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ORIGINAL PAPER

Leaching of dissolved organic and inorganic nitrogen from legume-based grasslands

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Abstract Leaching of dissolved inorganic N (DIN) and dissolved organic N (DON) is a considerable loss pathway in grassland soils. We investigated the white clover (*Trifolium repens*) contribution to N transport and temporal N dynamics in soil solution under a pure stand of white clover and white clover-ryegrass (*Lolium perenne*) mixed stand. The temporal white clover contribution to N leaching was analysed by ^{15}N incorporation into DIN and DON in percolating soil solution collected at 25-cm depth following white clover ^{15}N leaf labelling that was applied at different times during the growing season. The white clover contribution to N transport in the soil profile was investigated over 2 years by analysing ^{15}N in DIN and DON in percolating soil solution collected at 25-, 45- and 80-cm depth following ^{15}N leaf labelling of white clover. The results showed that clover was a source of both DIN and DON. White clover autumn deposition contributed the most to N leaching. The leaching of DIN from the white clover in pure stand exceeded that of the mixed stand and confirmed that leaching of DIN is a function of N loadings and N demand. The DON leaching was unaffected by the presence of a companion grass, suggesting that the DON leaching from our grassland derived from the lysis of soil microbial biomass living on recent white clover deposits. White clover contributed to the leaching of DIN and DON at all depths, and the fact that the contents of DI ^{15}N and DO ^{15}N did not change with depth indicated that surplus of DIN and DON, formed in the uppermost soil layer, was transported in the soil profile.

Keywords DIN · DON · ^{15}N · Leaching · White clover · Grasslands

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Introduction

Optimising the use efficiency of legume-derived nitrogen (N) is a way of tightening the N cycle and thus a step towards the needed enhancement of sustainability in agricultural production. The ability of legumes to biologically fix N_2 (BNF) in symbiosis with *Rhizobium* bacteria gives them a huge advantage in grassland ecosystems. The extent of BNF is regulated by N availability (Soussana and Tallec 2010) and the presence of companion species competing for soil N (Rasmussen et al. 2012). The important forage legume, white clover (*Trifolium repens*), is known to have a high complementary effect by donating N to companion grasses through above- and below-ground N transfer (Fustec et al. 2010; Høgh-Jensen and Schjoerring 2000) and by improving soil N fertility via deposition of fixed N to the soil (e.g. (Rasmussen et al. 2012). However, high N input and internal N flows increase the risk of undesirable N losses.

White-clover-derived N enters the soil through deposition that includes rhizodeposits (root exudates, lysates and dead root cell material) and plant decay. The deposition of N from white clover to the soil has been reported to range from 20 up to 300 kg N ha^{-1} $year^{-1}$ (Ledgard and Steele 1992). Deposits, depending on their origin and composition, can be assimilated and dissimilated by microbial community, taken up by plants or transported down the soil profile (Pinton et al. 2007). The dominance of each of those processes is season-dependent due to changes in temperature and soil solution content affecting plant activity (Bouchart et al. 1998). The greatest white clover N allocation to leaves is reported at high temperatures, whereas the highest N content was observed in roots and stolons at low temperatures (Bouchart et al. 1998). Seasonal variation of white clover N deposition and N transfer (Dahlin and Stenberg 2010; Rasmussen et al. 2013) likely leads to the variation of N pool sizes in the rhizosphere. It is widely recognised that leaching of dissolved inorganic N (DIN) is related to high N

loads into the soil, management and the sward botanical composition, so that high grass N yields and removal reduce N leaching (Vinther et al. 2006). However, management-independent patterns of oscillation in DIN leaching from grassland soils have been observed (Eriksen et al. 2010). Therefore, in the present study, we investigated whether seasonal variation of white clover N deposition was directly related to DIN leaching.

It is evident that N leaches in considerable amounts in both inorganic and organic forms (Perakis and Hedin 2002; Rasmussen et al. 2008) and white clover contributes to the leaching of both DIN and dissolved organic N (DON) below the plough layer (Rasmussen et al. 2008). Moreover, DON leaching has been reported even in N-limited environments (Perakis and Hedin 2002), suggesting that other factors besides plant and microbial N demand affect DON movement through the soil profile. There are two main models explaining DON leaching in soil—dynamic exchange and continual stripping—based on studies in mainly forest ecosystems (Neff et al. 2003; Scott and Rothstein 2014). The N dynamic exchange model (Scott and Rothstein 2014) suggests that recently deposited DON displaces previously sorbed DON that migrates in the soil profile and leads to a greater pool of older DON in deeper layers. DON leaching according to the continual stripping model can be explained by partitioning DON into mainly microbial-derived hydrophilic (Qualls and Haines 1991) and mainly plant-derived hydrophobic pools (Gu et al. 1995). Hydrophobic DON can be easily sorbed to mineral soil due to its high-molecular-weight and polymeric structure, whereas hydrophilic DON, due to its low molecular weight, is easy soluble and therefore remains in soil solution. Thus, according to this model, hydrophobic DON replaces hydrophilic DON that is preferentially transported in the soil profile. While leaching of DIN and DON from grassland soils has already been demonstrated (Rasmussen et al. 2008; Vinther et al. 2006), there is still a lack of information on to what extent white clover contributes to the transport of N in the soil profile and how the composition of grass-clover mixed stands may control the extent of leaching and the underlying controls of N dynamics in the soil profile.

The overall objective was to investigate the contribution of white clover to the leaching of DIN and DON in soil profile, with three specific aims:

- Firstly, to investigate white clover's temporal contribution to the soil solution N. We hypothesised that higher N deposition from white clover in autumn (Rasmussen et al. 2013) results in greater soil solution N loading than in spring or summer.
- Secondly, to investigate the fate of white clover N in the soil profile. The hypothesis was that DIN from the uppermost layer is transported down the soil profile in water, while the recently deposited DON pool decreases in size with depth as proposed by the dynamic exchange model (Scott and Rothstein 2014).
- Thirdly, to study the effect of a companion grass. The hypothesis was that due to N uptake by ryegrass and thus lower N loading in the soil, less DIN and DON would be prone to leaching under the mixed stand compared to the pure stand.

Therefore, two parallel experiments were conducted in a pure stand of white clover and a ryegrass-white clover mixed stand to investigate the white clover contribution to transport of N in the soil profile and temporal deposition of N. The white clover temporal contribution to N leaching was investigated by using ^{15}N leaf labelling of white clover that was applied at different times during the growing season ('time experiment'). The white clover contribution to N transport in the soil profile was investigated over 2 years using ^{15}N leaf labelling of white clover ('transport experiment'). The N_2 fixation by white clover was estimated in adjacent plots.

Materials and methods

Experimental site and conditions

The field experiments were conducted on a sandy loam at the Foulumgård Experimental Station, Viborg, Denmark (55° 28 N, 09° 07 E). The soil is classified as a Typic Hapladult with 6.4 % clay, 8.5 % silt, 44 % fine sand and 39 % coarse sand. The site has been used for dairy farming with a grassland-arable crop rotation since 1987 (Eriksen et al. 2014). The soil contained 1.7 % C and 0.16 % N. The mean temperature and precipitation (Fig. 1) were 9 °C and 654 mm in the first experimental season (May 2011–April 2012) and 7 °C and 694 mm in the second experimental season (May 2012–April 2013), respectively. The field experiment was established in April 2011 by installing PVC cylinders (ϕ 30 cm) in a

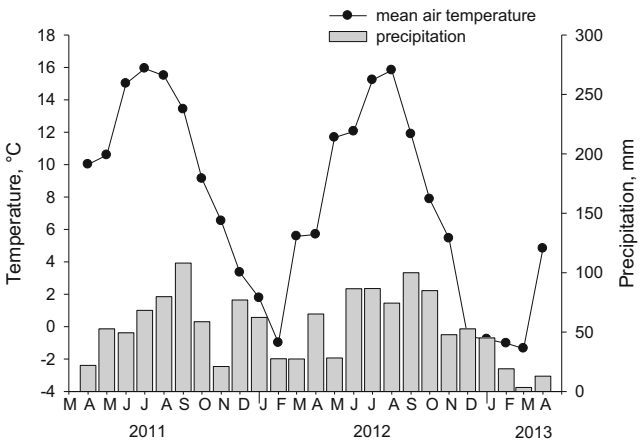

Fig 1 Monthly precipitation and mean air temperature during the experimental period

randomised block design with four replicates in pure stand of white clover (*T. repens* L., 6 kg ha^{-1}) and a ryegrass-white clover mixed stand (*L. perenne* L., 24 kg ha^{-1}, *T. repens* L., 4 kg ha^{-1}) seeded in April 2011. The PVC cylinders had a length allowing 5 cm of the cylinders to be above-ground. The cylinders were placed with a distance of at least 2 m in between.

Atmospheric N_2 fixation was estimated in adjacent plots with a size of 1 m^2. Plots were established in pure stands of white clover and ryegrass and in the ryegrass-white clover mixed stand. A ^{15}N-labelled $(NH_4)_2SO_4$ solution (60 at.% ^{15}N, 1.5 kg N ha^{-1}) was applied to the soil (Høgh-Jensen and Schjoerring 1994) before plant germination in April 2011. Plant shoots were harvested to a height of 5 cm two times in 2011 (July and October) and three times in 2012 (May, July and October). Plant material was divided into white clover and grass and analysed as described below.

Time experiment

White clover in PVC cylinders inserted at 20-cm depth was ^{15}N leaf-labelled (as described below) during the following four periods: autumn'11 (T1) in August–September 2011, spring'12 (T2) in April–May 2012, summer'12 (T3) in June–July 2012 and autumn'12 (T4) in August–September 2012. Harvests were conducted 2 weeks after the end of each labelling period. Sample preparation and analysis are described below.

The soil solution was sampled monthly from September to April at 25-cm depth using suction cups (as described below). Soil solution was collected during the two leaching seasons where cylinders had been labelled.

At the end of the experiment (May 2013), plant samples (shoots and roots) and soil were taken from all cylinders (total 32). Soil cores (one core per cylinder) were sampled with a soil auger (ϕ 8.75 cm) and divided into two sections (0–10 and 10–25 cm). Sample preparation and analysis are described below.

Transport experiment

White clover in PVC cylinders inserted at 20-, 40- and 75-cm depths was ^{15}N leaf-labelled (as described below) before each harvest over two growing seasons: (1) June–July and August–September in 2011 and (2) April–May, June–July and August–September in 2012.

The shoot biomass was harvested to a height of 5 cm 2 weeks after each labelling period. The shoot biomass was harvested two times in 2011 (August and October) and three times in 2012 (May, July and October). Sample preparation and analysis are described below.

Soil solution was sampled monthly from September to April at 25-, 45- and 80-cm depth during two leaching seasons. At the end of the 2-year experiment (May 2013), plant samples (shoots and roots) and soil were taken from the 75-cm length cylinders. Soil cores (one core per cylinder) were sampled with a soil auger (ϕ 8.75 cm) and divided into four sections (0–10, 10–25, 25–50 and 50–80 cm). Sample preparation and analysis are described below.

Labelling

The ^{15}N leaf labelling method (McNeill et al. 1997) was applied to introduce the ^{15}N tracer into the white clover. Briefly, a white clover leaf was inserted into a 2-mL vial with 1 mL of ^{15}N urea solution (99.6 at.% ^{15}N, 0.5 %*w*/*v*) and sealed with an inert plastic material (UNIGUM Sanitary putty, Unipak A/S, Galten, Denmark) to avoid ^{15}N loss. In each cylinder, four clover leaves were labelled per each labelling occasion. After 4 days of labelling, the tubes were removed and labelled leaves were dried with paper towels to avoid soil contamination by ^{15}N; the following four new leaves were then exposed to the labelling procedure. The same amount of ^{15}N was applied to each cylinder in each of the experiments with a total of 32 vials in the time experiment and 172 vials in the transport experiment.

Soil solution sampling

Soil solution was collected by Teflon suction cups (Prenart Super Quartz, pore size <1 μm, ϕ 21 mm, length 70 mm, Prenart Equipment Aps, Frederiksberg, Denmark) inserted 5 cm beneath the cylinders (Rasmussen et al. 2007). The preparation and installation of the sampling system were performed according to Grossmann and Udluft (1991). When sampling the soil solution, suction cups were connected to acid-washed 1-L glass bottles, and vacuum was applied (0.7 bars) for 2–3 days using a vacuum pump. Due to frost, sampling in February (2012) and March (2013) was not possible.

Sample preparation and analysis

Soil solution

Total DIN and total dissolved N (TDN) were determined in filtrated (0.6 μm, glass fibre filter) soil solution according to Ros et al. (2010). Briefly, 50 mL of soil solution was used to analyse DIN by adding 7.4 g of KCl, 0.2 g MgO and 0.4 g Devarda's reagent. A glass-fibre filter trap was acidified by adding 20 μL of 1.5 M H_2SO_4, packed in Teflon paper and placed in a bottle with the sample (Sørensen and Jensen 1991). The diffusion was processed for 5 days before the glass filter traps were dried and packed into tin capsules for ^{15}N and N measurement. Water samples for TDN analyses were treated

as for DIN after the oxidation of organically bound N to NO_3^- by 7.5-mL persulphate reagent and autoclaved at 121 °C for 30 min. Dissolved organic N was calculated as the difference between TDN and DIN (DON=TDN−DIN).

Plants and soil

Above-ground biomass (leaves and stolons) harvested during the experiment and below-ground biomass harvested at the end of the experiment (May 2013) were separated into ryegrass and white clover. Samples were dried at 60 °C, ground to a fine powder in a ball mill and packed into tin capsules for further analyses.

The soil cores sampled at the end of the experiment (May 2013) (sampling is described above) were manually separated and all visible roots collected. A subsample of the soil was gently washed in a 425-μm sieve and the remaining material washed at least three times to separate roots from soil mineral particles. Finally, non-root material was removed with tweezers.

A soil subsample was sieved (<2 mm), oven-dried at 60 °C, ground to a fine powder in a ball mill and packed into tin capsules for further analyses.

Analysis

The analysis of N in shoots, roots, soil and soil solution (DIN and TDN) and ^{15}N enrichment was carried out at the UC Davis Stable Isotope Facility (UC Davis, CA, USA) using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europe 20-20 isotope ratio mass spectrometer (Sercon Ltd. Cheshire, UK).

Statistics and calculations

The ^{15}N at.% excess of plant, soil and percolating soil solution was calculated as the difference between ^{15}N at.% in labelled plots and unlabelled controls sampled at the same time.

The percentage of N derived from the atmosphere (% Ndfa) was calculated according to McNeill et al. (1994):

$$\%\,\mathrm{Ndfa} = 1 - \frac{\text{white clover at. \% excess}}{\text{ryegrass at. \% excess}} \times 100\,\%$$

where white clover and ryegrass at.% excess is calculated by subtracting the ^{15}N at.% in ^{15}N dilution plots from the ^{15}N at.% in unlabelled (control) plots.

The specific concentration of ^{15}N incorporation into DIN and DON was calculated as the ratio between the contents of DIN and DI ^{15}N or DON and DO ^{15}N.

Statistical analyses were performed using R statistical software. Dry matter yields, N content and leaching of DIN and DON were analysed by a repeated measures analyses of variances using a linear mixed model, where (1) grassland composition (white clover pure stand and white clover-ryegrass mixed stand), depth (25, 45 and 80 cm) and sampling time were included as fixed effects in the transport experiment and (2) grassland composition (white clover pure stand and white clover-ryegrass mixed stand) and labelling time were included as fixed effects in the time experiment.

Results

In the time experiment, the white clover proportion of shoot biomass in the mixed stand was 31 and 34 % in 2011 and 2012, respectively. The average white clover biomass in the pure stand was significantly ($P<0.001$) higher than in the mixed stand, at 454±25 compared with 177±20 g DM m^{-2} $years^{-1}$, containing 16±1 and 6±0.8 g N m^{-2} $years^{-1}$, respectively. Average total harvested dry matter of ryegrass in the mixed stand was 344±20 g m^{-2} $years^{-1}$, containing 5±0.4 g N m^{-2} $years^{-1}$. Measurements of shoot biomass in the transport experiment showed that the white clover proportion in the mixed stand with ryegrass was 16 and 30 % in 2011 and 2012, respectively. Ryegrass in the mixed stand accumulated 171±16 g biomass m^{-2} $years^{-1}$, containing 4±0.2 g N m^{-2} $years^{-1}$, and white clover accumulated 53±19 and 264±32 g biomass m^{-2} $years^{-1}$, containing 2±0.6 and 10±1 g N m^{-2} $years^{-1}$ in the mixed and pure stands, respectively.

The N derived from the atmosphere (Ndfa), determined by the enriched ^{15}N dilution method, was 94 % in 2011 and 80 % in 2012 of total N in the mixed stand, whereas for the pure stand, it was 64 % in 2011 and 74 % in 2012. Thus, annual N yields derived from the atmosphere were 19 kg N ha^{-1} in 2011 and 41 kg N ha^{-1} in 2012 for the mixed stand and 65 kg N ha^{-1} in 2011 and 82 kg N ha^{-1} in 2012 for the pure stand.

Plant and soil ^{15}N enrichment

Time experiment

To study the temporal white clover contribution to N in the soil solution, white clover ^{15}N leaf labelling was conducted at four different times: autumn'11 (T1), spring'12 (T2), summer'12 (T3) and autumn'12 (T4). The results showed rapid ^{15}N label transfer from white clover to ryegrass in all treatments (T1, T2, T3 and T4) (Table 1). Recovery of ^{15}N in the first harvest after each labelling event was the largest ($P<0.001$) in spring'12 (T2) in the mixed stand and in autumn'11 (T1) in the pure stand. The lowest ^{15}N recovery was observed in the autumn'2012 labelling (T4) for both swards. Analysis of soil cores sampled at the end of the experiment (May 2013) showed high soil ^{15}N enrichment in the top 10 cm

Table 1 Time experiment: the ^{15}N content in plant above- (leaves and stolons) and below- (roots) ground biomass and soil, in the experiment where the label was added at different times (mean±SE, n=4)

Treatment	Time	Labelling time	Sward	^{15}N mg/cylinder									Sum
				Shoots					Roots		Soil		
				2011	2012			2013	2013		2013		
				Oct	May	Jul	Oct	May	May		May		
White clover-ryegrass	T1	Sep 2011	White clover	6±1.57	4±0.05	1.12±0.01	0.89±0.01	0.01±0.01	0.161±0.02	0.005±0.003	4±0.41	0.51±0.08	21
			Ryegrass	4±2.71	0.25±0.55	0.04±0.21	0.02±0.07	0.05±0.01					
	T2	May 2012	White clover		19±0.94	3±0.15	1.37±0.01	0.03±0.01	0.215±0.03	0.01±0.002	7±1.71	0.82±0.23	34
			Ryegrass		2±1.15	0.33±0.55	0.04±0.28	0.13±0.009					
	T3	July 2012	White clover			6±0.58	2±0.02	0.02±0.01	0.069±0.01	0.003±0.004	3±1.18	0.23±0.06	15
			Ryegrass			4±1.12	0.09±0.12	0.04±0.02					
	T4	Sep 2012	White clover				5±0.2	0.10±0.03	0.196±0.02	0.006±0.001	3±0.42	0.56±0.31	14
			Ryegrass				4±0.24	0.08±0.04					
White clover	T1	Sep 2011	White clover	7±1.91	1.11±0.18	0.38±0.09	0.17±0.04	0.03±0.002	0.047±0.005	0.005±0.001	13±4	1.73±0.53	16
	T2	May 2012	White clover		6±1.3	1.07±0.18	2±1.48	0.03±0.005	0.045±0.003	0.009±0.004	6±0.55	1.01±0.18	16
	T3	July 2012	White clover			6±0.77	0.24±0.04	0.03±0.01	0.036±0.01	0.002±0.001	5±0.42	0.52±0.03	12
	T4	Sep 2012	White clover				4±0.26	0.08±0.03	0.107±0.01	0.006±0.002	6±0.91	1.18±0.45	11

of soil. Total ^{15}N recovery (sum of ^{15}N in shoots over the whole period, roots and soil) in the mixed stand was 32 mg ^{15}N per cylinder, and in the pure stand, it was 16 mg ^{15}N per cylinder.

Transport experiment

Leaves of white clover, in the pure and in the mixed stands, were ^{15}N-labelled over two growing seasons to investigate the white clover contribution to N in the soil profile. The overall ^{15}N recovery (sum of ^{15}N in shoots, roots and soil) per cylinder was 63 and 67 mg ^{15}N under the mixed and pure stands, respectively (Table 2). The accumulated ^{15}N recovery in soil was similar for the two swards and amounted to 31 mg ^{15}N. The ^{15}N enrichment of the white clover shoot material in the mixed stand averaged 5 and 2 at.% excess in 2011 and 2012, respectively, and the ryegrass shoot material in the mixed stand averaged 0.5 and 1.4 at.% excess in 2011 and 2012, respectively. Shoot material from the white clover pure stand had ^{15}N enrichments averaging 0.6 and 1.2 at.% excess in 2011 and 2012, respectively. At termination of the experiment in May 2013, root material had the highest ^{15}N enrichment in the uppermost soil layer (0–10 cm) for both the white clover-ryegrass mixed stand (1.2 at.% excess) and the white clover pure stand (0.8 at.% excess), with the enrichment of roots in deeper layers being fairly similar for both treatments ranging from 0.2 to 0.5 at.% excess. The ^{15}N enrichment of the soil was similar under the two treatments, being 0.1 at.% excess in the upper most soil layer (0–10 cm) and about one order of magnitude lower in the deeper soil layers.

DIN and DON concentrations

The DIN and DON concentrations in percolating soil solution collected at 25 cm in the time and transport experiments were, as expected, not statistically different (Table 3), and therefore, they were analysed as one data set. Analysis of percolating soil solution collected at 25, 45 and 80 cm under the mixed and pure stands showed that sward type and sampling time significantly ($P<0.001$) affected the DIN concentration (Fig. 2) with the highest concentration under the pure stand. Furthermore, the DIN concentration increased over the two leaching seasons under both swards at 25 cm, whereas at 45 and 80 cm, sampling time significantly affected DIN leaching ($P<0.05$) under the mixed stand. The DON concentration significantly ($P<0.01$) increased over time but was not affected by sward type and depth.

White clover temporal contribution to nitrogen leaching (time experiment)

The autumn'11 (T1) labelling caused ^{15}N enrichment of DIN and DON in both of the following leaching seasons, showing longer-term contribution of white clover to N leaching. White clover grown in a pure stand had a significantly ($P<0.001$) larger contribution to DIN leaching compared to the mixed stand (Fig. 3). The largest ($P<0.001$) white clover contribution to the leaching of DIN and DON was observed in the autumn'11 (T1) and autumn'12 (T4) labellings, but DI ^{15}N and DO ^{15}N were observed irrespective of when labelling was carried out. The specific concentrations of DI ^{15}N and DO ^{15}N followed the same pattern at all labelling times under both swards (Fig. 4).

Transport of white-clover-derived N in the soil profile

White clover in a pure stand gave significantly ($P<0.001$) larger contributions to DI ^{15}N content compared to the mixed stand (Fig. 5). DI ^{15}N was observed under the pure stand at all depths during both leaching seasons, whereas under the mixed stand, DI ^{15}N was detected only during the second leaching season. The DO ^{15}N concentration under both swards was not affected by sward type, whereas there was a significant effect of sampling time at 25- and 80-cm depths ($P<0.001$). There was a significant effect of depth for DI ^{15}N, but not for DO ^{15}N (Tables 3). The DO ^{15}N results from the first leaching season showed a lower white clover contribution to the leaching of DON than those from the second leaching season. During the second leaching season, an increase of DO ^{15}N was observed with a time delay with depth under both swards. The specific concentrations of DI ^{15}N and DO ^{15}N followed the same pattern at all depths under both swards (Fig. 6). The ^{15}N enrichments of DIN and DON in the three depths under the mixed sward were in the range 0.1–0.2 at.% excess, and under the pure stand, the range was 0.2–0.4 at.% excess.

Discussion

In this study, the contribution of white clover to N leaching under the white clover and the white clover-ryegrass mixed stand was investigated by leaf labelling with ^{15}N urea followed by DIN and DON analysis in percolating soil solution. The presence of ^{15}N in the soil solution showed white clover as an immediate source of N leaching and allowed investigation of the specific contribution of white clover to DIN and DON leaching.

DIN and DON leaching

The present study was conducted in low-N-input grassland under temperate climatic conditions, and therefore, low leaching levels of both DIN and DON were expected. Nitrogen leaching was estimated on the basis of N concentrations in suction cups and drainage calculated by the Evacrop

Table 2 Transport experiment: the ^{15}N content in plant above- (leaves and stolons) and below- (roots) ground biomass, in the experiment where the label was added over two growing seasons (mean±SE, n=4)

^{15}N mg/cylinder															
	Shoot						Root				Soil				Sum
	Clover			Ryegrass			May 2013				May 2013				
Time	2011[a]	2012[a]	2013	2011[a]	2012[a]	2013									
Depth (cm)							0–10	10–25	25–50	50–80	0–10	10–25	25–50	50–80	
White clover-ryegrass	8±1.1	11±0.6	0.18±0.004	2.5±0.1	10±0.5	0.09±0.009	0.1±0.002	0.03±0.003	0.009±0.001	0.003±0.0004	26±2	3±0.5	1.82±0.9	0.32±0.003	63
White clover	15±0.4	21±0.6	0.2±0.007				0.14±0.002	0.01±0.004	0.006±0.001	0.007±0.001	26±3	3±0.3	1.10±0.2	0.84±0.007	67
^{15}N at.% excess															
White clover-ryegrass	5±1.6	2±0.4	1.7±0.2	0.48±0.003	1.4±0.1	1.09±0.1	1.2±0.2	0.3±0.04	0.4±0.03	0.3±0.03	0.1±0.02	0.01±0.002	0.01±0.003	0.004±0.001	
White clover	0.62±0.1	1.16±0.1	0.8±0.2				0.8±0.1	0.2±0.05	0.4±0.1	0.5±0.2	0.1±0.02	0.01±0.001	0.01±0.001	0.01±0.004	

[a] Values are the sum of two harvests in 2011, three harvests in 2012 and final harvest in May 2013

Table 3 ANOVA table of *P* values on the effect of the following: (1) sampling time, depth and sward on total DIN and DON; (2) labelling time, depth and sward on DI ^{15}N and DO ^{15}N in time experiment; and (3) sampling time, depth and sward on DI ^{15}N and DO ^{15}N in transport experiment

Analyses of variances	Total		Time experiment				Transport experiment			
	DIN	DON	Concentration		Specific concentration		Concentration		Specific concentration	
			DI ^{15}N	DO ^{15}N	DI ^{15}N	DO ^{15}N	DI ^{15}N	DO ^{15}N	DI ^{15}N	DO ^{15}N
Sampling time	***	***	–	–	–	–	***	***	***	***
Labelling time	–	–	***	ns	**	ns	–	–	–	–
Depth	ns	ns	–	–	–	–	*	ns	***	ns
Sward	***	ns	***	ns	***	ns	***	ns	***	*
Sward*depth	**	ns	–	–	–	–	**	ns	**	ns
Sward*sampling time	ns	ns	–	–	–	–	***	ns	***	***
Sward*labelling time	–	–	*	ns	ns	ns	–	–	–	–

***$p<0.001$; **$p<0.01$; *$p<0.05$; ns $p>0.05$

model for which inputs were daily meteorological measurements, crop, sowing and cutting date and soil physical parameters according to Eriksen et al. (1999). The amount of DIN leaching at 80 cm during the first leaching season was 2 and 17 kg ha^{-1} year^{-1} under the mixed and pure stand, respectively, and during the second leaching season, it was 7 and

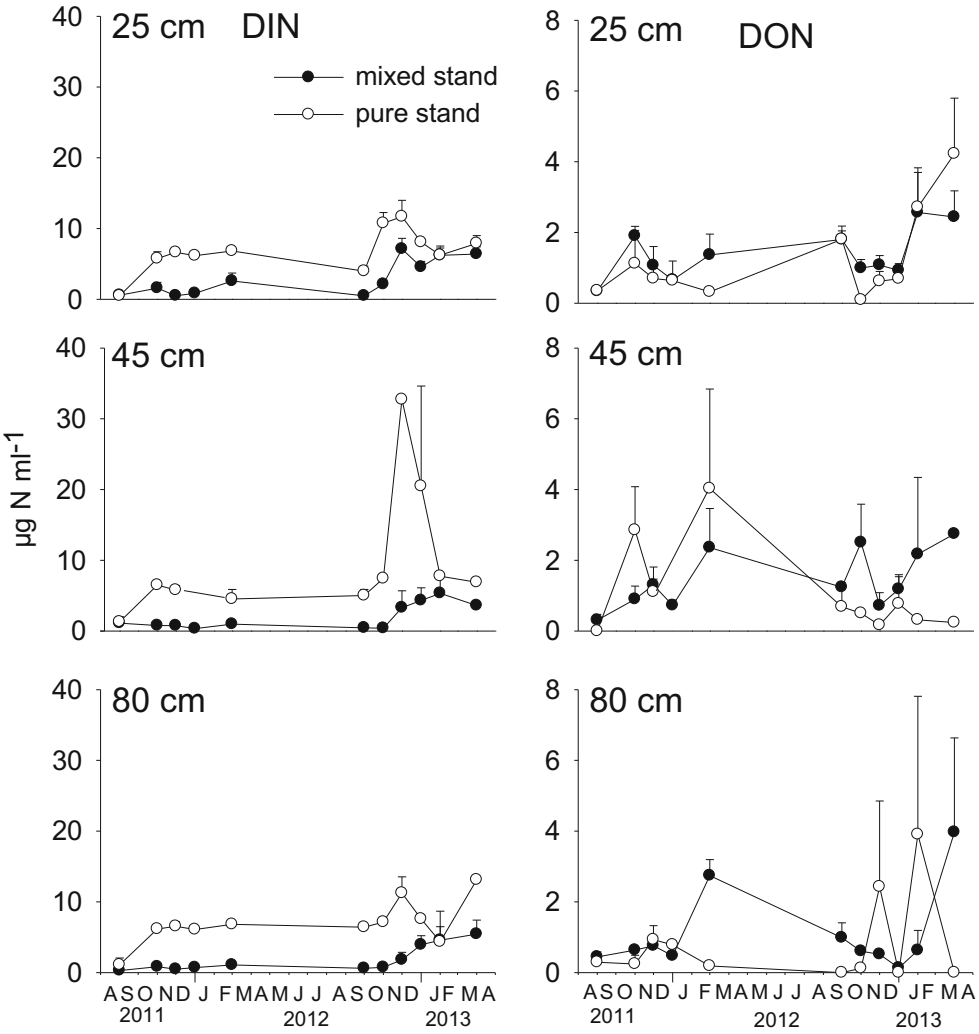

Fig 2 Dissolved inorganic N (DIN) and dissolved organic N (DON) contents in the soil solution at 25-, 45- and 80-cm depth under a pure stand of white clover and a white clover-ryegrass mixed stand (*bars* represent SE values, $n_{25\ cm}=20$, $n_{45\ and\ 80\ cm}=4$)

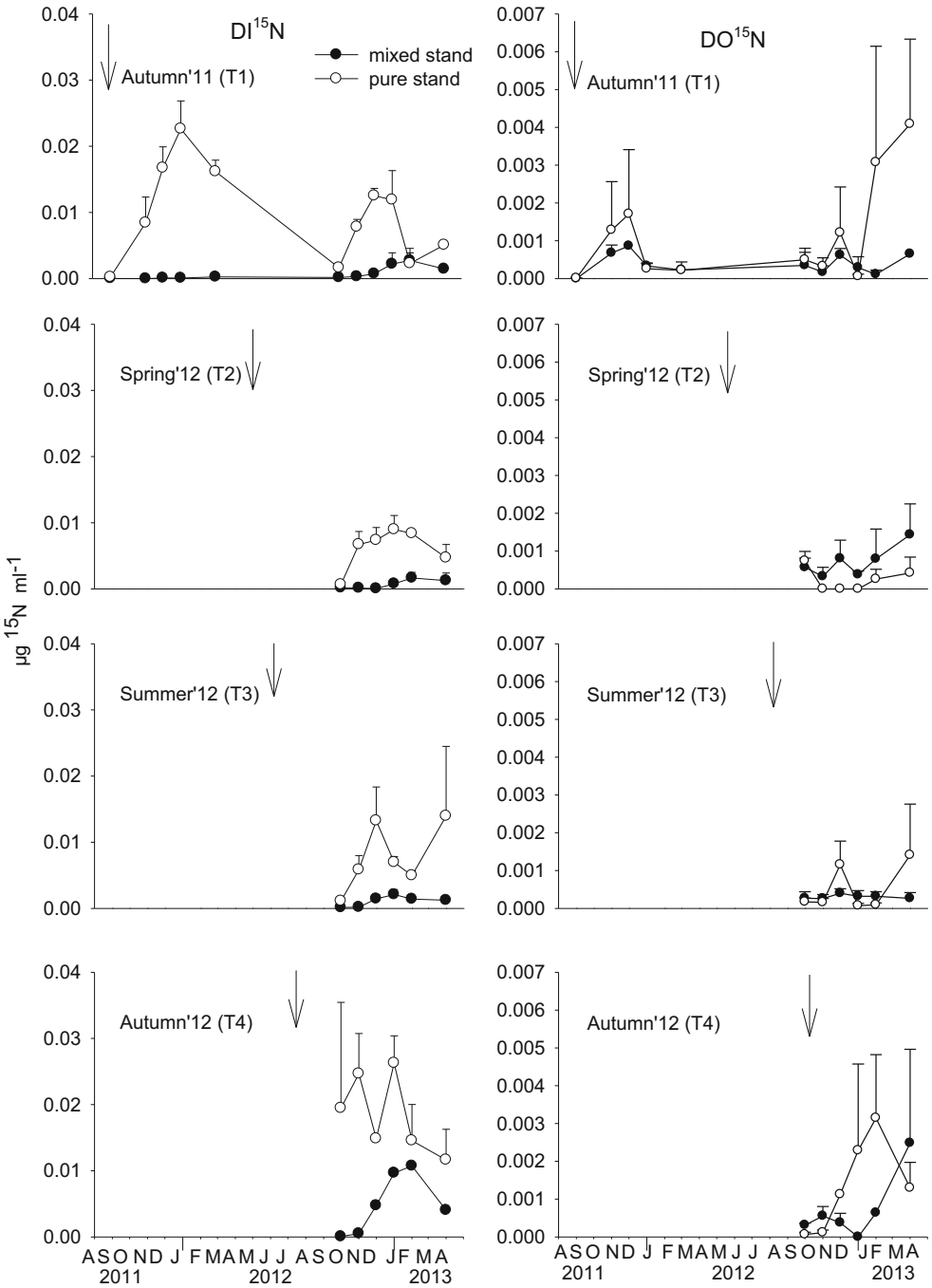

Fig 3 Dissolved inorganic ^{15}N (DI ^{15}N) and dissolved organic ^{15}N (DO ^{15}N) contents in the soil solution at 25-cm depth under a pure stand of white clover and a white clover-ryegrass mixed stand in an experiment with different times of labelling (time experiment) (*bars* represent SE values, $n=4$). The *arrow* indicates labelling time

25 kg ha^{-1} year^{-1}, respectively. The leaching of DON was 2–3 kg ha^{-1} year^{-1} under both swards during both leaching seasons. The DIN and DON leaching under the mixed stand was comparable to previous studies on leaching from grass-white clover mixed stands under comparable soil and climatic conditions (Vinther et al. 2006) and sward management (e.g. Anguelov et al. 2011; Eriksen et al. 2004; Eriksen et al. 2010). We found higher levels of DIN leaching under the white clover pure stand than under the mixed stand, which can be explained by a larger harvest removal of N by ryegrass from the mixed stand and greater N yield derived from the atmosphere in the pure stand. Given the clear difference between pure and mixed stands in DIN leaching, we were surprised not to find any difference between the two swards in relation to DON leaching. At first hand, this seems to fit with previous studies showing that DIN and DON do not originate from the same source (Neff et al. 2003; Scott and Rothstein 2014), but when looking at the results from ^{15}N labelling, the previous findings do not fit in the present system.

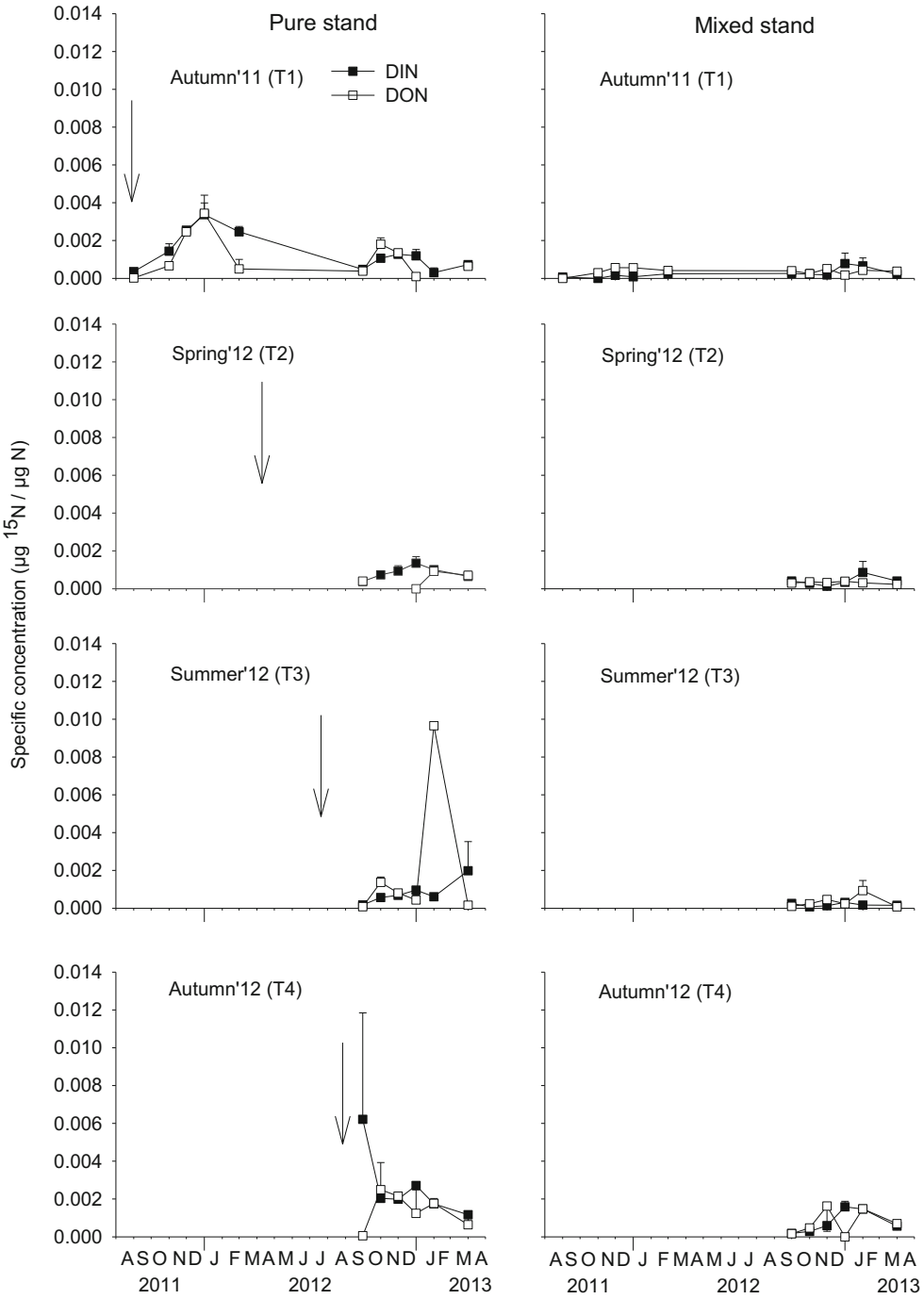

Fig 4 The specific concentrations of dissolved inorganic N (DIN) and dissolved organic N (DON) in the soil solution at 25-cm depth under a pure stand of white clover and a white clover-ryegrass mixed stand in an experiment with different times of labelling (time experiment) (*bars* represent SE values, n=4)

Temporal contribution of white clover to nitrogen leaching

The ^{15}N analyses showed that white clover contributed to both DIN and DON. In our study, white clover labelled in autumn contributed most to the leaching of DIN and DON and confirmed the first hypothesis that higher N deposition from white clover in autumn results in a higher N loading in the soil solution. The explanation may be a high white clover N deposition late in the growing season (Rasmussen et al. 2013) and changes in N allocation from leaves to stolons and roots at low temperatures (Bouchart et al. 1998) that cause an increase of N losses in autumn. The increase in total DIN leaching observed under the mixed stand in the second leaching season was white-clover-derived, where the autumn'12 (T4) treatment contributed roughly half of the DI ^{15}N and the other three treatments contributed the other half. In addition, the increased contribution from white clover to the leaching of DIN in the pure stand compared to the mixed stand confirmed that DIN leaching is a function of N loading and N demand.

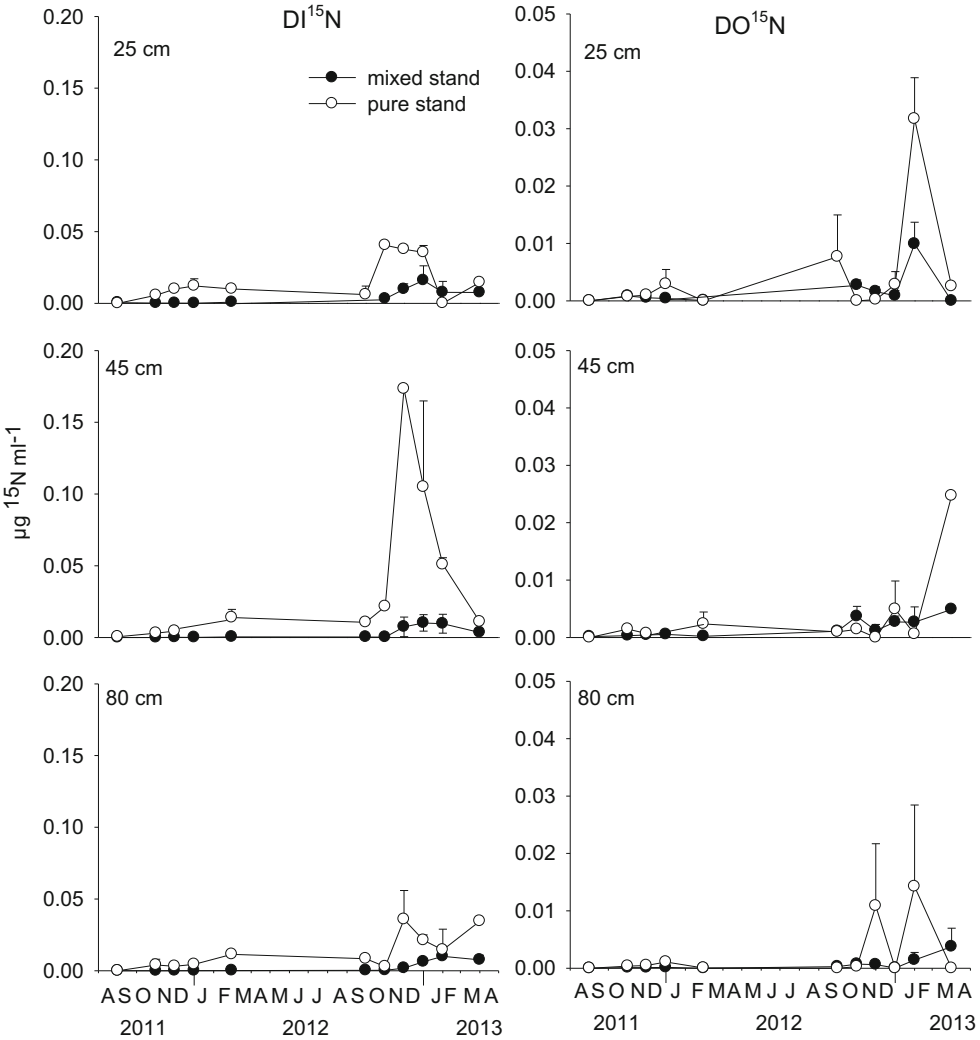

Fig 5 Dissolved inorganic ^{15}N (DI ^{15}N) and dissolved organic ^{15}N (DO ^{15}N) contents in the soil solution under a pure stand of white clover and a white clover-ryegrass mixed stand at 25-, 45- and 80-cm depth where the label was added over two growing seasons (transport experiment) (*bars* represent SE values, n=4)

The DI ^{15}N and DO ^{15}N leaching when the label was applied in autumn'11 (T1) showed that white clover continuously (over 2 years) contributed to the leaching of DIN and DON. When the label was applied in spring'12 (T2) and summer'12 (T3), there was less N leaching compared to other labelling times. This indicates that white clover spring deposits were either (i) immobilised in soil by incorporation into microbial biomass and subsequently built into the soil organic N (SON) pool and/or (ii) taken up by ryegrass and later deposited in the soil. Likewise, the clear presence of DI ^{15}N and DO ^{15}N when labelling in autumn'11 (T1) in the second leaching season was probably due to plant decay and remobilisation of ^{15}N from white clover deposits immobilised by microbial biomass during the first growing season.

Surprisingly, the DO ^{15}N leaching was not affected by the presence of a companion grass. Thus, the DO ^{15}N results did not confirm our third hypothesis that ryegrass N uptake would reduce DON leaching. Furthermore, the present results demonstrate that the source of DON is not only indigenous SON as suggested by Chantigny (2003), but recent plant deposits also contribute to the pool, since our findings clearly showed that labelled white clover contributed to DON leaching.

Nitrogen transport in the soil profile

White clover contributed to DIN leaching at all depths, and the fact that DI ^{15}N did not change with depth showed that DIN in the studied soil was formed in the uppermost soil layer and transported in the soil profile. This implies that studies of DIN leaching from the plough layer (e.g. Rasmussen et al. 2008) most likely represent the extent of DIN leaching below the rooting zone in white clover-ryegrass swards.

The results showed that white clover also contributed to the leaching of DON under both swards at all depths. The DO ^{15}N content did not significantly decrease with depth, which did not confirm our second hypothesis that the recently deposited DON pool decreases in size with depth, as proposed by the dynamic exchange model (Neff et al. 2003). We observed a temporal increase in DO ^{15}N content under both swards at all depths, indicating an increased white clover contribution to

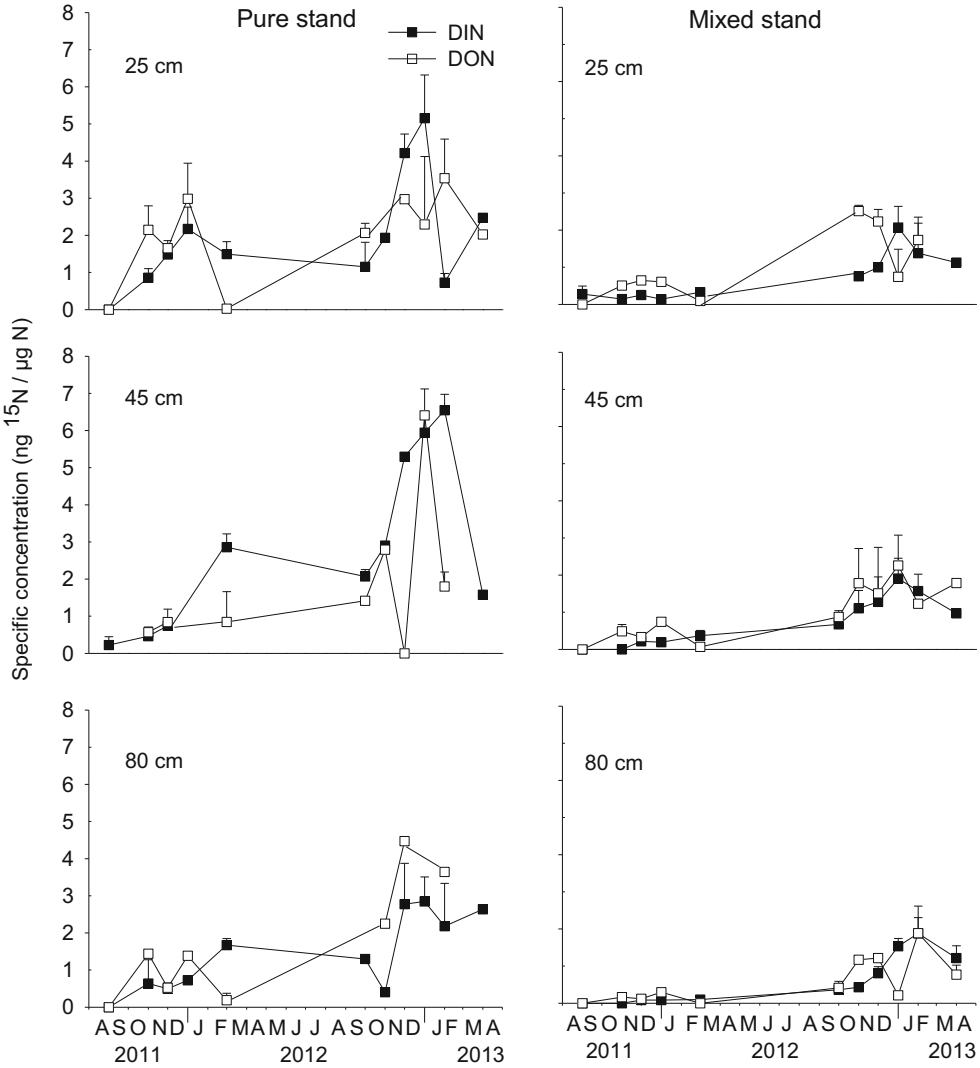

Fig 6 Specific concentrations of dissolved inorganic N (DIN) and dissolved organic N (DON) in the soil solution under a pure stand of white clover and a white clover-ryegrass mixed stand at 25-, 45- and 80-cm depth where the label was added over two growing seasons (transport experiment) (*bars* represent SE values, n=4)

the leaching of DON. It may be speculated that the greater content of DO ^{15}N in the second leaching season was due to the larger white clover biomass during the second growing season resulting in increased deposition of ^{15}N tracer to the soil.

The mechanism of N leaching from grassland soils

Our study showed that the specific concentrations of DI ^{15}N and DO ^{15}N followed similar patterns across swards type, time of labelling and depths, which point to that white clover contributed similarly to DIN and DON leaching irrespective of differences in harvested N, total N leaching and temporal contribution to N leaching under the two swards. However, the DI ^{15}N and DO ^{15}N specific concentrations do not say anything about the proportion of total N leaching originating from the labelled white clover. In order to evaluate the proportional contribution from the labelled white clover to total N leaching, one has to compare the ^{15}N enrichment of the DIN and DON leached with the ^{15}N enrichment of potential sources. If, for example, indigenous SON (organic N found in the soil prior to the growth of the present plants) was the sole source for DON, then the ^{15}N enrichment of DON would be similar to the background enrichment of the soil; i.e. no excess ^{15}N would be present. We will, in the following, focus on the leaching results of the 2012–2013 leaching season from the transport experiment where the highest amounts of ^{15}N were applied. The label of white clover in the transport experiment was applied over two growing seasons, and we cannot, in the present setup, separate whether leached ^{15}N came directly from e.g. white clover shoots or from ^{15}N that was deposited to the SON pool during the experimental period. Therefore, the leached ^{15}N will be the sum of shoot and root direct contributions to leaching and indirect contributions from these via shorter and longer-term deposition of ^{15}N to the

Table 4 Mean ^{15}N enrichment (at.% excess) of DIN and DON leached from the transport experiment in the 2012–2013 leaching season, mean± SE

Sward	Depth (cm)	DIN (at.% excess)	DON (at.% excess)
Mixed stand	25	0.13±0.02	0.19±0.02
	45	0.13±0.02	0.16±0.03
	80	0.10±0.02	0.09±0.02
Pure stand	25	0.28±0.05	0.29±0.06
	45	0.41±0.07	0.28±0.09
	80	0.20±0.04	0.35±0.07

SON pool (Rasmussen 2011; Mayer et al. 2003; Fustec et al. 2010).

The ^{15}N deposition over two growing seasons to the SON pool resulted in an enrichment of the soil of 0.1 at.% excess in the uppermost soil layer and 0.01 at.% excess in the lower layers. If new and old SON had contributed equally to N leaching, then the resulting enrichment of the leached N should correspond to the overall enrichment of the SON pool. The leached ^{15}N had across the three sampling depths an enrichment of 0.1–0.2 at.% excess under the mixed stand and 0.2–0.4 at.% excess under the white clover pure stand (Table 4), which initially demonstrates two things: (i) white-clover-derived N was more prone to leaching relative to indigenous SON; i.e. white-clover-derived N contributes relatively more to N leaching than SON, and (ii) leached ^{15}N most likely came from the uppermost soil layer as the soil ^{15}N enrichment in the deeper layers was much lower than the N leached or directly from roots in the deeper soil layers. Furthermore, higher ^{15}N enrichment of leached N under white clover pure stand than under the mixed stand in spite of the soil enrichment being similar under the two swards revealed that a significant proportion of leached ^{15}N came from white clover shoot and root die off during the leaching season. However, as ^{15}N enrichments of DIN and DON were similar, the N coming from white clover shoot and root die off must have passed through the same bottleneck before leaching, which most likely is the microbial community.

The mechanisms leading ^{15}N added via leaf labelling to white clover to be leached as DI ^{15}N and DO ^{15}N acted both in the shorter and the longer term, but always through the microbial community, with the shorter term being ^{15}N deposited from white clover during the leaching season and the longer term being the deposition over the two growing seasons. In both the shorter and the longer term, the deposited ^{15}N from shoot and root material was decomposed where ^{15}N was build-in to microbial tissue and surplus N released as inorganic N. During the growing season, inorganic ^{15}N was taken up by the plants and therefore did not give a significant contribution to DIN leaching, especially under the mixed stand where ^{15}N was removed in harvested grass biomass. We suggest that two main routes contributed to ^{15}N found lost as DIN and DON: (i) ^{15}N that was built into the microbial tissue over the growing season was during the colder leaching season released as part of the microbial community die off, and (ii) ^{15}N from microbial decomposition of white clover deposits during the leaching season. For both routes, DIN was formed when surplus N was released as the microbial community decomposed dying microbial tissue or white clover material, and DON was formed as cell lysates or cell wall release to the soil solution. In order to elucidate this, future studies could look into the $^{15}N/^{13}C$ enrichment of different plant and microbial biomarkers in the leaching soil solution, which could confirm the suggested DON formation route. Furthermore, future studies including greater variation in climatic, soil and botanical composition could give additional insight into potential management strategies to reduce N leaching from grasslands.

Conclusion

The present study investigated the white clover contribution to the leaching of DIN and DON in situ. The result showed that white clover significantly contributed to the leaching of DIN and DON. White clover deposition in autumn contributed more to N leaching than deposition in spring and summer. The leaching of DIN from white clover in the pure stand exceeded that of the mixed stand, confirming that DIN leaching is a function of N loading and N demand, whereas DON leaching was unaffected by the presence of a companion grass. White clover contributed to DIN and DON formation in the uppermost soil layer which was then freely transported down the soil profile with water movement. There were strong indications that white-clover-derived DIN and DON in the soil solution were of microbial origin. Hence, fluctuations in white clover content and its deposition and decomposition should be expected to influence total N leaching in grass-clover mixed stands.

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