes it more adapted to pastures. Red and white clover are the predominant N-fixing legumes in Norway. They differ in growth habit; red clover (*Trifolium pratense* L.) is tall and has a persistent deep taproot (e.g. Ergon et al. 2016), while white clover (*Trifolium repens* L.) forms stolon.

We hypothesized that mixtures of grasses and N-fixing clover (1) would overyield in herbage biomass and total N yield and (2) overyield in recovery of deep-placed ^{15}N (3a) through complementary vertical differentiation of root activity. Alternatively, (3b) increased N concentration in grasses due to presence of clovers in the mixture would suppress vertical niche differentiation and reduce deep N uptake by the grasses. Further, we expected perennial ryegrass, bluegrass and the clovers to decrease their uptake of deep-placed ^{15}N and the purportedly deep-rooting grasses to increase it when grown in mixtures rather than in monoculture. This article reports the overyielding and diversity effects in the two mixtures. The performance of the species in monoculture was presented in Byers et al. (2024).

Materials and methods

Site, experimental treatments and sward management

The experiment was established in an Umbric Epistagnic Retisol (IUSS 2015) at the NMBU research farm in Ås, Norway (59°39'49"N, 10°45'38"E, 69 m a.s.l.). The soil is a stone-free silty loam (~31% clay, 44% silt and 25% sand), artificially drained at 1 m depth. The bulk density is low in the top layer (1.15 g cm⁻³), but below the ploughing depth (20 cm) it increases markedly to 1.5 g cm⁻³ at 40 cm (Byers et al. 2024). The mean temperature normal (1991–2020) is 13.8 °C during the growing season from May through September, and 0.9 °C for October through April (Wolff et al. 2021). During the winter, freeze–thaw events and periods with snow cover are common. The normal annual precipitation is 892 mm, roughly evenly distributed through the year, but periods of drought can occur, especially in

early summer due to strong insolation and wind (Byers et al. 2024).

Average temperature was close to normal in 2017 (Suppl. Fig. S1). Plants experienced drought in April and July 2017. There was an exceptionally heavy rain event of ~55 mm in August 2017 between the second and third harvests. The winters were harsh, especially before spring 2018, with continuous snow cover from early January to early April.

Monocultures and mixtures of forage grass and clover species were sown in June 2014 in fully randomized plots (8 m×1.5 m) on 12 sowing rows per plot spaced 12 cm apart. Two mixtures were used. **Mix 4** was composed of timothy (*Phleum pratense* L. cv. Grindstad), perennial ryegrass (*Lolium perenne* L. cv. Figgjo), tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort. cv. Swaj), and red clover (*Trifolium pratense* L. cv. Lea). **Mix 10** in addition to the four species of Mix 4 included meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv. cv. Fure), Kentucky bluegrass (*Poa pratensis* L. cv. Knut), white clover (*Trifolium repens* L. cv. Milkanova), smooth brome-grass (*Bromus inermis* Leyss.), festulolium (Hybrid *Festuca x Lolium*), and alfalfa (*Medicago sativa* L.). The best locally adapted cultivars were chosen. However, festulolium, smooth brome-grass and alfalfa had not survived in the mixture by the time of this study, apart from a few brome-grass leaves. If any festulolium was present this was likely included among the fescues during sorting. In the case of alfalfa inoculation with a suitable *Rhizobium* had failed. Clover seed was successfully inoculated using soil from an organically managed crop rotation.

Starting in 2015, the swards were harvested three times per year following local recommendations for high forage digestibility. All treatments received moderate N fertilization, in total 200 kg N ha⁻¹ yr⁻¹ applied as NPK 22–3–10, distributed 40% in early spring (60% in 2018), 40% after the first harvest and 20% after the second harvest. More details are reported in Byers et al. (2024).

^{15}N Labeling

A slow-release label of 98 atom percent (AT%) $^{15}\text{NH}_4^+$ adsorbed to clinoptilolite was applied in 4 subplots distributed in 4 fully randomized sowing plots per treatment, before the onset of the spring regrowth (18–20 April 2017). Each subplot received

a dose of 36.0 mg ^{15}N , which translates to 68 mg m^{-2} of plant sampling area, placed at 41–43 cm depth in a pre-augured 4×4 array of 16 mm diameter, 0.43 m deep holes centered between sowing rows and spaced 12 cm apart. Care was taken to avoid contamination and holes were sealed with finely ground and compacted dry clayey soil, to hinder preferential root growth. See Byers et al. (2024) for an evaluation of the ^{15}N labeling method and results of the companion ^{15}N -labeling study in monocultures of timothy, ryegrass, tall and meadow fescue, bluegrass, red and white clover in the same field experiment.

Plant sampling, total N and ^{15}N uptake

We measured the dry matter (DM) and N yield, and uptake of ^{15}N in the herbage of the three harvests (H1, spring; H2, summer; and H3, autumn; on 6 June, 27 July and 18 September, respectively) during the growing season following the labeling event, and in the spring harvest of the following growing season (H4, on 30 May 2018). Herbage above ~5 cm stubble height was collected from a square sampling area (0.532 m^2) with a width of 5 sowing rows, which was superimposed and larger than the area delineating the ^{15}N label placement (Powlson and Barraclough 1993). The herbage of mixtures was sorted into individual species while fresh. Samples were dried at 40–60 °C, chopped, and a subsample was ground, ball-milled and analyzed for N content and excess ^{15}N above natural abundance to calculate uptake as detailed in Byers et al. (2024).

Per mixture subplot j , the total yield in herbage DM (g m^{-2}), N (g m^{-2}) or ^{15}N uptake (mg m^{-2}) is the summed values of the component species i :

$$(\text{Yield})_j = \sum_i (\text{Yield})_{ij} \quad (1)$$

The whole-season cumulative ^{15}N uptake per herbage DM (mg g^{-1}) was calculated per subplot j over three consecutive harvests h as:

$$(^{15}\text{Nuptake}/\text{DM})_j = \frac{\sum_{h,i} (^{15}\text{Nuptake})_{hij}}{\sum_{h,i} (\text{DMyield})_{hij}} \quad (2)$$

The same approach was used to calculate the yield-weighted average total N concentration in DM (g g^{-1}).

Diversity effects on mixtures' components

For each species, the *expected yield* (g m^{-2} for DM and N, mg m^{-2} for ^{15}N) and *diversity effect* (same units) were calculated from the mean DM or N yields and ^{15}N uptake in monoculture (Eqs. 3 and 4) weighted by the attained share of the DM yield in the mixture. This accounted for the marked shift in botanical composition by the third production year and differs from the expected yield based on sown proportions as used in otherwise similar studies (e.g. Kirwan et al. 2009).

$$(\text{Expected yield})_{ij} = (\text{DM share})_{ij} * (\text{Mean yield})_{\text{mono},i} \quad (3)$$

$$(\text{Diversity effect})_{ij} = (\text{Yield})_{ij} - (\text{Expected yield})_{ij} \quad (4)$$

The expected ^{15}N uptake (mg m^{-2}) at each single harvest was also weighted based on the amount of ^{15}N remaining in the soil after previous harvests, assuming that plant uptake into harvested herbage was the only removal of ^{15}N . This was done to account for the fact that the label amount remaining in the soil could differ between mixtures and monocultures (Eq. 5).

$$(\text{Expected } ^{15}\text{Nuptake})_{ij} = (\text{DMshare})_{ij} * \text{Mean} \left(\frac{^{15}\text{Nuptake}}{^{15}\text{Nremaining}} \right)_{\text{mono},i} * (^{15}\text{Nremaining})_j \quad (5)$$

However, the whole season cumulative diversity effect on ^{15}N uptake summed over three harvests (h) was based on the amount of applied ^{15}N .

The diversity effect on ^{15}N uptake can result from changes in DM yield or changed deep root activity. To explore this, at each harvest we estimated the ^{15}N

recovery per dry matter (RDM, Eq. 6) relative to the amount of ^{15}N label not yet removed in the harvested herbage. Similarly, to explore how the plants' utilization of N_{min} from deeper soil contributes to their total N acquisition, we estimated a relative deep uptake index (RDUI, Eq. 7).

$$RDM_{ij} = \left(\frac{{}^{15}\text{Nuptake} / {}^{15}\text{Nremaining}}{\text{DMyield}} \right)_{ij} \quad (6)$$

$$RDUI_{ij} = \left(\frac{{}^{15}\text{Nuptake} / {}^{15}\text{Nremaining}}{\text{Nyield}} \right)_{ij} \quad (7)$$

Thus, RDM and RDUI express the strength of ${}^{15}\text{N}$ recovery per DM or N yield, weighted based on the amount of ${}^{15}\text{N}$ label assumed still in the soil in each subplot, and since they are analogous to concentrations they can be compared directly between species in mixture or monoculture. The diversity effects on RDM and RDUI were calculated as the difference from the observed value in mixture and the average value in monoculture. A positive or negative diversity

effect on RDUI indicates a niche response towards deeper or shallower N uptake.

Notice that a negative diversity effect on RDM or RDUI does not necessarily implies a negative effect on the contribution to total ${}^{15}\text{N}$ uptake in the mixture, as it could co-occur with increased growth vigor (positive diversity effect on DM) or increased nitrogen concentration.

Mixture overyielding

The mixtures' overyielding in DM yields, N yields and ${}^{15}\text{N}$ uptake m^{-2} was defined as the sum of the diversity effects on the components. It was calculated per subplot j as the difference between the yield of the mixture and the sum of the expected values of all species i present in the mixture subplot:

$$\text{Overyielding}_j = \text{Mixtureyield}_j - \sum_i (\text{Expectedyield})_{ij} = \sum_i (\text{diversityeffect})_{ij} \quad (8)$$

Overyielding was defined as *transgressive* when a mixture surpasses the performance of the best monoculture (Kirwan et al. 2009).

Bromegrass was found in small amounts in some Mix 10 subplots. Since no ${}^{15}\text{N}$ labeling experiment was carried out in bromegrass monocultures, mixture overyielding was calculated assuming that bromegrass ${}^{15}\text{N}$ *expected* values were equal to *realized* values (no diversity effects). Weeds were excluded from overyielding calculations.

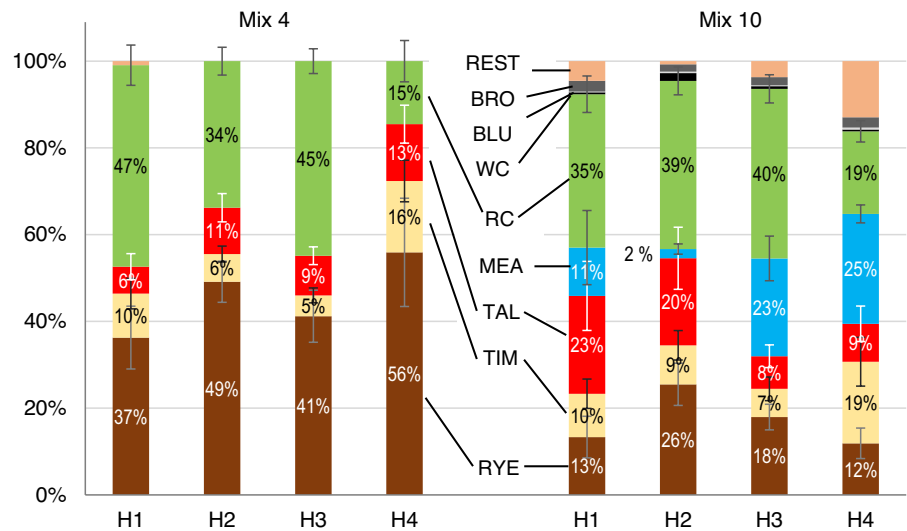
Soil mineral N content

Soil was sampled in the spring from the holes for the ${}^{15}\text{N}$ label placement (at 0–20, 20–30 and 30–43 cm depths), and again in the autumn (30 October 2017) in selected treatments using a hydraulic press outside the subplots (at 0–20, 20–40 and 40–60 cm depths, four soil cores per main plots hosting the subplots). The soil was immediately refrigerated to 4 °C, sieved to 2.0 mm, and analyzed for 1 M KCl-extractable NH_4 , NO_2 and $\text{NO}_3\text{-N}$ using colorimetric assays (Byers et al. 2024).

Statistics

One-way analyses of variance were used to detect significant effects of ley type (two mixtures and seven monocultures) on DM and N yield, ${}^{15}\text{N}$ uptake and other traits considered; homoscedasticity was checked and confirmed for all variables using the HOVTEST=BF option in the MEANS statements of the GLM procedure in the statistical package SAS 9.4 (Statistical Analysis System; SAS Institute, NC, USA). Post hoc Tukey tests to compare yields and ${}^{15}\text{N}$ uptake of mixtures and monocultures was used for multiple means comparison of significant effects. Mixture positive overyielding was assessed by one-sided t-tests separately for Mix 4 and Mix 10, and for both mixtures pooled together. Diversity effects were assessed separately per species by two-sided t-tests, by mixture and also with mixtures pooled together. In all cases, an effect was considered statistically significant when $p \leq 0.05$, and for brevity is referred to as 'significant'. However, for t-tests we also report probability values ≤ 0.15 , while larger probabilities are indicated as 'n.s.'

Fig. 1 Species' share of DM yield ($\pm$ SE) in Mix 4 and Mix 10, by harvest. SE is not shown for species present only in small amounts in Mix 10. RYE: perennial ryegrass; TIM: timothy; TAL: tall fescue; MEA: meadow fescue; BLU: bluegrass; RC: red clover; WC: white clover; REST: weeds. H1: spring 2017; H2: summer 2017; H3: autumn 2017, H4: spring 2018



Results

For detailed results of the species in monocultures and an evaluation of the ^{15}N labeling method see Byers et al. (2024).

Mixture composition

At the time of the labeling experiment in the third production year, the contribution of single components to the total mixture DM yield diverged strongly from the original seeding proportion, and changed from harvest to harvest (Fig. 1). Mix 4 was dominated by red clover and perennial ryegrass, with timothy and tall fescue comprising only around 16% of the herbage (H1 to H3). Mix 10 was also dominated by red clover, while timothy, ryegrass and both fescues (hereafter called the tall-growing grasses) were more evenly distributed. Ryegrass doubled its DM yield in both mixtures from H1 to the drought-affected summer harvest H2 (Suppl. Table S2); red clover also yielded strongly in H2 and only showed a reduced proportion in Mix 4 due to ryegrass dominating. Timothy contributed about the same DM share per harvest in both mixtures.

After a harsh winter and prolonged early spring snow cover, several red clover plants died in mixtures and well as in monoculture, and both fescues and timothy increased their share in the harvest (H4) of the second spring after ^{15}N labeling. However, while in Mix 4 ryegrass remained the dominating species, Mix 10

became dominated by timothy and the fescues along with an increased weed presence. While only sparse ryegrass tillers survived in monocultures, ryegrass overwintered relatively well in the mixtures. Only small amounts of bluegrass, white clover and brome-grass survived early competition and were found in Mix 10 in at least two replicate plots at each harvest.

Overyielding of mixtures

Considering the whole growing season (H1-H3), mixtures showed a tendency towards transgressive DM overyielding at over 900 g m^{-2} and transgressively overyielded in N yield by $8\text{--}9 \text{ g m}^{-2}$ compared to the best monospecific grass (Table 1). Further, Mix 4 recovered 52% of the applied deep ^{15}N , similarly to the best-recovering monocultures (timothy; 55%), while Mix 10 recovered 43%, similarly to tall and meadow fescue monocultures (Tables 1 and 2).

Compared to expected values (Eqs. 3 and 5), mixtures together overyielded significantly (Eq. 9), with +10% DM yield, +25% N yield, and +16% ^{15}N recovery (Table 2A) over three harvests. Including H4 (first harvest of the following year, Table 2B) gave similar results. Mix 10 exhibited weaker overyielding than Mix 4, particularly with respect to deep ^{15}N recovery, which while significant was only 5% more than expected, compared to 27% more in Mix 4 (Table 2A). In Mix 10 the DM overyielding ($\sim 8\%$) was very variable, linked to patchiness of its herbage ($\text{SE} \pm 60 \text{ g m}^{-2}$, Table 2).

Table 1 Whole-season DM yield (g DM m⁻²), N yield (g N m⁻²), and total ¹⁵N herbage uptake (mg ¹⁵N m⁻²) of three consecutive harvests after ¹⁵N labeling in early spring 2017, and related yield-weighted (Eq. 2) N concentration in the DM (%), ¹⁵N herbage uptake to DM yield (mg ¹⁵N g⁻¹ DM), and ¹⁵N herbage uptake to N yield (mg ¹⁵N g⁻¹ N)

	DM Yield (g DM m ⁻²)	N yield (g N m ⁻²)	¹⁵ N herbage uptake (mg ¹⁵ N m ⁻²)	N conc. (%)	¹⁵ N herbage uptake/DM yield (mg ¹⁵ N g ⁻¹ DM)	¹⁵ N herbage uptake/N yield (mg ¹⁵ N g ⁻¹ N)
Mix 4	933 (± 9.4) ^A	24.7 (± 0.6) ^A	35.3 (± 0.8) ^{AB}	2.64 (± 0.07) ^B	0.038 (± 0.001) ^{ABC}	1.43 (± 0.02) ^{CD}
<i>Expected</i>	836 (± 9.9)	19.3 (± 0.4)	27.8 (± 0.6)			
Mix 10	908 (± 60.7) ^{AB}	23.8 (± 2.1) ^A	29.2 (± 0.3) ^B	2.61 (± 0.07) ^{BC}	0.033 (± 0.002) ^{CDE}	1.25 (± 0.10) ^{DE}
<i>Expected</i>	842 (± 13.4)	19.5 (± 0.6)	27.8 (± 0.5)			
Monocultures						
Per. Ryegrass	749 (± 28.1) ^{BCD}	12.3 (± 0.4) ^B	33.5 (± 0.9) ^{AB}	1.64 (± 0.02) ^D	0.045 (± 0.002) ^A	2.73 (± 0.11) ^A
Timothy	851 (± 17.8) ^{ABC}	15.7 (± 0.6) ^B	37.6 (± 1.5) ^A	1.84 (± 0.05) ^D	0.044 (± 0.002) ^{AB}	2.42 (± 0.14) ^{AB}
Tall fescue	880 (± 27.9) ^{ABC}	15.6 (± 1.0) ^B	29.2 (± 0.9) ^B	1.78 (± 0.12) ^D	0.033 (± 0.002) ^{BCD}	1.90 (± 0.17) ^{BC}
Meadow fescue	742 (± 24.6) ^{CD}	14.2 (± 0.7) ^B	31.0 (± 2.6) ^{AB}	1.91 (± 0.04) ^D	0.042 (± 0.004) ^{ABC}	2.21 (± 0.23) ^{AB}
Bluegrass	645 (± 8.5) ^D	14.9 (± 0.4) ^B	21.0 (± 2.2) ^C	2.31 (± 0.06) ^C	0.032 (± 0.003) ^{CDE}	1.41 (± 0.14) ^{CD}
Red clover	892 (± 49.8) ^{ABC}	27.8 (± 2.2) ^A	19.5 (± 0.5) ^C	3.10 (± 0.07) ^A	0.022 (± 0.001) ^E	0.71 (± 0.05) ^E
White clover	686 (± 39.2) ^D	22.1 (± 1.3) ^A	16.3 (± 0.6) ^C	3.22 (± 0.03) ^A	0.024 (± 0.002) ^{DE}	0.75 (± 0.07) ^E

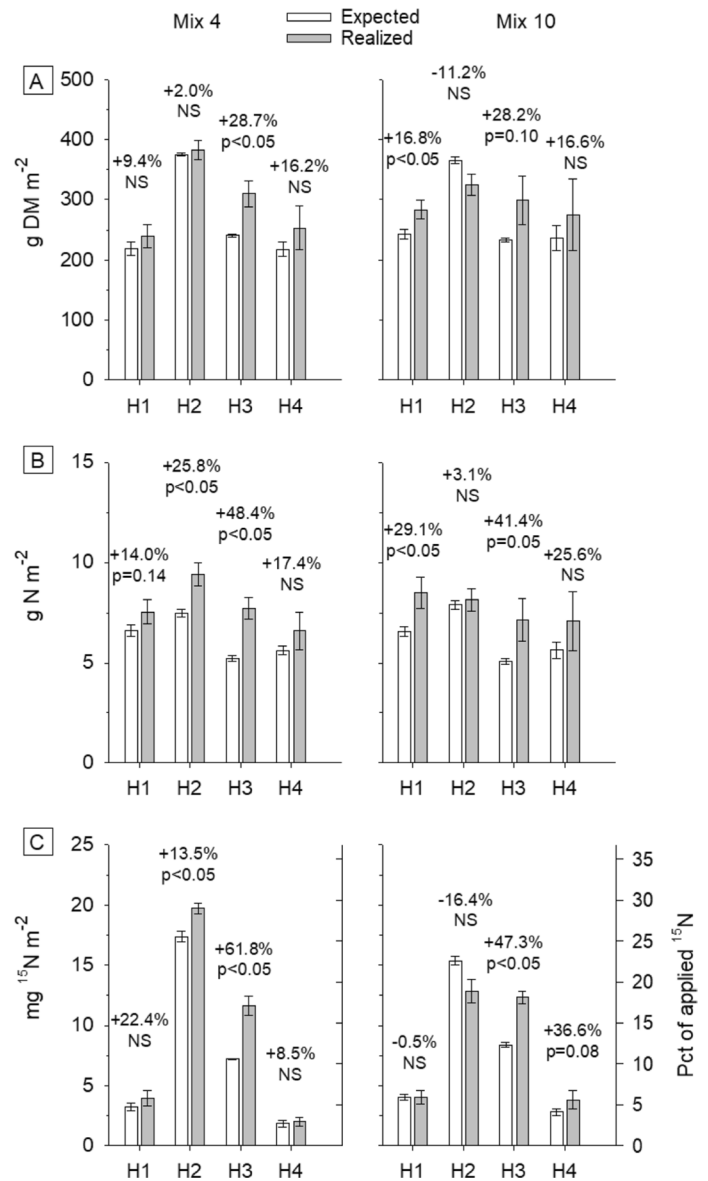
Table 2 Cumulative overyielding as percent increase from expected values in DM yield (g DM m⁻²), N yield (g N m⁻²) and total ¹⁵N herbage uptake (mg ¹⁵N m⁻²) of three (A) and four (B) harvests after ¹⁵N labeling

Mixture	Overyielding (± SE) as % increase from expected value			¹⁵ N recovered, % of label applied	
	DM yield	N yield	¹⁵ N Recovery	In total	Due to overyielding
A) First year, spring, summer and autumn harvest in 2017, (H1 to H3)					
Both	+ 9.7% (± 2.8%), <i>p</i> = 0.006	+ 24.8% (± 4.0%), <i>p</i> < 0.001	+ 16.1% (± 4.7%), <i>p</i> = 0.006	47.4	6.6
Mix 4	+ 11.6% (± 1.2%), <i>p</i> = 0.001	+ 27.8% (± 3.1%), <i>p</i> = 0.001	+ 27.0% (± 4.7%), <i>p</i> = 0.005	51.9	11.0
Mix 10	+ 7.8% (± 5.8%), <i>p</i> = 0.135	+ 21.8% (± 7.6%), <i>p</i> = 0.032	+ 5.1% (± 1.5%), <i>p</i> = 0.020	42.9	2.1
B) All harvests, including spring harvest in 2018, (H1 to H4)					
Both	+ 11.1% (± 4.3%), <i>p</i> = 0.018	+ 24.1% (± 4.9%), <i>p</i> = 0.001	+ 16.8% (± 3.8%), <i>p</i> = 0.002	51.7	7.4
Mix 4	+ 12.6% (± 2.7%), <i>p</i> = 0.009	+ 25.5% (± 2.1%), <i>p</i> = 0.001	+ 25.9% (± 4.0%), <i>p</i> = 0.004	54.9	11.3
Mix 10	+ 9.7% (± 8.8%), <i>p</i> = 0.175	+ 22.7% (± 10.2%), <i>p</i> = 0.056	+ 8.0% (± 1.8%), <i>p</i> = 0.010	48.5	3.6

Herbage overyielding changed during the growing season. Herbage DM overyielding was moderate in the springs (significant only in Mix 10), absent or negative in the drought-affected summer harvest and large in autumn (~28% above expected) (Fig. 2). Nitrogen overyielding was present in every harvest,

except for Mix 10 in the summer harvest H2, and was strongest in autumn (48% more than expected in Mix 4, 41% in Mix 10). Overyielding in ¹⁵N uptake was non-significant or small in spring and summer, when only Mix 4 overyielded, and very large in the autumn: 62% and 47% more than expected in Mix 4 and Mix

Fig. 2 Expected and realized herbage DM and N (g m^{-2}) and ^{15}N uptake (mg m^{-2}) in mixtures in each of four consecutive harvests (H1 to H4). Mean values of 4 replicates ($\pm \text{SE}$). Percent change and probability values of one-sided t -test for positive overyielding are shown above bars. NS: $p > 0.15$. Brome grass is included assuming no diversity effect. Excludes weeds. NS: $p > 0.15$. Figure table shows the ^{15}N recovery in the herbage by mixture and harvest, in percent of ^{15}N assumed remaining in the soil, and the percent point contribution by the mixture effect (overyielding)



Harvest	^{15}N recovered (% of ^{15}N in soil)			
	Total uptake		Contribution by overyielding	
	Mix 4	Mix 10	Mix 4	Mix 10
H1	6	6	1	0
H2	31	20	3	-4
H3	26	24	7	6
H4	6	10	0	2

10, respectively. Autumn ^{15}N overyielding was larger by percentage-point than N overyielding, which in turn exceeded DM overyielding.

Weighted relative to ^{15}N label assumed remaining in the soil, the recovery by mixtures was weakest in the springs (~6% of applied ^{15}N in H1, 6–10% in H4) and stronger in summer and autumn (~20–30% of remaining ^{15}N in H2 and H3 (Fig. 2)), as was also found in monocultures (Byers et al. 2024).

Mean values of 4 replicates ($\pm$ SE), weeds excluded. Letters indicate post hoc Tukey groupings ($\alpha=0.05$). Both realized and expected yields are shown for mixtures. Monocultures' values adapted from Byers et al. (2024).

Mean percent increase of 4 replicates relative to expected values ($\pm$ SE). Note that probability values (p) of one-sided t-tests for positive overyielding were calculated on absolute differences rather than percentages. Also: Percent of ^{15}N label recovered in harvested herbage in total, and due to overyielding. H1: spring 2017; H2: summer 2017; H3: autumn 2017, H4: spring 2018.

The growing season average N concentration (Eq. 2) of the mixtures was ~2.6% of DM, closer to that of clover monocultures than tall-growing grasses, and consequently the mixture N yields (~25 g N m⁻²) were similar to the N yields of the clovers (Table 1). Consequently, the average ratio of ^{15}N per total N in both mixtures was significantly smaller than in the monocultures of ryegrass, timothy and meadow fescue, similar to that of bluegrass and somewhat higher than in clovers (Table 1). Notice that reputed deep rooting tall fescue in monoculture had a lower ^{15}N per total N ratio than ryegrass. On average the uptake of ^{15}N per herbage DM in Mix 4 was nearly the same as in the best monocultures (ryegrass, timothy and meadow fescue), while in Mix 10 it was lower, yet about 50% greater than in clovers.

Species diversity effects contributing to overyielding

The diversity effects (Eq. 4) express the species' contribution to mixture overyielding (Eq. 8). Those on DM and N yields were mostly positive or neutral, except in the drought affected summer harvest (Fig. 3A–B, *p*-values available in Suppl. Table S2, cumulative values for the growing season (H1_H3 available in table Suppl. Table S1 A & B). Ryegrass followed by red clover showed the strongest positive

diversity effects on DM and N yields. In the autumn harvest also the fescues and timothy contributed to total DM and N overyielding. Timothy, which in monoculture had the best spring yield (Byers et al. 2024), responded negatively in mixtures in the spring harvests and generally showed negative or non-significant diversity effects on N yield. Bluegrass and white clover were found in small amounts in Mix 10 and no substantial diversity effects on DM or N yield could be discerned.

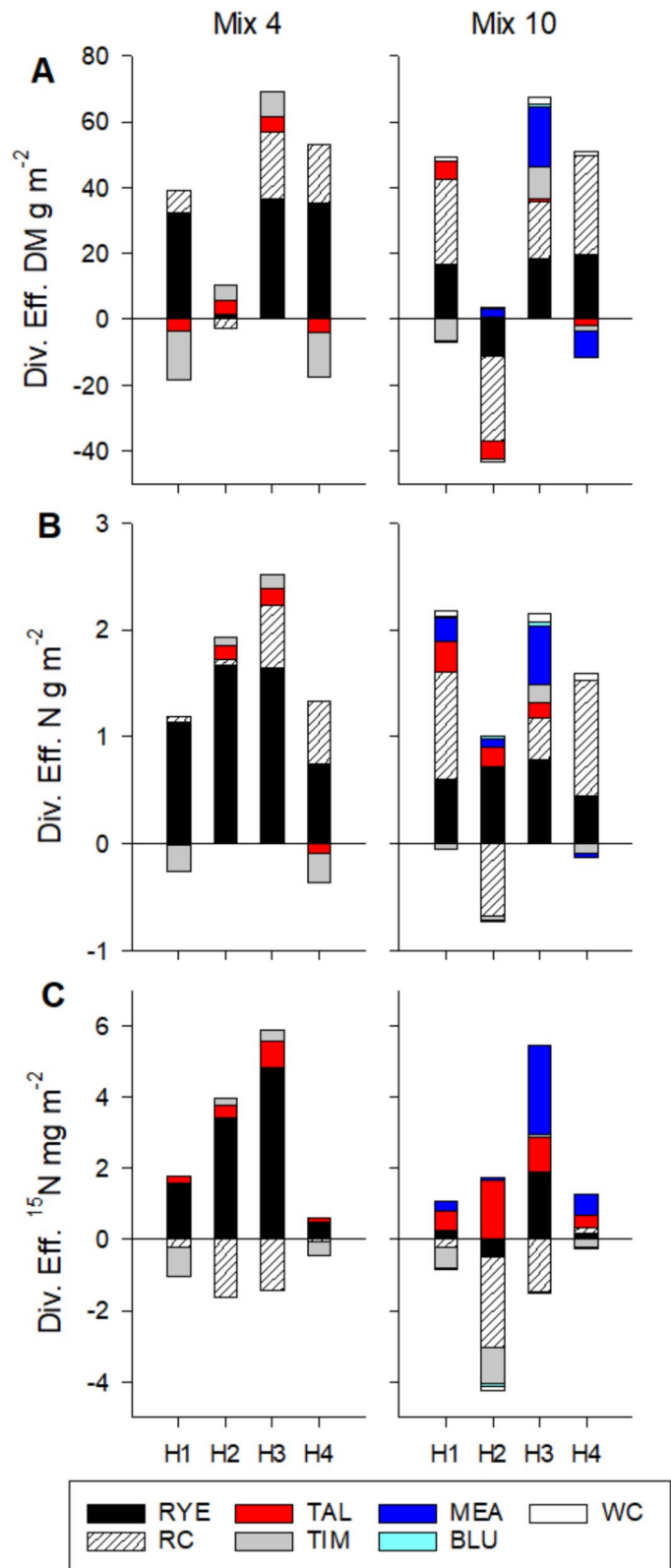
The diversity effects on ^{15}N uptake (Eqs. 4 and 5) discriminated species with positive response, ryegrass, tall and meadow fescue, from other species which showed overall negative responses, mainly red clover and timothy (Fig. 3C, Suppl. Table S2). Ryegrass dominated the positive diversity effects on ^{15}N recovery in Mix 4, whereas it contributed little in Mix 10, where the fescues together had the most positive effects. White clover and bluegrass were scarcely present in Mix 10 and expressed no or small negative diversity effects.

Diversity effects on N and ^{15}N concentrations in the herbage and N acquisition profile

The mixture components exhibited clearly different responses in average total N and ^{15}N concentrations in the herbage yield of the whole growing season (H1 – H3) (Eq. 2, Fig. 4). The fescues and ryegrass combined increased total N concentration with enhanced ^{15}N uptake per DM, while Timothy, on the contrary, showed no benefit in N concentration and a decrease in ^{15}N uptake per DM. Short growing bluegrass showed a strong increase in total N concentration and a marked decrease in ^{15}N per DM. Clovers showed no overall response in N concentration and a decrease in ^{15}N uptake per DM.

To explore in more detail which factor contributed throughout the growing season to the diversity effect in deep ^{15}N uptake we examined its relationship with the diversity effects RDM or RDUI at each harvest (^{15}N per DM or per N, weighted per ^{15}N remaining in the soil, Eqs. 6 and 7), as well as with diversity effects on DM and N yield (Fig. 5). Changes in RDUI can give an indication of how the presence of N-fixing clover and competition with other grasses influences the N uptake profile in the soil. The species showed distinct patterns: tall fescue most often exhibited an increase in RDUI, although the effect was significant

Fig. 3 Species' diversity effects contributing to overyielding in DM yield (g m^{-2}), N yield (g N m^{-2}) and total ^{15}N herbage uptake ($\text{mg } ^{15}\text{N m}^{-2}$) in Mix 4 and Mix 10, by harvest. Mean values of 4 replicates. Standard errors and significance of two-sided t-tests are shown in Suppl. Table S2. RYE: perennial ryegrass; TIM: timothy; TAL: tall fescue; MEA: meadow fescue; BLU: bluegrass; RC: red clover; WC: white clover. H1: spring 2017; H2: summer 2017; H3: autumn 2017, H4: spring 2018



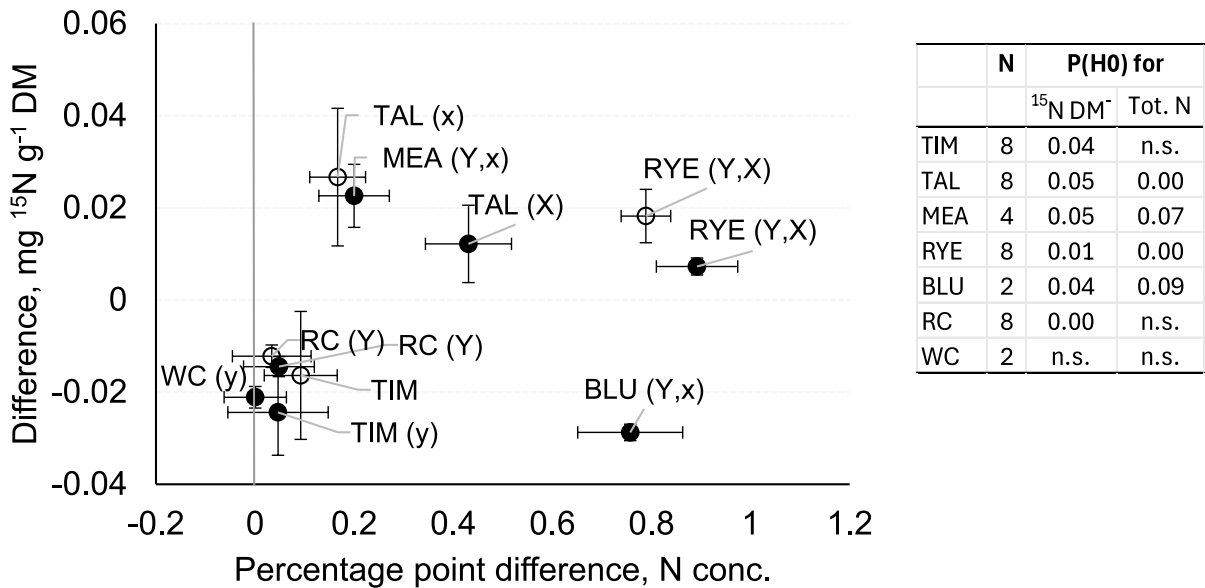


Fig. 4 Diversity effect (value in mixture – value in monocultures) on deep ^{15}N uptake per herbage biomass ($\text{mg } ^{15}\text{N g}^{-1}\text{ DM}$) versus diversity effect on total N concentration (%). Weighted means of three consecutive harvests (H1, H2 and H3) in Mix 4 (open circles) and Mix 10 (filled circles), $\pm$ standard error. In parentheses: statistical significance in the x and/or y direction (two-sided t-test, capital letters indicate $p \leq 0.05$, small letters indicate $0.5 < p < 0.1$). Species name abbreviations as in Fig. 3.

Some species were found in fewer than 4 replicates in Mix 10: tall fescue (3 replicates in H1), meadow fescue (2 in H1, 3 replicates in H2), bluegrass (2 in all 3 harvests), and white clover (3 in H1, 2 replicates in H3). Figure table shows probability for H0 with species pooled from both mixtures, when relevant. (See Suppl. Table S1 A and B for values and probability of diversity effects)

only for the cumulative data of the whole growing season, and timothy and blue grass showed a significant reduction in RDUI, while ryegrass and meadow fescues varied in their response (Fig. 5C, Suppl. Table S2, Suppl. Fig. S2). However, niche differentiation as indicated by RDUI did not explain the diversity effects on the amount of ^{15}N taken up, which was greatest in ryegrass as it combined enhanced total N concentration with enhanced RDM.

Considering RDM, positive (most samples of tall fescue and ryegrass, some samples of meadow fescue) and negative (timothy, bluegrass and clovers) diversity often corresponded with positive and negative diversity effects on ^{15}N uptake (Fig. 5D, Suppl. Table S2, Suppl. Fig. S2). However, RDM alone could not predict the size of the diversity effect on ^{15}N uptake, which depended also on the diversity effect on DM yield, (Fig. 5B and D). This will be analyzed more deeply in the Discussion.

Soil mineral N

The soil mineral N was measured at the beginning and end of the growing season; in the mixtures it was low ($< 1.8 \text{ g m}^{-2}$ in the upper 43 cm soil layer in the spring and $< 1.3 \text{ g m}^{-2}$ in the upper 60 cm in autumn), about the same as in grass monocultures and about half that of clover monocultures (Suppl. Table S2).

Discussion

Mixtures overyielding

In total, the grass-clover mixtures surpassed the weighted yields of their components with respect to biomass and nitrogen yield as well as deep ^{15}N uptake, confirming the overyielding Hypotheses 1 and 2 (Table 1). The forage crude protein yield also

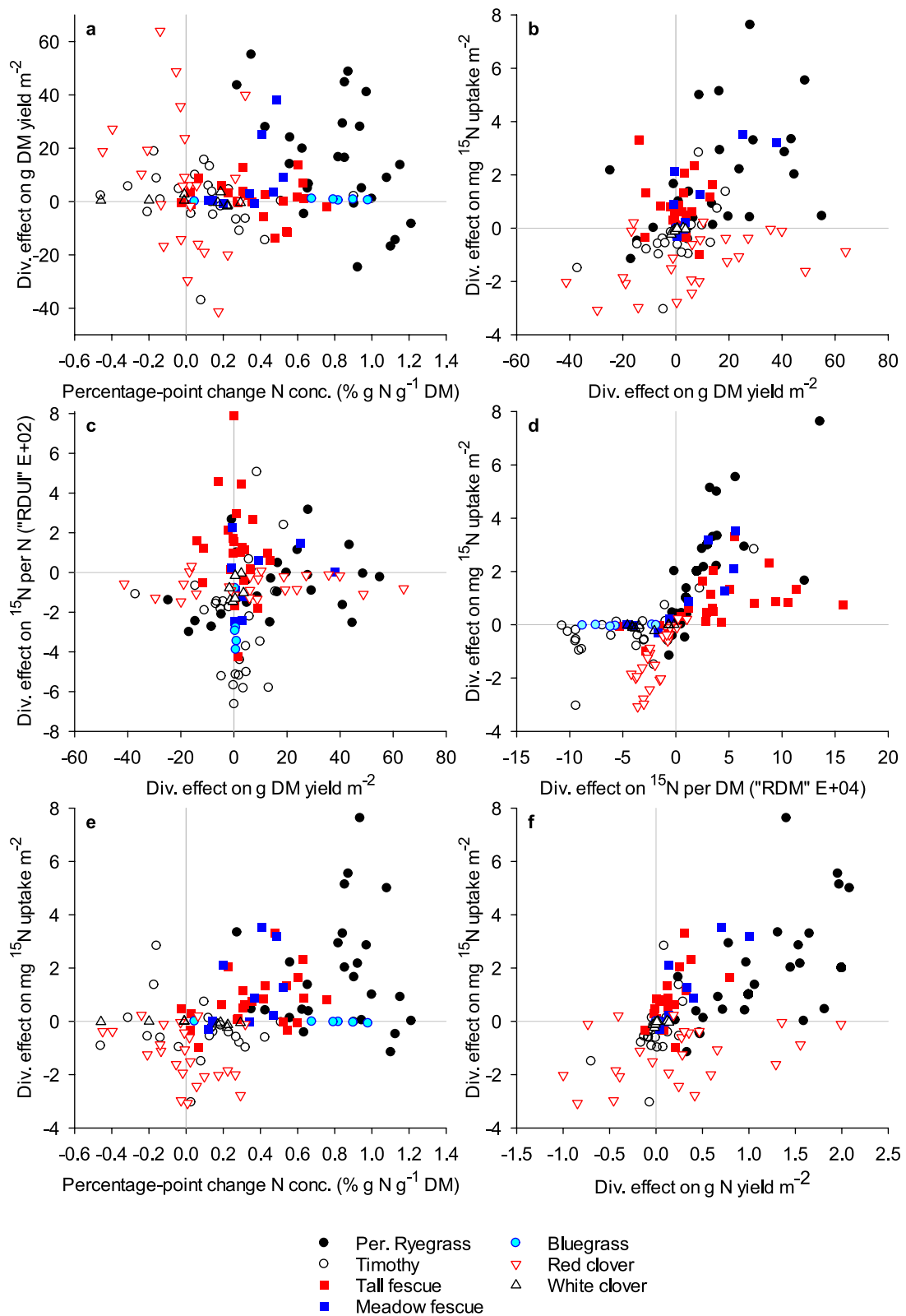


Fig. 5 Relationships between selected diversity effects (*realized—expected* values, Δ for brevity) on: (A) Δ DM yield (g m^{-2}) versus Δ N concentration in DM (percentage-point change); (B) Δ ^{15}N uptake (mg m^{-2}) versus Δ DM yield (g m^{-2}); (C) Δ RDUI ($\text{g}^{-1} \text{m}^2 \cdot \text{E03}$) versus DM yield (g m^{-2}); (D) Δ ^{15}N uptake (mg m^{-2}) versus Δ RDM ($\text{g}^{-1} \text{m}^2 \cdot \text{E04}$); (E) Δ ^{15}N uptake (mg m^{-2}) versus Δ N concentration in DM (percentage-point change); (F) Δ ^{15}N herbage uptake (mg m^{-2}) versus Δ N yield (g m^{-2}). Values for all replicates of both mixtures at each of three harvests (H1–H3). Corresponding graphs with symbols per-harvest are in Suppl. Fig. S2

increased since it is directly proportional to N content. Even though the mixtures did not achieve transgressive annual overyielding in any of these traits, the mixtures were superior to the monocultures as they combined the best DM and N yields and deep ^{15}N uptake in one sward. Positive DM and N overyielding of well assorted grass and clover species is well documented (Kirwan et al. 2007; Ergon et al. 2016; Hooper et al. 2005; Jørgensen et al. 2023). Our study demonstrates that the exposure to competition by other species can promote vertical root differentiation and enhance the recovery of deep mineral N even when the N nutritional state of the plants is substantially increased by N-fixing legumes, confirming Hypothesis 3a and rejecting Hypothesis 3b as discussed in more detail later.

Noteworthy, in the autumn, when grass species in monoculture showed the most severe N deficiency (Byers et al. 2024), ^{15}N overyielding was transgressive and surpassed N overyielding (Fig. 2). In monocultures the ^{15}N uptake increased during the growing season (Byers et al. 2024). In addition to this, ^{15}N overyielding was largest in the autumn (47%–62% more than expected) (Fig. 2). This indicates a development of roots' vertical niche differentiation during the growing season which harmonizes with the fact that during the winter many tillers and associated roots die, and new roots must develop from new tillers in the spring. This was well exemplified by poor winter hardy ryegrass.

It is reasonable to assume that the positive complementarity effect observed on $^{15}\text{NH}_4^+$ uptake would also apply to the more mobile NO_3^- . Thus, the overyielding in ^{15}N recovery indicates a heightened ability of mixtures to recover nutrients and in particular mineral N leached below the ploughed layer. Soil analysis in the early spring and at the end of the growing season in autumn confirmed that mixtures exploited

soil mineral N similarly to the grasses and better than the clovers (Suppl. Table S2). Thus, the grasses in the mixtures alleviated the exacerbating effect clovers have on N leaching and residual mineral N in the soil, which is also expected to impact the emissions of the greenhouse gas nitrous oxide, N_2O . This is important especially in the autumn, because winter freeze–thaw enhances N_2O formation (Bleken et al. 2022; Byers et al. 2021), and the winter period contributes a majority of total annual emissions under hemiboreal conditions (Wagner-Riddle et al. 2017).

Factors contributing to overyielding, other than nitrogen

Synergy, complementarity and facilitation other than N uptake

The three overyielding parameters considered, DM, total N and deep ^{15}N uptake, are intrinsically interconnected in addition to being focal for the agronomic outcome and N losses. Synergy, complementarity and facilitation effects contribute to overyielding (Hooper 1998) and thus enhance N uptake, e.g. increased light interception and use efficiency due to better foliar architecture and temporal distribution. In our experiment there was also a marked reduction of foliar diseases which affected grasses in the autumn (data not shown).

Facilitation was also observed in the less winter hardy ryegrass and white clover, which survived the winters better when grown in mixtures, where hardy species modified the microclimate and provided protection from frost damage and also from early drought when the soil was cold ($< 5^\circ \text{C}$) and the evaporative demand high due to strong radiation and wind. The latter was corroborated by soil moisture measurements before the onset of the growing season (data not shown, notice that dead leaves of ryegrass and clovers in monoculture had mostly decomposed under the snow and left the soil largely barren). By contrast, winter hardy timothy, which in the year of the labeling experiment (third production year) had high DM and N yields and highest ^{15}N uptake among monocultures (Byers et al. 2024), it was outcompeted by ryegrass when grown in mixtures. This illustrates the continuous dynamics in a mixture, where diversity effects that contribute to overyielding lead also to competition and potentially to the depression of some

species, with environmental conditions influencing the outcome (Cardinale et al. 2007).

The fact that mixtures overyielded despite strong dominance by red clover in Mix 10 and by ryegrass and red clover in Mix 4 (Fig. 1) is in accordance both with the theory that diversity effects outweigh species dominance over time (Fargione et al. 2007), and with previous observations that although productivity generally increases with species diversity, the choice of species may be more important than the number (Hooper et al. 2005; Jørgensen et al. 2023). Slowly establishing species were rapidly outcompeted in Mix 10 (i.e. smooth brome grass and bluegrass), and so was alfalfa for which the inoculum with N-fixing rhizobium had failed, leaving patches open for weeds invasion. Thus, the mixing of slowly with rapidly establishing grasses may not be an advantage (Jørgensen et al. 2023), contrary to the generalized expectation that functional trait dispersion increases resource capture and weed control in forage (Hofer et al. 2017). Our experiment was established in early summer without a cover crop, contrary to locally recommended practice of sowing leys under a spring cereal, and this probably favored the faster establishing species.

Diversity effects on N uptake and vertical niche differentiation

Biological nitrogen-fixation – N supply

The presence of N-fixing legumes increased the N yield of the mixtures compared to the average of the grasses by nearly 10 g N m⁻². About 4–5 g N m⁻² came from increased N concentration in the grass, similarly to results from Ergon et al. (2016) and Nyfeler et al. (2011). The increased N concentration when grown with clovers can itself enhance growth. However, only in ryegrass and meadow fescue a positive diversity effect on N concentration was mostly associated with a positive diversity effect on DM yield (Fig. 5A). Light competition was probably a limiting factor for other grasses.

Contribution of increased biomass or increased ¹⁵N concentration to ¹⁵N uptake

Diversity effects on DM yield or ¹⁵N concentration in the herbage can both contribute to ¹⁵N overyielding.

RDM measures the ¹⁵N concentration weighted per remaining ¹⁵N in the soil. In general, positive (fescues and ryegrass) and negative effects (mainly red clover but also timothy) on RDM corresponded with positive and negative diversity effects on ¹⁵N uptake (Fig. 5D). Furthermore, the diversity effect on RDUI was very specific and apparently unrelated to the diversity effect on plant vigor (Fig. 5C). These responses show a species-specific change in the deep N acquisition: a preference for deeper N uptake by tall fescue, and the opposite by timothy, while ryegrass and meadow fescue often combined enhanced total N uptake with enhanced deep N uptake.

However, the effect size on deep ¹⁵N uptake depended on the effect on plant vigor and thus biomass production, which played an important role in the case of ryegrass and meadow fescue but not tall fescue (Fig. 5B). This is illustrated also by red clover for which the relatively strong negative diversity effect on deep ¹⁵N uptake depended on increased plant vigor combined with a minor decline in RDM.

The contribution of temporal complementarity (Husse et al. 2017, 2016) is supported by the fact that during the growing season variations in diversity effects on ¹⁵N uptake in mixtures and ¹⁵N recovery in monoculture were not synchronous (Suppl. Table S2).

N nutrition and vertical niche differentiation

We suspected (Hypothesis 3b) that better N nutrition due to the presence of N-fixing clovers would eliminate the competitive pressure for deep N uptake and weaken the recovery of deep ¹⁵N. This was disproved in our mixtures for the fescues and ryegrass, which combined a positive diversity effect on concentration and yield of total N with a positive effect on deep N uptake (Fig. 5E and 5F).

These results indicate that nitrogen deficiency by itself was not fundamental for the diversity effects on deep ¹⁵N acquisition. This agrees with observations by Husse et al. (2017), and Mommer et al. (2010) who attributed changes in root distributions to inter- and intraspecific root recognition rather than nutritious cues and echoes the conclusion by Hoekstra et al. (2015) that including legumes reduces N limitation and allows for enhanced root exploration and nutrient uptake by other species. Ryegrass benefited the most, as it combined the strongest alleviation of N deficiency with the strongest increase in deep N uptake

both in the summer and autumn harvests, confirming its competitiveness for N (Maire et al. 2009).

We used the parameter RDUI to estimate the uptake of deep N relative to total N uptake. A positive diversity effect on RDUI indicates a niche effect towards deeper root activity, especially when the total N concentration in the shoot DM also increases. Vice versa, a negative diversity effect indicates a niche effect towards shallower root activity. Tall fescue exhibited the clearest niche differentiation towards deeper root activity, as indicated by a positive diversity effect on RDUI (Fig. 5C and D) concomitant with an increase in total N (Fig. 4), especially in summer and autumn. Timothy, on the other hand, responded with a shallower N uptake profile (negative diversity effect on RDUI) and no overall effect on total N concentration, almost a specular behavior to that of tall fescue (Fig. 5C and D). Bluegrass showed an even stronger niche response towards shallower N uptake, as it strongly increased its N concentration despite being the grass species with the highest N concentration in monoculture, and decreased ^{15}N uptake per DM (RDM, Figs. 4 and 5A, C, D). Interestingly, the ability to recover deep ^{15}N shown by the grasses in monoculture was not a good predictor of the species response to competition: in monoculture timothy had the largest deep ^{15}N uptake of all species and its preference for deep N, indicated by the whole season $^{15}\text{N}/\text{N}$ ratio, was larger than in tall fescue ($P < 0.1$) and significantly larger than in bluegrass (Table 1). In conclusion, differentiation of root activity towards relatively deeper or shallower N uptake is species-specific and it is not easily related to root performance in monoculture.

As expected, red clover consistently recovered less deep ^{15}N in mixture than in monoculture. However, non-N-fixing timothy and bluegrass showed a more negative diversity effect on RDUI than red clover. This can indicate an increased reliance of red clover on non-deep sources such as BNF (see effects on RDUI in H2 and H3, Suppl. Fig. S3 S4.), in line with observations by Pirhofer-Walzl et al. (2013), but it is also compatible with the fact that the persistent tap-root contributes to uptake of deep N (Husse et al. 2017) and water (Hoekstra et al. 2014) when exposed to competition by grass species. Also, NO_3^- and water are more easily taken up by thicker roots than NH_4^+ , which can influence the competition between clover and grass species with thinner roots. Maire

et al. (2009) observed that timothy is less competitive for NH_4^+ than perennial ryegrass but more competitive for NO_3^- and NH_4^+ than tall fescue. Roots of ryegrass are characterized as thin, while tall fescue has deep thick roots adapted to water transport.

Notice that niche differentiation was only a partial contributor to the mixture overyielding in total N uptake, and by itself was insufficient to explain a species' contribution to deep N overyielding, as illustrated by tall fescue, ryegrass, timothy and bluegrass (Fig. 5, compare C with D–F). Ryegrass showed a predominantly negative effect on the RDUI, due to a relatively larger gain in total N from the upper soil horizon(s), in line with observation by Mamolos et al. (1995) on the behavior of the most competitive species after surface application of mineral N. Also, in the case of meadow fescue, the diversity effect on RDUI was at times negative. Nevertheless, ryegrass contributed to most of the 15 N overyielding of Mix 4 and, when present, meadow fescue consistently contributed positively to 15 N overyielding.

About the methodology

The $^{15}\text{NH}_4^-$ adsorbed to clinoptilolite was mainly released and transported upwards by plant activity, with relatively little loss of label out of the soil–plant system. On average, after two growing season 88% of applied ^{15}N was accounted for in the herbage plus soil above and around the clinoptilolite in the tall growing grasses monocultures (Byers et al. 2024). This enabled us to follow the diversity effects of deep roots activity during several successive harvests, which was important as deep N uptake increased during the growing season. A challenge is given by the fact that treatments affect the amount of ^{15}N present in the soil after the first harvest. To account for this, all by-harvest diversity effects related to ^{15}N were calculated relative to the ^{15}N assumed remaining in the soil. Thus, if leaching took place, our method underestimated the effective recovery in later harvests of the label present in the soil. Notice however, that the diversity effects cumulated over the whole growing season were related to the single label application in the spring, which gives a fair comparison also in the case of losses by leaching.

Seldom the space occupied by the components corresponds to the sown seed proportions. Therefore, we used attained species proportions as a proxy for the space occupied in the mixture. This enabled

to split the factors contributing to deep ^{15}N diversity effects between impacts on plant vigor or ^{15}N concentration in the herbage and analyze separately how they related to changes in total N concentration. Thus, our approach for estimating diversity effects and overyielding differs from that based on seed proportions at sowing (Ergon et al. 2016, Kirwan et al. 2009 and Nyfeler et al. 2011), which is linked to the concept of replacing a group of monocultures. Although easy to grasp, the rationale of that approach can be questioned in the case of species which are not suitable for use in monoculture, e.g. the clovers. A drawback of our method is the labor needed for sorting each harvested sample in its entirety.

Agronomical relevance

Timothy and meadow fescue are the most commonly used grass species in areas with challenging winter conditions in Fennoscandia and Canada. Recently Jørgensen et al. (2023) confirmed the high agronomic value of traditional mixtures with timothy, meadow fescue and red clover in climatically contrasting environments in Norway and questioned the advantage of including perennial ryegrass. However, they did not study the recovery of deeply placed N, nor did they present the results of mixtures with ryegrass to support their statement. In the present study, perennial ryegrass had a pivotal role in the recovery of deep N in the grass-clover mixtures, and its contribution increased during the growing season, potentially moderating N dissipation while increasing high-quality forage production with moderate application of N fertilizer. Thus, our results support a cautious inclusion of perennial ryegrass in clover-grass leys in combination with the more winter hardy timothy. Tall fescue proved capable of recovering N that has leached below the ploughed layer, but its value in ley mixtures is curtailed by a relatively low palatability and digestibility, therefore its use is more adapted in areas where its drought tolerance is most beneficial (Cougnon et al. 2014).

Conclusions

Mixtures of grasses and clovers overyielded in deep N uptake, herbage biomass and total N yield. N-fixing legumes increased the N-yield of the leys by about

10 g m^{-2} without reducing the ability of the leys to recover deep mineral N. Enhanced uptake of deeply placed mineral N coexisted with enhanced N nutrition brought by the presence of N fixing clovers.

Stimulation of deeper N uptake depended on both the species-specific root responses and growth vigor. Niche differentiation was found towards deeper root N uptake in tall fescue, and towards shallower uptake in timothy and Kentucky bluegrass. Ryegrass growth was strongly facilitated through a better overwintering when grown in mixture and proved to be as deep-rooted as tall and meadow fescue when in competition with them, and more competitive for N and thus a major contributor to overyielding in both total N and deep N uptake. Timothy displayed low competitiveness for N and especially deep N when grown in mixture. It was confirmed that bluegrass and white clover have a shallow root system compared to common tall ley species and that red clover has a higher uptake of deep N than white clover.

Niche differentiation of N uptake and facilitation of species with a strong competition for N can play an important role in preventing N losses from legume-grass mixtures. To exploit this effect properly, a targeted choice of species and cultivars is necessary. Our study with species of highest agricultural value under hemi-boreal conditions indicates that functional traits as observed in monoculture are not always good predictors of the diversity effects realized in mixtures and supports a careful introduction of less winter hardy ryegrass in mixtures in this region.

To our knowledge, this is the first study applying a slow-release ^{15}N label to explore root niche differentiation in response to competition pressure by roots of other species, including N-fixing legumes, during the whole production season. We opted to study some of the species with highest agricultural value under hemi-boreal conditions. Deep root activity increased from spring to autumn and the mixtures overyielded on deep N uptake was largest in autumn. This seasonal effect has received little attention and deserves further studies.

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Author contributions Erin Byers, carried out field and lab work included ^{15}N analysis, performed synthesis/results analyses and was the primary contributor to writing and editing the manuscript. Marina A. Bleken found financial support, contributed the conception of the experimental methods and experiment design, initiated the field trial, participated to field and lab work, data statistical analysis and interpretation, and was responsible for the final writing and editing. Peter Dörsch provided the competence for the ^{15}N analysis method via IRMS and contributed to writing and editing. Susanne Eich-Greatorex contributed to conceiving the ^{15}N label application method, prepared the label, help in the label application and contributed to revising the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. A permanent dataset is under preparation – and will be available at <https://dataverse.no/dataverse/nmbu>.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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References

- Bessler H, Temperton VM, Roscher C, Buchmann N, Schmid B, Schulze E-D, Weisser WW, Engels C (2009) Above-ground overyielding in grassland mixtures is associated with reduced biomass partitioning to belowground organs. *Ecology* 90:1520–1530. <https://doi.org/10.1890/08-0867.1>
- Bleken MA, Rittl TF, Nadeem S, Hansen S (2022) Roots and other residues from leys with or without red clover: quality and effects on N_2O emission factors in a partly frozen soil following autumn ploughing. *Sci Total Environ* 831:154582. <https://doi.org/10.1016/j.scitotenv.2022.154582>
- Byers E, Bleken MA, Dörsch P (2021) Winter N_2O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH. *Environ Res Commun* 3:015001. <https://doi.org/10.1088/2515-7620/abd623>
- Byers E, Dörsch P, Eich-Greatorex S, Bleken MA (2024) Deep n acquisition in cultivated grasslands: uptake of slow-release ^{15}N -labeled ammonium in hemiboreal monospecific leys. *Plant Soil* 499:393–408. <https://doi.org/10.1007/s11104-023-06455-z>
- Cahill JF, McNickle GG (2011) The behavioral ecology of nutrient foraging by plants. *Annu Rev Ecol Evol Syst* 42:289–311. <https://doi.org/10.1146/annurev-ecolsys-102710-145006>
- Cardinale BJ, Wright JP, Cadotte MW, Carroll IT, Hector A, Srivastava DS, Loreau M, Weis JJ (2007) Impacts of plant diversity on biomass production increase through time because of species complementarity. *Proc Natl Acad Sci U S A* 104:18123–18128. <https://doi.org/10.1073/pnas.0709069104>
- Cougnon M, Baert J, Van Waes C, Reheul D (2014) Performance and quality of tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) and mixtures of both species grown with or without white clover (*Trifolium repens* L.) under cutting management. *Grass Forage Sci* 69:666–677. <https://doi.org/10.1111/gfs.12102>
- Ergon Å, Kirwan L, Bleken MA, Skjelvåg AO, Collins RP, Rognli OA (2016) Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies: 1. dry-matter yield and dynamics of species composition. *Grass Forage Sci* 71:667–682. <https://doi.org/10.1111/gfs.12250>
- IUSS Working Group WRB (2015) World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome. <https://openknowledge.fao.org/server/api/core/bitstreams/bcdecec7-f45f-4dc5-beb1-97022d29fab4/content>
- Fargione J, Tilman D, Dybzinski R, Lambers JHR, Clark C, Harpole WS, Knops JMH, Reich PB, Loreau M (2007) From selection to complementarity: shifts in the causes of biodiversity–productivity relationships in a long-term biodiversity experiment. *Proc R Soc Lond B Biol Sci* 274:871–876. <https://doi.org/10.1098/rspb.2006.0351>
- Finn JA, Kirwan L, Connolly J, Sebastià MT, Helgadottir A, Baadshaug OH, Bélanger G, Black A, Brophy C, Collins RP, Čop J, Dalmannsdóttir S, Delgado I, Elgersma A, Fothergill M, Frankow-Lindberg BE, Ghesquiere A, Golinska B, Golinski P, Grieu P, Gustavsson A-M, Höglind M, Huguenin-Elie O, Jørgensen M, Kadziuliene Z, Kurki P, Llurba R, Lunnán T, Porqueddu C, Suter M, Thumm U, Lüscher A (2013) Ecosystem function enhanced by combining four functional types of

- plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. *J Appl Ecol* 50:365–375. <https://doi.org/10.1111/1365-2664.12041>
- Hernandez P, Picon-Cochard C (2016) Presence of *Trifolium repens* Promotes Complementarity of Water Use and N Facilitation in Diverse Grass Mixtures. *Front Plant Sci* 7. <https://doi.org/10.3389/fpls.2016.00538>
- Hodge A (2004) The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytol* 162:9–24. <https://doi.org/10.1111/j.1469-8137.2004.01015.x>
- Hoekstra NJ, Finn JA, Hofer D, Lüscher A (2014) The effect of drought and interspecific interactions on depth of water uptake in deep- and shallow-rooting grassland species as determined by $\delta^{18}\text{O}$ natural abundance. *Biogeosciences* 11:4493–4506. <https://doi.org/10.5194/bg-11-4493-2014>
- Hoekstra NJ, Suter M, Finn JA, Husse S, Lüscher A (2015) Do belowground vertical niche differences between deep- and shallow-rooted species enhance resource uptake and drought resistance in grassland mixtures? *Plant Soil* 394:21–34. <https://doi.org/10.1007/s11104-014-2352-x>
- Hofer D, Suter M, Buchmann N, Lüscher A (2017) Nitrogen status of functionally different forage species explains resistance to severe drought and post-drought overcompensation. *Agric Ecosyst Environ* 236:312–322. <https://doi.org/10.1016/j.agee.2016.11.022>
- Hooper DU (1998) The role of complementarity and competition in ecosystem responses to variation in plant diversity. *Ecology* 79:704–719. [https://doi.org/10.1890/0012-9658\(1998\)079\[0704:TROCAC\]2.0.CO;2](https://doi.org/10.1890/0012-9658(1998)079[0704:TROCAC]2.0.CO;2)
- Hooper DU, Chapin FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B, Setälä H, Symstad AJ, Vandermeer J, Wardle DA (2005) Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecol Monogr* 75:3–35. <https://doi.org/10.1890/04-0922>
- Husse S, Huguenin-Elie O, Buchmann N, Lüscher A (2016) Larger yields of mixtures than monocultures of cultivated grassland species match with asynchrony in shoot growth among species but not with increased light interception. *Field Crops Res* 194:1–11. <https://doi.org/10.1016/j.fcr.2016.04.021>
- Husse S, Lüscher A, Buchmann N, Hoekstra NJ, Huguenin-Elie O (2017) Effects of mixing forage species contrasting in vertical and temporal nutrient capture on nutrient yields and fertilizer recovery in productive grasslands. *Plant Soil* 420:505–521. <https://doi.org/10.1007/s11104-017-3372-0>
- Jing J, Sjøgaard K, Cong W-F, Eriksen J (2017) Species diversity effects on productivity, persistence and quality of multispecies swards in a four-year experiment. *PLoS ONE* 12:e0169208. <https://doi.org/10.1371/journal.pone.0169208>
- Jørgensen M, Bakken AK, Østrem L, Brophy C (2023) The effects of functional trait diversity on productivity of grass-legume swards across multiple sites and two levels of nitrogen fertiliser. *Eur J Agron* 151:126993. <https://doi.org/10.1016/j.eja.2023.126993>
- Kirwan L, Lüscher A, Sebastià MT, Finn JA, Collins RP, Porqueddu C, Helgadottir A, Baadshaug OH, Brophy C, Coran C, Dalmannsdóttir S, Delgado I, Elgersma A, Fothergill M, Frankow-Lindberg BE, Golinski P, Griew P, Gustavsson AM, Höglind M, Huguenin-Elie O, Iliadis C, Jørgensen M, Kadziulienė Z, Karyotis T, Lunnan T, Malengier M, Maltoni S, Meyer V, Nyfeler D, Nykanen-Kurki P, Parente J, Smit HJ, Thumm U, Connolly J (2007) Evenness drives consistent diversity effects in intensive grassland systems across 28 European sites. *J Ecol* 95:530–539. <https://doi.org/10.1111/j.1365-2745.2007.01225.x>
- Kirwan L, Connolly J, Finn JA, Brophy C, Lüscher A, Nyfeler D, Sebastià M-T (2009) Diversity–interaction modeling: estimating contributions of species identities and interactions to ecosystem function. *Ecology* 90:2032–2038. <https://doi.org/10.1890/08-1684.1>
- Kristensen HL, Thorup-Kristensen K (2004) Root growth and nitrate uptake of three different catch crops in deep soil layers. *Soil Sci Soc Am J* 68:529. <https://doi.org/10.2136/sssaj2004.0529>
- Lüscher A, Finn JA, Connolly J, Sebastià MT, Collins R, Fothergill M, Porqueddu C, Brophy C, Huguenin-Elie O, Kirwan L, Nyfeler D, Helgadóttir A (2008) Benefits of sward diversity for agricultural grasslands. *Biodiversity* 9:29–32. <https://doi.org/10.1080/14888386.2008.9712877>
- Lüscher A, Mueller-Harvey I, Soussana JF, Rees RM, Peyraud JL (2014) Potential of legume-based grassland-livestock systems in Europe: a review. *Grass Forage Sci* 69:206–228. <https://doi.org/10.1111/gfs.12124>
- Maire V, Gross N, da Silveira Pontes L, Picon-Cochard C, Soussana J-F (2009) Trade-off between root nitrogen acquisition and shoot nitrogen utilization across 13 co-occurring pasture grass species. *Funct Ecol* 23:668–679. <https://doi.org/10.1111/j.1365-2435.2009.01557.x>
- Malcolm BJ, Moir JL, Cameron KC, Di HJ, Edwards GR (2015) Influence of plant growth and root architecture of Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) on N recovery during winter. *Grass Forage Sci* 70:600–610. <https://doi.org/10.1111/gfs.12157>
- Mamolos AP, Veresoglou DS (2000) Patterns of root activity and responses of species to nutrients in vegetation of fertile alluvial soil. *Plant Ecol* 148:245–253. <https://doi.org/10.1023/A:1009890630391>
- Mamolos AP, Elisseeu GK, Veresoglou DS (1995) Depth of root activity of coexisting grassland species in relation to N and P additions, measured using nonradioactive tracers. *J Ecol* 83:643. <https://doi.org/10.2307/2261632>
- Mommer L, Van Ruijven J, De Caluwe H, Smit-Tiekstra AE, Wagemaker CAM, Joop Ouborg N, Bøgemann GM, Van Der Weerden GM, Berendse F, De Kroon H (2010) Unveiling below-ground species abundance in a biodiversity experiment: a test of vertical niche differentiation among grassland species: below-ground species distributions in a biodiversity experiment. *J Ecol* 98:1117–1127. <https://doi.org/10.1111/j.1365-2745.2010.01702.x>
- Nyfeler D, Huguenin-Elie O, Suter M, Frossard E, Lüscher A (2011) Grass–legume mixtures can yield more nitrogen than legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-symbiotic sources. *Agric Ecosyst Environ* 140:155–163. <https://doi.org/10.1016/j.agee.2010.11.022>
- Østrem L, Rapacz M, Larsen A, Dalmannsdóttir S, Jørgensen M (2015) Influences of growth cessation and

- photoacclimation on winter survival of non-native *Lolium-Festuca* grasses in high-latitude regions. *Environ Exp Bot* 111:21–31. <https://doi.org/10.1016/j.envexpbot.2014.10.008>
- Pirhofer-Walzl K, Eriksen J, Rasmussen J, Høgh-Jensen H, Sørensen K, Rasmussen J (2013) Effect of four plant species on soil ¹⁵N-access and herbage yield in temporary agricultural grasslands. *Plant Soil* 371:313–325. <https://doi.org/10.1007/s11104-013-1694-0>
- Powlson DS, Barraclough D (1993) Mineralization and Assimilation in Soil-Plant Systems. In: *Nitrogen Isotope Techniques*. Elsevier, pp 209–242. <https://doi.org/10.1016/B978-0-08-092407-6.50013-4>
- Ravenek JM, Bessler H, Engels C, Scherer-Lorenzen M, Gessler A, Gockele A, De Luca E, Temperton VM, Ebeling A, Roscher C, Schmid B, Weisser WW, Wirth C, de Kroon H, Weigelt A, Mommer L (2014) Long-term study of root biomass in a biodiversity experiment reveals shifts in diversity effects over time. *Oikos* 123:1528–1536. <https://doi.org/10.1111/oik.01502>
- Robinson D, Hodge A, Griffiths BS, Fitter AH (1999) Plant root proliferation in nitrogen-rich patches confers competitive advantage. *Proc R Soc Lond B Biol Sci* 266:431–435. <https://doi.org/10.1098/rspb.1999.0656>
- Schmid B, Hector A, Saha P, Loreau M (2008) Biodiversity effects and transgressive overyielding. *J Plant Ecol* 1:95–102. <https://doi.org/10.1093/jpe/rtn011>
- von Felten S, Schmid B (2008) Complementarity among species in horizontal versus vertical rooting space. *J Plant Ecol* 1:33–41. <https://doi.org/10.1093/jpe/rtn006>
- von Felten S, Niklaus PA, Scherer-Lorenzen M, Hector A, Buchmann N (2012) Do grassland plant communities profit from N partitioning by soil depth? *Ecology* 93:2386–2396. <https://doi.org/10.1890/11-1439.1>
- Wagner-Riddle C, Congreves K, Abalos D et al (2017) Globally important nitrous oxide emissions from croplands induced by freeze-thaw cycles. *Nat Geosci* 10:279–283. <https://doi.org/10.1038/ngeo2907>
- Weisser WW, Roscher C, Meyer ST, Ebeling A, Luo G, Allan E, Beßler H, Barnard RL, Buchmann N, Buscot F, Engels C, Fischer C, Fischer M, Gessler A, Gleixner G, Halle S, Hildebrandt A, Hillebrand H, de Kroon H, Lange M, Leimer S, Le Roux X, Milcu A, Mommer L, Niklaus PA, Oelmann Y, Proulx R, Roy J, Scherber C, Scherer-Lorenzen M, Scheu S, Tschardt T, Wachendorf M, Wagg C, Weigelt A, Wilcke W, Wirth C, Schulze E-D, Schmid B, Eisenhauer N (2017) Biodiversity effects on ecosystem functioning in a 15-year grassland experiment: patterns, mechanisms, and open questions. *Basic Appl Ecol* 23:1–73. <https://doi.org/10.1016/j.baee.2017.06.002>
- Wolff M, Thue-Hansen V, Grimenes AA (2021) Meteorologiske data for Ås 2020. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: <https://www.nmbu.no/forskning/grupper/meteorologiske-data>. Accessed 2022-12-08

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