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

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10

11 **Abstract**

12 Leaching of dissolved inorganic N (DIN) and dissolved organic N (DON) is a considerable loss pathway in  
13 grassland soils. We investigated the white clover (*Trifolium repens*) contribution to N transport and temporal N  
14 dynamics in soil solution under a pure stand of white clover and white clover-ryegrass (*Lolium perenne*) mixed  
15 stand. The temporal white clover contribution to N leaching was analysed by <sup>15</sup>N incorporation into DIN and DON  
16 in percolating soil solution collected at 25 cm depth following white clover <sup>15</sup>N leaf-labelling that was applied at  
17 different times during the growing season. The white clover contribution to N transport in the soil profile was  
18 investigated over two years by analysing <sup>15</sup>N in DIN and DON in percolating soil solution collected at 25, 45, and  
19 80 cm depth following <sup>15</sup>N leaf labelling of white clover. The results showed that clover was a source of both DIN  
20 and DON. White clover autumn deposition contributed the most to N leaching. The leaching of DIN from the white  
21 clover in pure stand exceeded that of the mixed stand and confirmed that leaching of DIN is a function of N loadings  
22 and N demand. The DON leaching was unaffected by the presence of a companion grass, suggesting that the DON  
23 leaching from our grassland derived from the lysis of soil microbial biomass living on recent white clover deposits.  
24 White clover contributed to the leaching of DIN and DON at all depths, and the fact that the contents of DI<sup>15</sup>N and  
25 DO<sup>15</sup>N did not change with depth indicated that surplus of DIN and DON, formed in the uppermost soil layer, was  
26 transported in the soil profile.

27

28 Keywords: DIN, DON, <sup>15</sup>N, leaching, white clover, grasslands

29

30 **Introduction**

31

32 Optimising the use efficiency of legume-derived nitrogen (N) is a way of tightening the N cycle and thus a step  
33 towards the needed enhancement of sustainability in agricultural production. The ability of legumes to biologically  
34 fix N<sub>2</sub> (BNF) in symbiosis with *Rhizobium* bacteria gives them a huge advantage in grassland ecosystems. The  
35 extent of BNF is regulated by N availability (Soussana and Tallec 2010) and the presence of companion species  
36 competing for soil N (Rasmussen et al. 2012). The important forage legume, white clover (*Trifolium repens*), is  
37 known to have a high complementary effect by donating N to companion grasses through above- and below-ground

38 N transfer (Fustec et al. 2010; Høgh-Jensen and Schjoerring 2000) and by improving soil N fertility via deposition  
39 of fixed N to the soil (e.g. (Rasmussen et al. 2012). However, high N input and internal N flows increase the risk of  
40 undesirable N losses.

41  
42 White-clover-derived N enter the soil through deposition that includes rhizodeposits (root exudates, lysates, and  
43 dead root cell material) and plant decay. The deposition of N from white clover to the soil has been reported to range  
44 from 20 up to 300 kg N ha<sup>-1</sup> yr<sup>-1</sup> (Ledgard and Steele 1992). Deposits, depending on their origin and composition,  
45 can be assimilated and dissimilated by microbial community, taken up by plants or transported down the soil profile  
46 (Pinton et al. 2007). The dominance of each of those processes is season-dependent due to changes in temperature  
47 and soil solution content affecting plant activity (Bouchart et al. 1998). The greatest white clover N allocation to  
48 leaves is reported at high temperatures, whereas the highest N content was observed in roots and stolons at low  
49 temperatures (Bouchart et al. 1998). Seasonal variation of white clover N deposition and N transfer (Dahlin and  
50 Stenberg 2010; Rasmussen et al. 2013) likely leads to the variation of N pool sizes in the rhizosphere. It is widely  
51 recognised that leaching of dissolved inorganic N (DIN) is related to high N loads into the soil, management and the  
52 sward botanical composition, so that high grass N yields and removal reduce N leaching (Vinther et al. 2006).  
53 However, management-independent patterns of oscillation in DIN leaching from grassland soils have been observed  
54 (Eriksen et al. 2010). Therefore in the present study we investigated whether seasonal variation of white clover N  
55 deposition was directly related to DIN leaching.

56  
57 It is evident that N leaches in considerable amounts in both inorganic and organic forms (Perakis and Hedin 2002;  
58 Rasmussen et al. 2008) and white clover contributes to the leaching of both DIN and DON below the plough layer  
59 (Rasmussen et al. 2008). Moreover, DON leaching has been reported even in N-limited environments (Perakis and  
60 Hedin 2002), suggesting that other factors besides plant and microbial N demand affect DON movement through the  
61 soil profile. There are two main models explaining DON leaching in soil – dynamic exchange and continual  
62 stripping – based on studies in mainly forest ecosystems (Neff et al. 2003; Scott and Rothstein 2014). The N  
63 dynamic exchange model (Scott and Rothstein 2014) suggests that recently deposited DON displaces previously  
64 sorbed DON that migrates in the soil profile and leads to a greater pool of older DON in deeper layers. DON  
65 leaching according to the continual stripping model can be explained by partitioning DON into mainly microbial-  
66 derived hydrophilic (Qualls and Haines 1991) and mainly plant-derived hydrophobic pools (Gu et al. 1995).  
67 Hydrophobic DON can be easily sorbed to mineral soil due to its high molecular weight and polymeric structure,  
68 whereas hydrophilic DON, due to its low molecular weight is easy soluble and therefore remains in soil solution.  
69 Thus, according to this model, hydrophobic DON replace hydrophilic DON that is preferentially transported in the  
70 soil profile. While leaching of DIN and DON from grassland soils has already been demonstrated (Rasmussen et al.  
71 2008, Vinther et al. 2006), there is still a lack of information on to what extent white clover contribute to the  
72 transport of N in the soil profile and how the composition of grass-clover mixed stands may control the extent of  
73 leaching and the underlying controls of N dynamics in the soil profile.

74

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

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

86

87 Therefore two parallel experiments were conducted in a pure stand of white clover and a ryegrass–white clover  
88 mixed stand to investigate the white clover contribution to transport of N in the soil profile and temporal deposition  
89 of N. The white clover temporal contribution to N leaching was investigated by using <sup>15</sup>N leaf-labelling of white  
90 clover that was applied at different times during the growing season ('Time experiment'). The white clover  
91 contribution to N transport in the soil profile was investigated over two years using <sup>15</sup>N leaf-labelling of white clover  
92 ('Transport experiment'). The N<sub>2</sub> fixation by white clover was estimated in adjacent plots.

93

## 94 **Materials and Methods**

95

### 96 **Experimental site and conditions**

97

98 The field experiments were conducted on a sandy loam at Foulumgård Experimental Station, Viborg, Denmark  
99 (55°28'N, 09°07'E). The soil is classified as a Typic Hapludult with 6.4% clay, 8.5% silt, 44% fine sand and 39%  
100 coarse sand. The site has been used for dairy farming with a grassland-arable crop rotation since 1987 (Eriksen et al.  
101 2014). The soil contained 1.7% C and 0.16% N. The mean temperature and precipitation (Fig. 1) were 9°C and 654  
102 mm in the first experimental season (May 2011 – April 2012) and 7°C and 694 mm in the second experimental  
103 season (May 2012 – April 2013). The field experiment was established in April 2011 by installing PVC cylinders (Ø  
104 30 cm) in a randomised block design with four replicates in pure stand of white clover (*Trifolium repens* L., 6 kg ha<sup>-1</sup>)  
105 and a ryegrass-white clover mixed stand (*Lolium perenne* L., 24 kg ha<sup>-1</sup>, *Trifolium repens* L., 4 kg ha<sup>-1</sup>) seeded in  
106 April 2011. The PVC cylinders had a length allowing 5 cm of the cylinders to be above ground. The cylinders were  
107 placed with a distance of at least 2 meters in between.

108

109 Atmospheric N<sub>2</sub> fixation was estimated in adjacent plots with a size of 1 m<sup>2</sup>. Plots were established in pure  
110 stands of white clover and ryegrass, and in the ryegrass-white clover mixed stand. A <sup>15</sup>N-labelled (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
111 solution (60 at% <sup>15</sup>N, 1.5 kg N ha<sup>-1</sup>) was applied to the soil (Høgh-Jensen and Schjoerring 1994) before plant

112 germination in April 2011. Plant shoots were harvested to a height of 5 cm two times in 2011 (July and October) and  
113 three times in 2012 (May, July and October). Plant material was divided into white clover and grass and analysed as  
114 described below.

115

116 Time experiment

117

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

122

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

125

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

129

130 Transport experiment

131

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

135

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

139

140 Soil solution was sampled monthly from September to April at 25, 45, and 80 cm depth during two leaching  
141 seasons. Sample preparation and analysis are described in section 2.6. At the end of the two-year experiment (May  
142 2013) plant samples (shoots and roots) and soil were taken from the 75-cm length cylinders. Soil cores (one core per  
143 cylinder) were sampled with a soil auger (Ø 8.75 cm) and divided into four sections (0-10, 10-25, 25-50, and 50-80  
144 cm). Sample preparation and analysis are described below.

145

146 Labelling

147

148 The <sup>15</sup>N leaf-labelling method (McNeill et al. 1997) was applied to introduce the <sup>15</sup>N tracer into the white clover.  
149 Briefly, a white clover leaf was inserted into a 2 ml vial with 1 ml of <sup>15</sup>N urea solution (99.6 atom% <sup>15</sup>N, 0.5% w/v)  
150 and sealed with an inert plastic material (UNIGUM Sanitary putty, Unipak A/S, Galten, Denmark) to avoid <sup>15</sup>N loss.  
151 In each cylinder four clover leaves were labelled per each labelling occasion. After four days of labelling, the tubes  
152 were removed and labelled leaves were dried with paper towels to avoid soil contamination by <sup>15</sup>N; following four  
153 new leaves were then exposed to the labelling procedure. The same amount of <sup>15</sup>N was applied to each cylinder in  
154 each of the experiments with a total of 32 vials in the Time experiment and 172 vials in the Transport experiment.

155

#### 156 Soil solution sampling

157

158 Soil solution was collected by Teflon suction cups (Prenart Super Quartz, pore size <1 µm, Ø 21 mm, length 70 mm,  
159 Prenart Equipment Aps, Frederiksberg, Denmark) inserted 5 cm beneath the cylinders (Rasmussen et al. 2007). The  
160 preparation and installation of the sampling system were performed according to Grossmann and Udluft (1991).  
161 When sampling the soil solution, suction cups were connected to acid-washed 1L glass bottles and vacuum was  
162 applied (0.7 bars) for two-three days using a vacuum pump. Due to frost, sampling in February (2012) and March  
163 (2013) was not possible.

164

#### 165 Sample preparation and analysis

166

##### 167 Soil solution

168 Total DIN and total dissolved N (TDN) were determined in filtrated (0.6µm, glass fibre filter) soil solution  
169 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  
170 MgO and 0.4 g Devardas reagent. A glass-fibre filter trap was acidified by adding 20 µl of 1.5 M H<sub>2</sub>SO<sub>4</sub>, packed in  
171 Teflon paper and placed in a bottle with the sample (Sørensen and Jensen 1991). The diffusion was processed for  
172 five days before the glass filter traps were dried and packed into tin capsules for <sup>15</sup>N and N measurement. Water  
173 samples for TDN analyses were treated as for DIN after the oxidation of organically bound N to NO<sub>3</sub><sup>-</sup> by 7.5 ml  
174 persulfate reagent and autoclaved at 121°C for 30 min. Dissolved organic N was calculated as the difference between  
175 TDN and DIN (DON=TDN-DIN).

176

##### 177 Plants and soil

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

181

182 The soil cores sampled at the end of the experiment (May 2013) (sampling is described above) were manually  
183 separated and all visible roots collected. A subsample of the soil was gently washed in a 425-µm sieve and the

184 remaining material washed at least three times to separate roots from soil mineral particles. Finally, non-root  
185 material was removed with tweezers.

186

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

189

## 190 Analysis

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

194

195 Statistics and calculations

196

197 The <sup>15</sup>N at% excess of plant, soil and percolating soil solution was calculated as the difference between <sup>15</sup>N at% in  
198 labelled plots and unlabelled controls sampled at the same time.

199

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

$$201 \quad \%Ndfa = 1 - \frac{\text{white clover at\%excess}}{\text{ryegrass at\%excess}} \times 100\%$$

202

203 where white clover and ryegrass at% excess is calculated by subtracting the <sup>15</sup>N at% in <sup>15</sup>N-dilution plots from the  
204 <sup>15</sup>N at% in unlabelled (control) plots.

205

206 The specific concentration of <sup>15</sup>N incorporation into DIN and DON was calculated as the ratio between the  
207 content of DIN and DI<sup>15</sup>N or DON and DO<sup>15</sup>N.

208

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

215

## 216 **Results**

217

218 In the Time experiment the white clover proportion of shoot biomass in the mixed stand was 31 and 34% in 2011  
219 and 2012, respectively. The average white clover biomass in the pure stand was significantly ( $P<0.001$ ) higher than  
220 in the mixed stand, at  $454 \pm 25$  compared with  $177 \pm 20$  g DM m<sup>-2</sup> yr<sup>-1</sup>, containing  $16 \pm 1$  and  $6 \pm 0.8$  g N m<sup>-2</sup> yr<sup>-1</sup>,

221 respectively. Average total harvested dry matter of ryegrass in the mixed stand was  $344 \pm 20 \text{ g m}^{-2} \text{ yr}^{-1}$ , containing  
222  $5 \pm 0.4 \text{ g N m}^{-2} \text{ yr}^{-1}$ . Measurements of shoot biomass in the Transport experiment showed that the white clover  
223 proportion in the mixed stand with ryegrass was 16 and 30% in 2011 and 2012, respectively. Ryegrass in the mixed  
224 stand accumulated  $171 \pm 16 \text{ g biomass m}^{-2} \text{ yr}^{-1}$ , containing  $4 \pm 0.2 \text{ g N m}^{-2} \text{ yr}^{-1}$ , and white clover accumulated  $53 \pm 19$   
225 and  $264 \pm 32 \text{ g biomass m}^{-2} \text{ yr}^{-1}$ , containing  $2 \pm 0.6$  and  $10 \pm 1 \text{ g N m}^{-2} \text{ yr}^{-1}$  in the mixed and pure stands, respectively.  
226

227 The N derived from the atmosphere (Ndfa), determined by the enriched  $^{15}\text{N}$  dilution method, was 94% in 2011  
228 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.  
229 Thus, annual N yields derived from the atmosphere were  $19 \text{ kg N ha}^{-1}$  in 2011 and  $41 \text{ kg N ha}^{-1}$  in 2012 for the  
230 mixed stand and  $65 \text{ kg N ha}^{-1}$  in 2011 and  $82 \text{ kg N ha}^{-1}$  in 2012 for the pure stand.

231

232 Plant and soil  $^{15}\text{N}$  enrichment

233

#### 234 Time experiment

235 To study the temporal white clover contribution to N in the soil solution, white clover  $^{15}\text{N}$  leaf-labelling was  
236 conducted at four different times: autumn'11 (T1), spring'12 (T2), summer'12 (T3), and autumn'12 (T4). The  
237 results showed rapid  $^{15}\text{N}$  label transfer from white clover to ryegrass in all treatments (T1, T2, T3, and T4) (Table 1).  
238 Recovery of  $^{15}\text{N}$  in the first harvest after each labelling event was the largest ( $P < 0.001$ ) in spring'12 (T2) in the  
239 mixed stand and in autumn'11 (T1) in the pure stand. The lowest  $^{15}\text{N}$  recovery was observed in the autumn'2012  
240 labelling (T4) for both swards. Analysis of soil cores sampled at the end of the experiment (May 2013) showed high  
241 soil  $^{15}\text{N}$  enrichment in the top 10 cm of soil. Total  $^{15}\text{N}$  recovery (sum of  $^{15}\text{N}$  in shoots over the whole period, roots  
242 and soil) in the mixed stand was  $32 \text{ mg}^{15}\text{N}$  per cylinder, and in the pure stand it was  $16 \text{ mg}^{15}\text{N}$  per cylinder.  
243

244

#### 244 Transport experiment

245 Leaves of white clover, in the pure and in the mixed stands, were  $^{15}\text{N}$ -labelled over two growing seasons to  
246 investigate the white clover contribution to N in the soil profile. The overall  $^{15}\text{N}$  recovery (sum of  $^{15}\text{N}$  in shoots,  
247 roots and soil) per cylinder was 63 and  $67 \text{ mg}^{15}\text{N}$  under the mixed and pure stands, respectively (Table 2). The  
248 accumulated  $^{15}\text{N}$  recovery in soil was similar for the two swards and amounted to  $31 \text{ mg}^{15}\text{N}$ . The  $^{15}\text{N}$  enrichment of  
249 the white clover shoot material in the mixed stand averaged 5 and 2 at% excess in 2011 and 2012, respectively, and  
250 the ryegrass shoot material in the mixed stand averaged 0.5 and 1.4 at% excess in 2011 and 2012, respectively.  
251 Shoot material from the white clover pure stand had  $^{15}\text{N}$  enrichments averaging 0.6 and 1.2 at% excess in 2011 and  
252 2012, respectively. At termination of the experiment in May 2013 root material had the highest  $^{15}\text{N}$  enrichment in  
253 the uppermost soil layer (0-10cm) for both the white clover – ryegrass mixed stand (1.2 at% excess) and the white  
254 clover pure stand (0.8 at% excess), with the enrichment of roots in deeper layers being fairly similar for both  
255 treatments ranging from 0.2-0.5 at% excess. The  $^{15}\text{N}$  enrichment of the soil was similar under the two treatments,  
256 being 0.1 at% excess in the upper most soil layer (0-10cm) and about one order of magnitude lower in the deeper  
257 soil layers.

258

259 DIN and DON concentrations

260

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

269

270 White clover temporal contribution to nitrogen leaching (Time experiment)

271

272 The autumn'11 (T1) labelling caused  $^{15}\text{N}$  enrichment of DIN and DON in both of the following leaching seasons,  
273 showing longer-term contribution of white clover to N leaching. White clover grown in a pure stand had a  
274 significantly ( $P<0.001$ ) larger contribution to DIN leaching compared to the mixed stand (Fig. 3). The largest  
275 ( $P<0.001$ ) white clover contribution to the leaching of DIN and DON was observed in the autumn'11 (T1) and  
276 autumn'12 (T4) labellings, but  $\text{DI}^{15}\text{N}$  and  $\text{DO}^{15}\text{N}$  were observed irrespective of when labelling was carried out. The  
277 specific concentrations of  $\text{DI}^{15}\text{N}$  and  $\text{DO}^{15}\text{N}$  followed the same pattern at all labelling times under both swards (Fig.  
278 4).

279

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

281

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

292

293 **Discussion**

294



295 In this study the contribution of white clover to N leaching under the white clover and the white clover-ryegrass  
296 mixed stand was investigated by leaf-labelling with <sup>15</sup>N-urea followed by DIN and DON analysis in percolating soil  
297 solution. The presence of <sup>15</sup>N in the soil solution showed white clover as an immediate source of N leaching and  
298 allowed investigation of the specific contribution of white clover to DIN and DON leaching.

299

300 DIN and DON leaching

301

302 The present study was conducted in low-N-input grassland under temperate climatic conditions and therefore low  
303 leaching levels of both DIN and DON were expected. Nitrogen leaching was estimated on the basis of N  
304 concentrations in suction cups and drainage calculated by the Evacrop model for which inputs were daily  
305 meteorological measurements, crop, sowing and cutting date and soil physical parameters according to Eriksen et al.  
306 (1999). The amount of DIN leaching at 80 cm during the first leaching season was 2 and 17 kg ha<sup>-1</sup> yr<sup>-1</sup> under the  
307 mixed and pure stand, respectively, and during the second leaching season it was 7 and 25 kg ha<sup>-1</sup> yr<sup>-1</sup>, respectively.  
308 The leaching of DON was 2-3 kg ha<sup>-1</sup> yr<sup>-1</sup> under both swards during both leaching seasons. The DIN and DON  
309 leaching under the mixed stand was comparable to previous studies on leaching from grass-white clover mixed  
310 stands under comparable soil and climatic conditions (Vinther et al. 2006) and sward management (e.g. Anguelov et  
311 al. 2011; Eriksen et al. 2004; Eriksen et al. 2010). We found higher levels of DIN leaching under the white clover  
312 pure stand than under the mixed stand, which can be explained by a larger harvest removal of N by ryegrass from  
313 the mixed stand and greater N yield derived from the atmosphere in the pure stand. Given the clear difference  
314 between pure and mixed stands in DIN leaching, we were surprised not to find any difference between the two  
315 swards in relation to DON leaching. At first hand this seems to fit with previous studies showing that DIN and DON  
316 does not originate from the same source (Neff et al. 2003; Scott and Rothstein 2014), but when looking at the results  
317 from <sup>15</sup>N labelling the previous findings does not fit in the present system.

318

319 Temporal contribution of white clover to nitrogen leaching

320

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

330

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

339

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

345

346 Nitrogen transport in the soil profile

347

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

352

353 The results showed that white clover also contributed to the leaching of DON under both swards at all depths.  
354 The  $DO^{15}N$  content did not significantly decrease with depth, which did not confirm our second hypothesis that the  
355 recently deposited DON pool decreases in size with depth, as proposed by the dynamic exchange model (Neff et al.  
356 2003). We observed a temporal increase in  $DO^{15}N$  content under both swards at all depths, indicating an increased  
357 white clover contribution to the leaching of DON. It may be speculated that the greater content of  $DO^{15}N$  in the  
358 second leaching season was due to the larger white clover biomass during the second growing season resulting in  
359 increased deposition of  $^{15}N$  tracer to the soil.

360

361 The mechanism of N leaching from grassland soils

362

363 Our study showed that the specific concentrations of  $DI^{15}N$  and  $DO^{15}N$  followed similar patterns across swards type,  
364 time of labelling and depths, which point to that white clover contributed similarly to DIN and DON leaching  
365 irrespective of differences in harvested N, total N leaching and temporal contribution to N leaching under the two  
366 swards. However, the  $DI^{15}N$  and  $DO^{15}N$  specific concentration do not say anything about the proportion of total N  
367 leaching originating from the labelled white clover. In order to evaluate the proportional contribution from the

368 labelled white clover to total N leaching one has to compare the  $^{15}\text{N}$  enrichment of the DIN and DON leached with  
369 the  $^{15}\text{N}$  enrichment of potential sources. If for example indigenous SON (organic N found in the soil prior to the  
370 growth of the present plants) was the sole source for DON, then the  $^{15}\text{N}$  enrichment of DON would be similar to the  
371 background enrichment of the soil, i.e. no excess  $^{15}\text{N}$  would be present. We will in the following focus on the  
372 leaching results of the 2012-2013 leaching season from the “Transport experiment” where the highest amounts of  
373  $^{15}\text{N}$  were applied. The label of white clover in the transport experiment was applied over two growing seasons, and  
374 we cannot in the present setup separate whether leached  $^{15}\text{N}$  came directly from e.g. white clover shoots or from  $^{15}\text{N}$   
375 that were deposited to the SON pool during the experimental period. Therefore, the leached  $^{15}\text{N}$  will be the sum of  
376 shoot and root direct contributions to leaching and indirect contributions from these via shorter and longer term  
377 deposition of  $^{15}\text{N}$  to the SON pool (Rasmussen 2011; Mayer et al. 2003; Fustec et al. 2010).

378  
379 The  $^{15}\text{N}$  deposition over two growing seasons to the SON pool resulted in an enrichment of the soil of 0.1 at%  
380 excess in the uppermost soil layer, and 0.01 at% excess in the lower layers. If new and old SON had contributed  
381 equally to N leaching, then the resulting enrichment of the leached N should correspond to the overall enrichment of  
382 the SON pool. The leached  $^{15}\text{N}$  had across the three sampling depths an enrichment of 0.1-0.2 at% excess under the  
383 mixed stand and 0.2-0.4 at% excess under the white clover pure stand, which initially demonstrates two things: (i)  
384 white clover derived N was more prone to leaching relative to indigenous SON, i.e. white clover derived N  
385 contribute relatively more to N leaching than SON, and (ii) leached  $^{15}\text{N}$  most likely came from the uppermost soil  
386 layer as the soil  $^{15}\text{N}$  enrichment in the deeper layers was much lower than the N leached or directly from roots in the  
387 deeper soil layers. Furthermore, higher  $^{15}\text{N}$  enrichment of leached N under white clover pure stand than under the  
388 mixed stand in spite of the soil enrichment being similar under the two swards revealed that a significant proportion  
389 of leached  $^{15}\text{N}$  came from white clover shoot and root die off during the leaching season. However, as  $^{15}\text{N}$   
390 enrichments of DIN and DON were similar the N coming from white clover shoot and root die off must have passed  
391 through the same bottleneck before leaching, which most likely is the microbial community.

392  
393 The mechanisms leading  $^{15}\text{N}$  added via leaf-labelling to white clover to be leached as  $\text{DI}^{15}\text{N}$  and  $\text{DO}^{15}\text{N}$  acted both  
394 in the shorter and the longer term, but always through the microbial community; with the shorter-term being  $^{15}\text{N}$   
395 deposited from white clover during the leaching season and the longer-term being the deposition over the two  
396 growing seasons. In both the shorter and the longer-term the deposited  $^{15}\text{N}$  from shoot and root material was  
397 decomposed where  $^{15}\text{N}$  was build-in to microbial tissue and surplus N released as inorganic N. During the growing  
398 season inorganic  $^{15}\text{N}$  was taken up by the plants and therefore did not give a significant contribution to DIN leaching,  
399 especially under the mixed stand where  $^{15}\text{N}$  was removed in harvested grass biomass. We suggest that two main  
400 routes contributed to  $^{15}\text{N}$  found lost as DIN and DON: (i)  $^{15}\text{N}$  that was built into the microbial tissue over the  
401 growing season was during the colder leaching season released as part of the microbial community die off, and (ii)  
402  $^{15}\text{N}$  from microbial decomposition of white clover deposits during the leaching season. For both routes DIN was  
403 formed when surplus N was released as the microbial community decomposed dying microbial tissue or white  
404 clover material, and DON was formed as cell lysates or cell wall release to the soil solution. In order to elucidate this,

405 future studies could look into the  $^{15}\text{N}/^{13}\text{C}$  enrichment of different plant and microbial biomarkers in the leaching soil  
406 solution, which could confirm the suggested DON formation route. Furthermore, future studies including greater  
407 variation in climatic, soil and botanical composition could give additional insight in potential management strategies  
408 to reduce N leaching from grasslands.

409

#### 410 Conclusion

411

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

421

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423

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427

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501 Figure legends:

502

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

504

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

508

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

512

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

516

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

520

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

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526

527

Fig 1

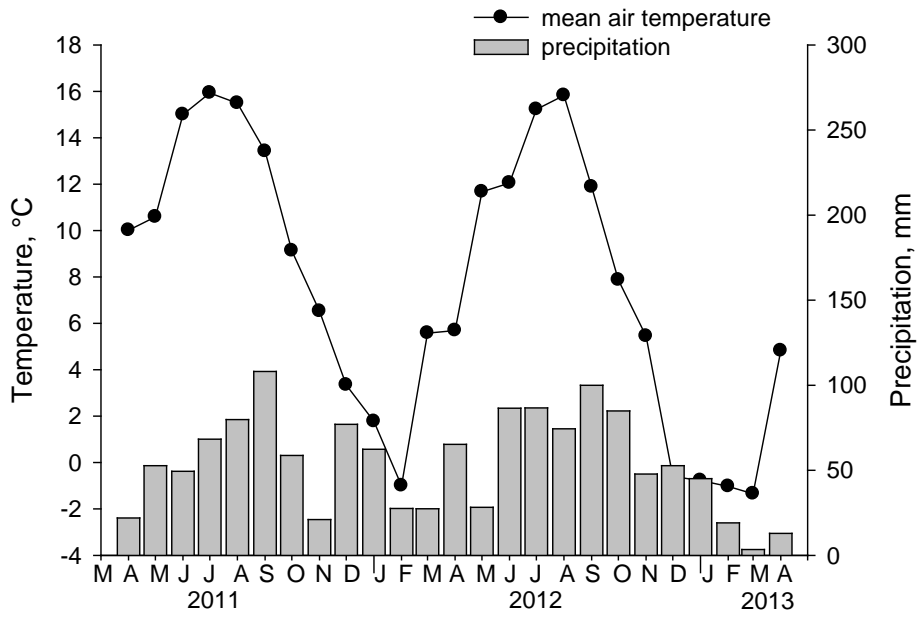






Fig 3

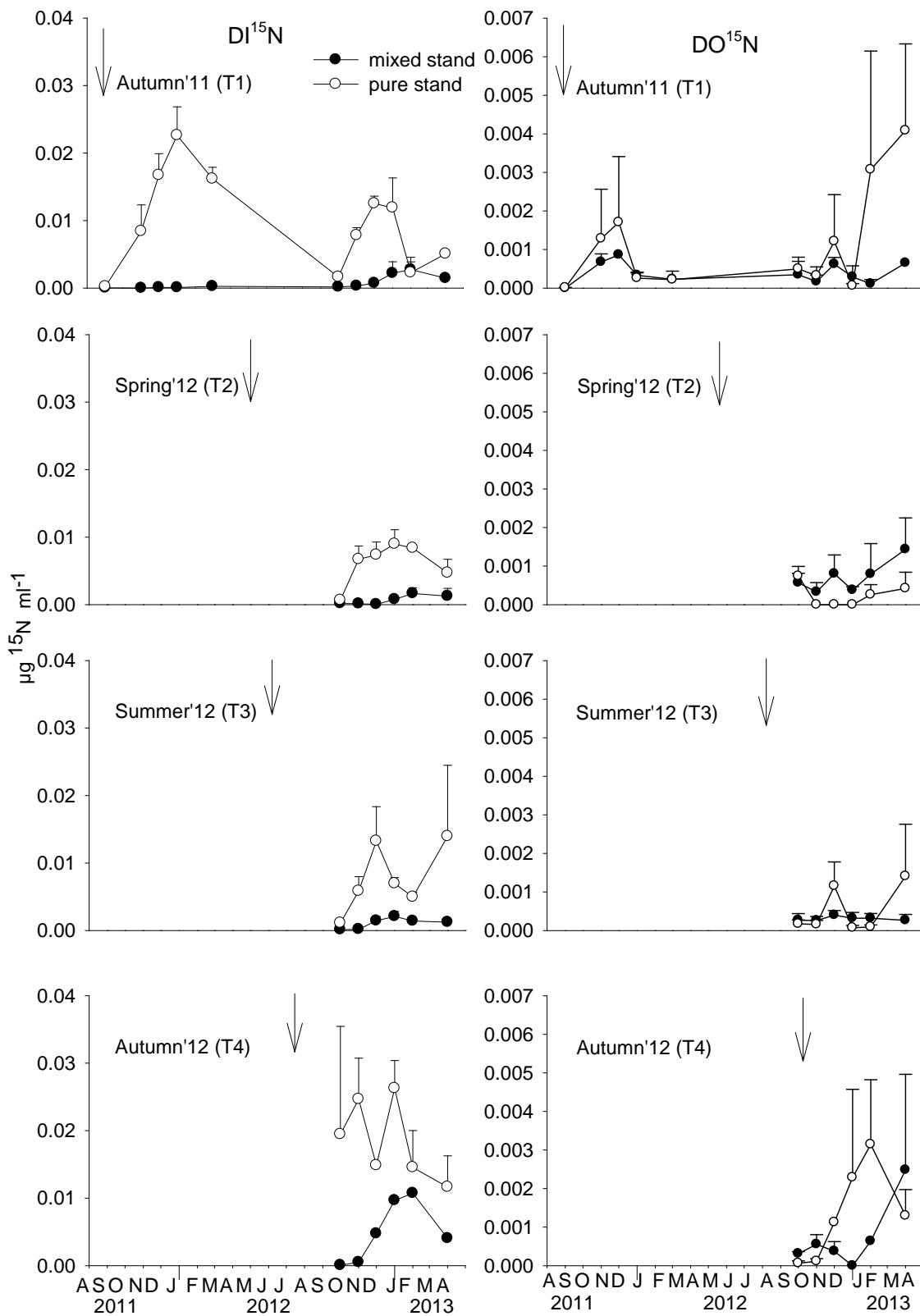




Fig 5

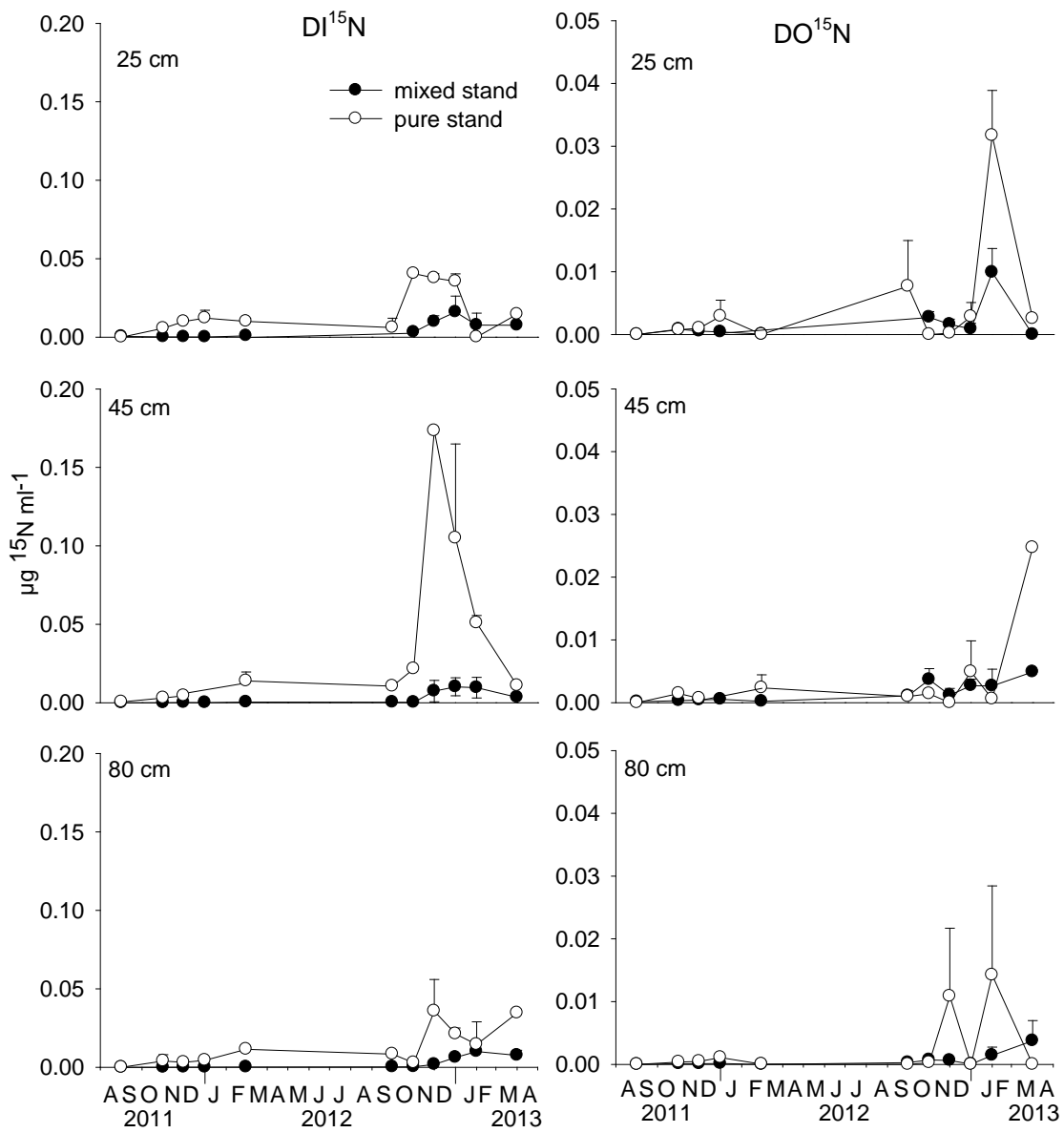
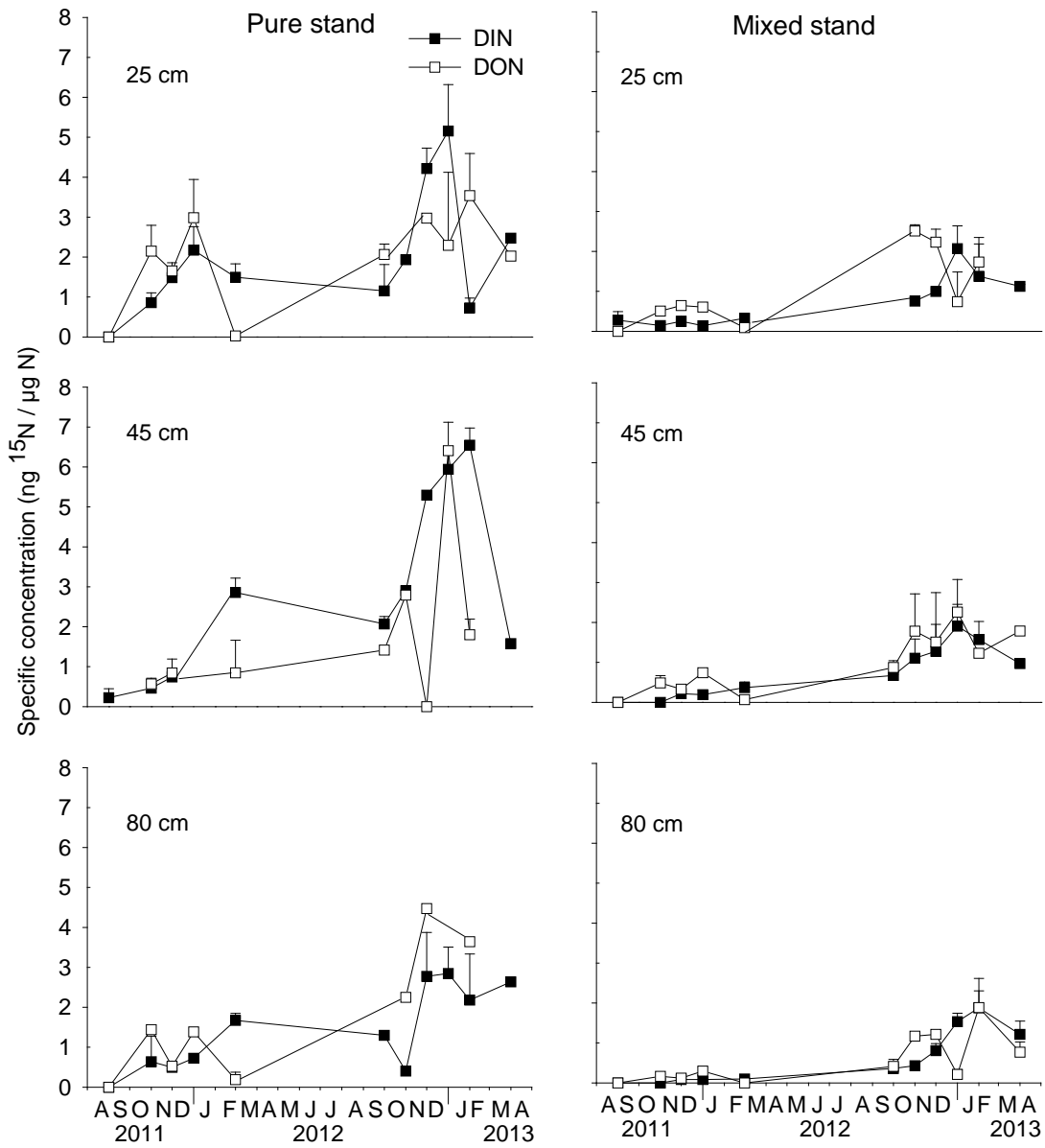


Fig 6



**Table 1.** Time experiment. The  $^{15}\text{N}$  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 $\pm$ SE, n=4).

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

**Table 2.** Transport experiment. The  $^{15}\text{N}$  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 $\pm$ SE, n=4).

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

\*Values are the sum of two harvests for 2011, three harvests in 2012, and final harvest in May 2013.

**Table 3.** Anova table of P values on the effect of: 1) sampling time, depth and sward on total DIN and DON, 2) labelling time, depth and sward on DI<sup>15</sup>N and DO<sup>15</sup>N in time experiment, and 3) sampling time, depth and sward on DI<sup>15</sup>N and DO<sup>15</sup>N in transport experiment.

Analyses of variances	Total		Time experiment				Transport experiment			
	DIN	DON	Concentration		Specific concentration		Concentration		Specific concentration	
			DI <sup>15</sup> N	DO <sup>15</sup> N	DI <sup>15</sup> N	DO <sup>15</sup> N	DI <sup>15</sup> N	DO <sup>15</sup> N	DI <sup>15</sup> N	DO <sup>15</sup> 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	-	-	-	-

\* Signif. codes \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ , ns  $p > 0.05$



**Table 4.** Mean  $^{15}\text{N}$  enrichment (at% excess) of DIN and DON leached from the Transport experiment in the 2012-2013 leaching season. Mean  $\pm$  SE.

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

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