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5 **Biologically fixed $N_2$ as a source for $N_2O$ production in a grass-clover mixture,**

6 **measured by $^{15}N_2$**

7

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## Abstract

The contribution of biologically fixed dinitrogen ($N_2$) to the nitrous oxide ($N_2O$) production in grasslands is unknown. To assess the contribution of recently fixed $N_2$ as a source of $N_2O$ and the transfer of fixed N from clover to companion grass, mixtures of white clover and perennial ryegrass were incubated for 14 days in a growth cabinet with a $^{15}N_2$-enriched atmosphere (0.4 atom% excess). Immediately after labelling, half of the grass-clover pots were sampled for $N_2$ fixation determination, whereas the remaining half were examined for emission of $^{15}N$ labelled $N_2O$ for another eight days using a static chamber method. Biological $N_2$ fixation measured in grass-clover shoots and roots as well as in soil constituted 342, 38 and 67 mg N $m^{-2}$ $d^{-1}$ at 16, 26 and 36 weeks after emergence, respectively. The drop in $N_2$ fixation was most likely due to a severe aphid attack on the clover component. Transfer of recently fixed N from clover to companion grass was detected at 26 and 36 weeks after emergence and amounted to 0.7 ± 0.1 mg N $m^{-2}$ $d^{-1}$, which represented 1.7 ± 0.3 % of the N accumulated in grass shoots during the labelling period. Total $N_2O$ emission was 91, 416 and 259 µg $N_2O$-N $m^{-2}$ $d^{-1}$ at 16, 26 and 36 weeks after emergence, respectively. Only 3.2 ± 0.5 ppm of the recently fixed $N_2$ was emitted as $N_2O$ on a daily basis, which accounted for 2.1 ± 0.5 % of the total $N_2O$-N emission. Thus, recently fixed N released via easily degradable clover residues appears to be a minor source of $N_2O$.

## Key words

emission factor, nitrogen fixation, nitrogen transfer, nitrous oxide, $^{15}N_2$, white clover

38 **Abbreviations**

39 CONT – control

40 EMI – determination of $N_2O$ EMIssion

41 FIX – determination of $N_2$ FIXation

42 START – sampled at the start of labelling

43

44 ## Introduction

45 In temperate organic farming, biological $N_2$ fixation in grass-legume swards provides a
46 major N input to the system, but knowledge is sparse regarding the amount of fixed $N_2$ lost
47 from the grasslands as $N_2O$. Agricultural soils are known to be a considerable source of
48 $N_2O$ (Kroeze et al. 1999) and at present this source accounts for 5 % of the European
49 release of anthropogenic derived greenhouse gases (EEA 2002). Furthermore, $N_2O$ is
50 involved in ozone depletion of the stratosphere (Crutzen 1981). In soils, $N_2O$ is mainly
51 produced in the bacterial processes of nitrification and denitrification (Firestone and
52 Davidson 1989). Thus, legumes may give rise to $N_2O$ by supplying the microbial
53 community in the soil with N compounds. In addition, many strains of the symbiotic $N_2$
54 fixing bacteria *Rhizobium* are able to denitrify nitrate that moves into the root nodules from
55 the soil (O'Hara and Daniel 1985). However, this ability was not found among the strains
56 that form symbiosis with white clover (de Klein et al. 2001).

57 According to the guidelines issued by the Intergovernmental Panel on Climate
58 Change, inventories for $N_2O$ emissions from agricultural soils should be based on the
59 assumption that 1.25 % of the total N supply is emitted as $N_2O$ (IPCC 1997). This emission
60 factor is used as a standard for all N inputs, although the factor relies on experiments with
61 fertilizer and manure only (Bouwman 1996). Input to the systems via biological $N_2$ fixation
62 in grass-legume swards is currently not considered as a source of $N_2O$ in the IPCC

63 guidelines (IPCC 1997), partly due to uncertainties in quantifying the $N_2$ fixation in
64 grasslands (Mosier et al. 1998). Hence, the agricultural greenhouse gas release may
65 presently be underestimated. As organic farming to a very large extent utilises grass-
66 legume mixtures as N source, the contribution from organic farming systems in particular
67 may be underestimated. However, countries are allowed to develop their own inventory
68 methodology based on local measurement data. Some countries, e.g. Denmark and
69 Switzerland, include the contribution from biological $N_2$ fixation in grasslands in the
70 national $N_2O$ inventory, using the standard emission factor of 1.25 % (Schmid et al. 2001;
71 Mikkelsen et al. 2005). This factor nonetheless seems to overestimate the contribution
72 from biologically fixed $N_2$, as substituting fertilizer N with biological $N_2$ fixation is often
73 found to reduce $N_2O$ emissions from grasslands (e.g. Garrett et al. 1992; Ruz-Jerez et al.
74 1994).

75 So far, the $N_2O$ emission factor for biologically fixed $N_2$ in grass-legume swards
76 has only been estimated via modelling (e.g. Schmid et al. 2001) or determined indirectly
77 by relating total $N_2O$ emission to measured $N_2$ fixation (e.g. Ruz-Jerez et al. 1994).
78 Therefore a $^{15}N_2$-tracer-experiment was initiated on grass-clover to assess the contribution
79 of recently fixed $N_2$ as a source of $N_2O$ and the transfer of fixed N from clover to
80 companion grass. The $^{15}N_2$-labelling technique is the sole direct measure of $N_2$ fixation,
81 and in many cases it is the only method to assess the fate of biologically fixed $N_2$
82 (Warembourg 1993). To our knowledge the present study is the first where the $^{15}N_2$-
83 labelling technique is used to determine the contribution of $N_2$ fixation to the $N_2O$
84 production.
85

86 **Materials and methods**

87

88 *Establishment of grass-clover*

89 Air-dried and sieved (1 cm) topsoil from an organic crop rotation was packed in 15 × 15
90 cm pots to a bulk density of 1.46 g $cm^{-3}$. The soil was a loamy sand with total N content of
91 0.12 %, total C content of 1.4 %, pH in water of 7.6 and water-holding capacity of 0.23 g
92 water $g^{-1}$ dry soil. Each pot was either sown with a mixture of white clover (*Trifolium*
93 *repens* L. cv. Klondike) and perennial ryegrass (*Lolium perenne* L. cv. Fanda) or with
94 perennial ryegrass only. All pots were placed in a glasshouse with a day/night regime of
95 16/8 h, minimum temperature 21/16 ºC and minimum light intensity of 120 µmol $m^{-2}$ $s^{-1}$
96 (PAR) provided by fluorescent tubes. Seedlings emerged around 21 March 2002, and after
97 three weeks the plant density was reduced to 14 seedlings per pot (grass:clover, 1:1).
98 Grazing was simulated by cutting to a height of 6 cm every second week. Six weeks after
99 emergence, the pots were transferred outdoors. Ammonium sulphate corresponding to 25
100 kg N $ha^{-1}$ was added at 8, 14, 21 and 26 weeks after emergence. At 28 weeks, pots were
101 transferred to a growth chamber with a day/night regime of 16/8 h, temperature 20/15 ºC
102 and light intensity of 300 µmol $m^{-2}$ $s^{-1}$ (PAR). From 26 weeks after emergence, attempts
103 were made to control aphids on clover via smothering agents and biological pest control by
104 the Asian lady beetle (*Harmonia axyridis*), an aphid midge (*Aphidoletes aphidimyza*) and a
105 parasitic wasp (*Aphidius colemani*).

106

107 *Growth cabinet for $^{15}N_2$-labelling*

108 The $^{15}N$-labelling approach consisted of introducing $^{15}N_2$ into the atmosphere in a
109 minimum-volume closed-system growth cabinet in order to trace the symbiotic $N_2$ fixation.
110 The labelling cabinet (Figure 1) was a modified chest freezer (model TMW300, Frigor,

111 Viborg, DK) in which the volume was reduced to 48 (width) × 86 (length) × 42 (depth) cm
112 by installing a raised floor (4 mm aluminium sheeting). The cabinet could host twelve 15 ×
113 15 cm pots, which were placed in plastic bags and elevated slightly above the floor to
114 hinder water exchange between pots. External growth lamps supplied light through a
115 transparent window of 12 mm plexiglas mounted above a 44 × 82 cm hole cut into the lid
116 of the freezer. To improve the seal between lid and casket, an EPDM rubber gasket was
117 fitted to the sealing edge of the freezer. Circulation of air within the cabinet was achieved
118 using a fan (60 × 60 mm) to blow air from the bottom to the top of the cabinet through a
119 7.5 cm diameter PVC Flex Pipe.

120 Temperature was maintained by a computer, which controlled the compressor of
121 the freezer. The computer also controlled light on/off as well as the supply of $CO_2$ during
122 defined periods in order to keep $CO_2$ near ambient levels. The concentration of $CO_2$ in the
123 cabinet was monitored by an infra-red gas analyser (IRGA; EGM-2, PP Systems, Hitchin,
124 UK). The atmosphere of the system was circulated externally around a closed loop made
125 from copper tubing ($^1/_8$" OD) by a timer-controlled diaphragm pump. A $CO_2$ scrub could
126 be integrated in the closed loop in order to remove excess $CO_2$ produced during night. The
127 scrub consisted of 1 *M* potassium hydroxide (KOH) in a 0.5 litre screw capped serum
128 bottle, mounted with a rubber stopper pierced by two tubes. This scrub was later replaced
129 by a 0.6 litre solid-state soda lime scrub (75 % $CaOH_2$, 3.5 % NaOH), as KOH foam had
130 started to corrode the rubber stopper. A 12 litres tedlar bag attached to the closed loop
131 prevented over-pressure in the system. The closed gas loop was equipped with a sampling
132 port for collecting gas samples and introducing $^{15}N_2$ to the system. Water was provided
133 through a silicon tube to each pot connected to a valve on the outside. The irrigation was
134 adjusted to obtain a soil water content slightly below the water-holding capacity based on
135 initial transpiration measurements, experience from the former labelling event and water

136 status of control pots. Condensate that accumulated at the floor of the cabinet was sucked
137 out daily via a silicone tube connected to a valve on the outside.
138

139 *$^{15}N_2$-labelling*

140 Three 14-day incubations were conducted with grass-clover mixtures at 16, 26 and 36
141 weeks of age. At each labelling event, 16 grass-clover and 12 grass pots were cut to a
142 height of 6 cm. Eight grass-clover and eight grass pots were placed in an ordinary growth
143 chamber with a day/night regime of 16/8 h, temperature at 20/15 ºC and light intensity of
144 300 µmol $m^{-2}$ $s^{-1}$ (PAR). The remaining eight grass-clover and four grass pots were placed
145 in the labelling cabinet under similar conditions. The following day (Day 1), four grass-
146 clover and four grass pots from the growth chamber were sampled to establish the amount
147 of N in the plant material at the start of the labelling period (START pots - soil not
148 analysed). The remaining four grass-clover and four grass pots in the growth chamber were
149 controls and were sampled on day 14 (CONT pots). On day 1, two litres 98 atom% $^{15}N_2$
150 were added to the labelling cabinet and on day 8, a volume of 0.7 litres was added,
151 resulting in a mean enrichment of the atmosphere over the 14-day incubation period of 0.4
152 atom% excess. To compensate for a leaky diaphragm pump during the incubation at 36
153 weeks after emergence, the $^{15}N_2$ addition on day 8 was substituted by addition of about 0.5
154 litres on day 4, 7 and 11. A sample of the cabinet atmosphere was taken daily and stored in
155 an evacuated 120 ml serum bottle fitted with rubber stopper before analysis for $^{15}N$
156 abundance of $N_2$, concentration of $N_2O$, and sometimes (5/14 days) $^{15}N$ abundance of $N_2O$.
157 On day 14, four grass-clover and four grass pots from the labelling cabinet were sampled
158 to establish the $N_2$ fixation during the labelling period (FIX pots).

159

160 *Measurement of* $^{15}N_2O$ *emission*

161 The remaining four grass-clover pots from the labelling cabinet (EMI pots) were
162 transferred to the ordinary growth chamber. During the following eight days, emission of
163 $^{15}N$ labelled $N_2O$ was measured daily from these pots using a static chamber method.
164 Beforehand, water-holding capacity was determined on a set of pots by removing plant
165 shoots and saturating the soil with water. The pots were covered by plastic to hinder
166 evaporation and were then allowed to drain for two days before weighing and
167 determination of gravimetric water content (oven drying at 105 °C for 24 h). At least one
168 hour before onset of gas measurements, the EMI pots were irrigated to reach 60-65 % of
169 the water-holding capacity. For analysis of initial $N_2O$ concentration and $^{15}N$ abundance,
170 two evacuated 3.5 ml Venoject vials and two evacuated 120 ml serum bottles were filled
171 with samples of growth chamber atmosphere using 5 and 60 ml Plastipak syringes,
172 respectively. The same procedures were used when sampling headspace gas during the
173 following cover period. Each pot was placed on an 11.5 × 11.5 × 1.3 cm platform above a
174 shallow (1 cm) tray of water. The pot was then enclosed within an 18 × 18 × 29 cm plastic
175 cover fitted with a rubber stopper to allow sampling, and weighted down to ensure a
176 complete water-seal. After 45, 90, 135 and 180 minutes of cover period, a 3.5 ml sample of
177 the headspace gas was removed through the rubber stopper for analysis of $N_2O$
178 concentration. At the end of the cover period (180 minutes), a 120 ml sample was taken for
179 analysis of $^{15}N$ abundance of $N_2O$. The EMI pots were harvested after eight days of gas
180 measurement. At 36 weeks after emergence, emission of $N_2O$ was also measured for
181 unlabelled grass pots. Once during each experiment, $^{15}N$ abundance of emitted $N_2O$ was
182 determined on unlabelled grass-clover pots. The result was at natural abundance or slightly
183 below, thus 0.3663 atom% was used as the background value in the calculations.

184 The 3.5 ml gas samples were added 2 ml $N_2$ before they were analysed for $N_2O$ in a
185 gas chromatograph (GC-14B, Shimadzu, Kyoto, JP) fitted with a HaySep Q column and an
186 electron capture detector (column and detector temperature were 30 °C and 300 °C,
187 respectively). Concentration of $N_2O$ in gas samples from the labelling cabinet was
188 determined in the same way. Gas samples of 100 µl from the labelling cabinet and from the
189 120 ml samples taken during the cover periods at 16 weeks were analysed manually for
190 $^{15}N$ abundance of $N_2$ using an elemental analyser (EA 1110, Carlo Erba, Milano, IT) fitted
191 with an injection port and coupled in continuous flow mode to an isotope-ratio mass
192 spectrometer (IRMS; Finnigan MAT Delta E or Finnigan MAT Delta Plus, Bremen, DE).
193 All 120 ml samples from the cover periods as well as selected samples from the labelling
194 cabinet were analysed for $^{15}N$ abundance of $N_2O$ following removal of $CO_2$ and cryogenic
195 focusing of $N_2O$ on a trace gas concentration unit (PreCon Finnigan MAT, Bremen, DE) in
196 continuous flow mode to an IRMS (Finnigan MAT Delta Plus, Bremen, DE).
197
198 *Sampling of pots*
199 Shoot material was harvested and sorted into clover and grass. At 26 and 36 weeks after
200 emergence, a dead shoot fraction was also determined for the grass-clover pots. Fresh
201 weight of shoot material from FIX pots was established in order to calculate needed
202 irrigation of EMI pots to reach 60-65 % of water-holding capacity. A root and a soil
203 fraction were obtained by sieving (6 mm), and the root fraction was subsequently cleaned
204 of soil by repeatedly being immersed in water then washed into a fine sieve.
205
206 *Analyses of plants and soil*
207 Dry matter of plant samples was determined (oven dried at 80 °C for 24 h). Plant samples
208 and samples of air-dried soil were finely ground and analysed for total N and $^{15}N$ on the

209 elemental analyser and IRMS (Finnigan MAT Delta Plus, Bremen, DE). In addition, total
210 carbon was measured on soil from grass-clover CONT pots sampled at 16 weeks after
211 emergence. This treatment was also used to determine soil pH in a 10:25 (w:vol)
212 suspension of fresh soil in distilled water. Within eight hours of pot sampling, 10 g
213 portions of fresh soil were extracted in 2 *M* KCl (1:10, w:vol), stirred on a horizontal
214 shaker for one hour. The extracts were filtered through Whatman 40 filters and kept at -20
215 °C until $NO_3^-$ and $NH_4^+$ were analysed colorimetrically on an autoanalyzer (Bran+Luebbe,
216 Norderstedt, DE). Nitrogen-15 abundance of inorganic N was determined in extracts by the
217 diffusion method, where $NO_3^-$ and $NH_4^+$ are converted into $NH_3$, which is trapped on an
218 acidified filter paper (Sørensen and Jensen 1991). The filters were subsequently analysed
219 for $^{15}N$ as described for plant and soil samples.
220
221 *Calculations*
222 When calculating the $N_2$ fixation, the proportion of total N in plants derived from a $^{15}N_2$-
223 enriched atmosphere (*P*) is determined as
224
225 $$P = N_L^*/N_P^* \qquad (1)$$
226
227 where $N_L^*$ is the $^{15}N$ atom% excess enrichment of the legume after exposure to an
228 atmosphere with a $^{15}N$ atom% excess enrichment of $N_P^*$ (Warembourg 1993). Wood and
229 McNeill (1993) show that this *P* value is independent of the plant N pool at the start of the
230 labelling period, which makes the equation suitable for calculating $N_2$ fixation for the FIX
231 pots. However, by extending their argumentation it can be established that the *P* value is
232 also valid in cases where the plants accumulate N both before and after the labelling
233 period. This makes the equation suitable for calculating $N_2$ fixation for the EMI pots as

234 well. The equation is based on the assumptions that N supplied by soil, fertilizer and non-
235 labelled atmosphere have the same $^{15}N$ abundance and that no $^{15}N_2$ remains during the last
236 period. The amount of N originating from fixation during the labelling period ($N_LP$) is
237
238 $$N_LP = N_LN_L^*/N_P^* \quad (2)$$
239
240 where $N_L$ is the N accumulated by the plants during their full growth. For the FIX pots, the
241 amount of N derived from fixation was calculated for each plant-soil fraction (*viz.* clover
242 shoot, grass shoot, dead shoot, root and soil total N), provided that $^{15}N$ abundance of the
243 fraction increased significantly between CONT and FIX pots. Total $N_2$ fixation was
244 determined in the same way for the EMI pots. Additionally, the amount of fixed $N_2$ lost as
245 $N_2O$ during the eight days emission measurement was calculated, and included in the total
246 $N_2$ fixation for the EMI pots.
247 Flux of $N_2O$ was calculated from the linear increase in $N_2O$ concentration in the
248 headspace during the cover period. Emission of $N_2O$-N derived from biologically fixed $N_2$
249 was calculated from the emission of $^{15}N$ labelled $N_2O$, which was determined in two ways.
250 If a significant $N_2O$ emission ($R^2$ of $N_2O$ concentration vs. time $\geq 0.7$) and increase in $^{15}N$
251 abundance of $N_2O$ (end-value $\geq 0.3689$ atom%) were detected, then emission of $^{15}N$
252 labelled $N_2O$ ($CC^*$) was calculated as
253
254 $$CC^* = C_tC_t^* - C_0C_0^* \quad (3)$$
255
256 where $C_0$ and $C_t$ are the $N_2O$ concentration calculated from the regression equation for the
257 start and end of the cover period, respectively, and $C_0^*$ and $C_t^*$ are the atom% excess
258 enrichment of $N_2O$ at the start and end of the cover period, respectively. If only a

259 significant increase in $^{15}N$ abundance of $N_2O$ was detected then the emission of $^{15}N$
260 labelled $N_2O$ was calculated as
261
262 $$CC^{*} = (C_t^{*} - C_0^{*})C_0 \quad (4)$$
263
264 Emission of $N_2O$-N derived from fixed $N_2$ (*CP*) was then established as
265
266 $$CP = CC^{*}/N_P^{*} \quad (5)$$
267
268 which corresponds to equation 2. The estimates were subsequently converted from
269 concentration of $N_2O$ to amount of N. The fraction of fixed N, which was emitted as $N_2O$
270 (*FE*) was calculated as
271
272 $$FE = E_{N2O}/F_{tot} \quad (6)$$
273
274 where $E_{N2O}$ is the amount of fixed N emitted as $N_2O$ per day and $F_{tot}$ is the total $N_2$ fixation
275 during the labelling period per day determined for the EMI pots.
276
277 *Statistics*
278 ANOVAs and Tukey's multiple comparison tests ($\alpha = 0.05$) were performed using SAS
279 General Linear Model procedure (SAS Institute 1997). Homogeneity of variance was not
280 obtained despite transformation when testing soil inorganic N for all grass-clover
281 treatments. Hence, differences between median inorganic N content for each experiment
282 were assessed using the Kruskal-Wallis test. The same constraint appeared for three
283 fractions when testing $^{15}N$ abundance of grass-clover CONT pots against FIX and EMI

284 pots, *viz.* soil of EMI pots at 26 weeks after emergence and clover shoot of FIX and EMI
285 pots at 36 weeks after emergence. Thus, the medians were compared using the Mann-
286 Whitney U-test. In some cases variation was indicated as coefficient of variance (CV),
287 which is the standard deviation in percent of the mean.
288
289 **Results**
290
291 *Labelling cabinet atmosphere*
292 Nitrogen-15 abundance of $N_2$ in the labelling cabinet declined in an apparent exponential
293 pattern (Figure 2), probably because of diffusion of $N_2$ through the 12 mm plexiglas
294 window. This problem was also faced in other $^{15}N_2$ incubation studies (McNeill et al. 1994;
295 Wood and McNeill 1993), and was compensated for by multiple additions of $^{15}N_2$. Mean
296 $^{15}N_2$ enrichment over the labelling period was 0.4369, 0.4177 and 0.3724 atom% excess at
297 16, 26 and 36 weeks after emergence, respectively. Nitrogen-15 abundance of $N_2O$ in the
298 labelling cabinet gave no evidence for release of $N_2O$ derived from biologically fixed $N_2$
299 during the labelling events (data not shown). During nighttime, the $CO_2$ concentration in
300 the labelling cabinet increased to > 1200 ppm. After onset of light, the $CO_2$ decreased
301 assisted by the $CO_2$ scrub (3-5 hours) to near ambient concentrations with a mean of 344,
302 530 and 468 ppm at 16, 26 and 36 weeks after emergence, respectively.
303
304 *Biomass in grass-clover FIX pots*
305 At 16 weeks of age, the clover component made up a significantly larger proportion of the
306 living shoot biomass (82 %) than at 26 and 36 weeks after emergence (65 and 51 %).
307 However, total living biomass including roots did not differ significantly between labelling
308 events ($P = 0.6523$), and constituted 772 ± 38 g dry matter $m^{-2}$ on average (Table 1).

309 Despite the increased $CO_2$ level, conditions in the labelling cabinet had no significant
310 effect on the growth of plants measured as living shoot and root biomass compared to the
311 control ($P = 0.1176$) (Table 1).
312
313 *Nitrogen fixation*
314 Amount of N and $^{15}N$ abundance of the different plant-soil fractions appear in Table 2 and
315 3, respectively. The results on $N_2$ fixation revealed a significant effect of time ($P <$
316 $0.0001$). Accordingly, at 16 weeks after emergence $N_2$ fixation measured in grass-clover
317 FIX pots constituted 342 mg N $m^{-2}$ $d^{-1}$, which declined to 38 and 67 mg N $m^{-2}$ $d^{-1}$ at 26 and
318 36 weeks, respectively (Figure 3). Overall the $N_2$ fixation differed between the FIX and the
319 EMI pots ($P = 0.0171$), which mainly resulted from the higher $N_2$ fixation measured in the
320 EMI pots at 16 weeks after emergence (Figure 3). The difference was probably due to
321 variation in the clover biomass between pots randomly selected for the two treatments.
322 Also, at 16 weeks, $N_2$ fixation calculated for the EMI pots includes fixed N found in the
323 grass shoot and soil fractions, which had not yet reached a significant $^{15}N$ enrichment in
324 the FIX pots (Table 3). Therefore, the fraction of fixed $N_2$ emitted as $N_2O$ (*FE*, equation 6)
325 was calculated using $N_2$ fixation measured in EMI pots. Fixed N accounted for 90 and 63
326 % of the N, which accumulated in clover shoots during the labelling period at 16 and 36
327 weeks after emergence, respectively, but the difference was insignificant ($P = 0.2465$). A
328 percentage was not calculated for 26 week old plants because of negative N accumulation
329 between START and FIX pots due to a severe aphid attack on the clover component (Table
330 2 B).
331

332 *Distribution of fixed N*

333 The majority of fixed N was found in clover shoot biomass (Figure 4). However, the
334 proportion varied significantly between the labelling events, accounting for 96, 69 and 84
335 % of the fixed N at 16, 26 and 36 weeks after emergence, respectively. Transfer of fixed N
336 from clover to companion grass determined in FIX pots was insignificant when the plants
337 were 16 weeks old (Table 3 A). However, eight days after the labelling event when pots for
338 determination of $N_2O$ emission were sampled, a significant increase in $^{15}N$ abundance of
339 grass shoots was detected, demonstrating that N transfer had taken place. At 26 and 36
340 weeks after emergence, the uncorrected transfer of fixed N from clover to grass shoots
341 constituted 1.0 mg N $m^{-2}$ $d^{-1}$ ($P = 0.9761$), which represented 2.6 and 1.5 % of the fixed N
342 ($P = 0.0727$) (Figure 4). An apparent $N_2$ fixation of 0.4 ± 0.1 mg N $m^{-2}$ $d^{-1}$ was detected in
343 pots with grass. This apparent fixed $N_2$ was either supplied via free-living or associative $N_2$
344 fixing bacteria or an artefact due to absorption of $^{15}N$-labelled ammonia ($^{15}NH_3$) through
345 stomata (McNeill et al. 1994). Ammonia contamination of the $^{15}N_2$ gas cannot be excluded.
346 Because of the relatively low importance, these two $^{15}N$ sources may only have introduced
347 minor error in the calculated symbiotic $N_2$ fixation for the grass-clover pots. In contrast,
348 they might have caused overestimation of the determined transfer of symbiotically fixed
349 $N_2$. When the $^{15}N$ enrichment of grass shoots in mixture is corrected for $^{15}N$ enrichment of
350 grass shoots in pure stand, then the transfer of fixed N amount to 0.7 ± 0.1 mg N $m^{-2}$ $d^{-1}$,
351 which represented 1.7 ± 0.3 % of the N accumulated in grass shoot during the labelling
352 period.

353

354 *$N_2O$ emission and soil water content*

355 Total $N_2O$ emission changed significantly over time ($P = 0.0004$) and was 91, 416 and 259
356 µg $N_2O$-N $m^{-2}$ $d^{-1}$ at 16, 26 and 36 weeks after emergence, respectively (Figure 5 A).

357 Emission of $^{15}N$ labelled $N_2O$ was detected at 16 weeks after emergence only and could
358 theoretically derive from 1) biological $N_2$ fixation in clover, 2) biological $N_2$ fixation by
359 free-living or associative bacteria, or 3) $^{15}NH_3$ contamination of the $^{15}N_2$ gas. However, the
360 two latter sources appeared to be minor as no $^{15}N$ labelled $N_2O$ was detected at 26 and 36
361 weeks after emergence. At 16 weeks, emission of $N_2O$-N derived from biologically fixed
362 $N_2$ constituted 1.6 ± 0.2 µg $N_2O$-N $m^{-2}$ $d^{-1}$. Thus, 3.2 ± 0.5 ppm of the $N_2$ fixed by 16 week
363 old clover was emitted as $N_2O$, which accounted for 2.1 ± 0.5 % of the total $N_2O$ emission.
364 Loss of $N_2O$ from grass pots measured at 36 weeks after emergence was 22 µg $N_2O$-N $m^{-2}$
365 $d^{-1}$ and did not differ significantly from the emission measured from grass-clover pots at 16
366 weeks. The advancement of the $N_2O$ emission was found to be similar to that of the soil
367 water content (Figure 5 A, B). The intention was to keep soil water content of the pots at
368 60-65 % of the water-holding capacity during $N_2O$ emission measurements. However,
369 because of excessive irrigation in the labelling cabinet prior to gas measurements, and low
370 transpiration rate at 26 and 36 weeks, the mean soil water content of grass-clover pots was
371 65, 90 and 80 % of the water-holding capacity at 16, 26 and 36 weeks after emergence,
372 respectively ($P = 0.0031$) (Figure 5 B). Soil water content of grass pots at 36 weeks was 64
373 %. Emission of $^{15}N_2$ from the grass-clover pots was assessed at 16 weeks after emergence,
374 however it was found to be below the detection limit.
375
376 *Soil inorganic nitrogen in grass-clover pots*
377 The content of soil inorganic N varied between the experiments, *i.e.* the median of
378 inorganic N was significantly lower at 16 weeks after emergence than at 36 weeks (Figure
379 6). The change was mainly a result of increased ammonium content. Soil inorganic N in
380 the FIX pots corresponded to 75, 1582 and 327 mg N $m^{-2}$ (0-17 cm soil layer) at 16, 26 and
381 36 weeks after emergence, respectively. At 26 weeks, the content in FIX pots was

382 significantly higher than in the CONT and EMI pots. Attempt to determine the $^{15}N$
383 abundance of the inorganic N pool failed, since total amounts of inorganic N trapped from
384 the KCl extracts were inadequate for a proper $^{15}N$ analysis.
385
386 **Discussion**
387
388 *Nitrogen fixation*
389 For 16 week old plants, the total $N_2$ fixation determined in grass-clover shoots and roots as
390 well as in bulk soil constituted 342 mg N $m^{-2}$ $d^{-1}$, which dropped dramatically to 38 and 67
391 mg N $m^{-2}$ $d^{-1}$ at 26 and 36 weeks after emergence, respectively (Figure 3). The main reason
392 for this drop was a severe aphid attack on the clover component, which peaked during the
393 experiment at 26 weeks and was still present 36 weeks after plant emergence. The aphids
394 probably reduced the $N_2$ fixation by contributing to a decline in the clover content from 82
395 to 65 and 51 % of the total herbage dry weight. Clover content is known to be the major
396 factor determining $N_2$ fixation in grass-clover swards (Kristensen et al. 1995). In field
397 studies, $N_2$ fixation is usually determined in the harvested herbage only, e.g. $N_2$ fixation
398 was reported between 206 and 235 kg N $ha^{-1}$ $y^{-1}$ in first year white clover-ryegrass swards
399 having a mean clover content of about 50-60 % (Jørgensen et al. 1999; Vinther and Jensen
400 2000). Assuming a growth season of six months, the reported values correspond to daily
401 $N_2$ fixation rates between 113 and 129 mg N $m^{-2}$ $d^{-1}$. In conclusion, the determined $N_2$
402 fixation at 16 weeks after emergence was relatively high compared to annual field
403 measurements. However, clover content and $N_2$ fixation vary over the growing season (e.g.
404 Jørgensen et al. 1999; Vinther and Jensen 2000), thus results at 16 weeks represent $N_2$
405 fixation at optimal growth conditions. Furthermore, our studies estimate total amounts of
406 fixed N in all pools in contrast to the field measurements.

407

408 *$N_2O$ emission*

409 Total $N_2O$ emission from the grass-clover pots was 91, 416 and 259 µg $N_2O$-N $m^{-2}$ $d^{-1}$ at
410 16, 26 ad 36 weeks after emergence, respectively (Figure 5 A), which is in the same order
411 of magnitude as emissions determined for other extensively managed grasslands containing
412 legumes (e.g. Carran et al. 1995; Wang et al. 1997). The increase in $N_2O$ emission between
413 16 and 26 weeks after plant emergence might relate to the aphid-induced clover shoot
414 death. The reason is that shoot death leads to decay of roots and nodules (Butler et al.
415 1959), which may act as a carbon source for denitrifying bacteria. Also, Beck and
416 Christensen (1987) showed that $N_2O$ emission increased when all above-ground ryegrass
417 was removed or when grass leaves turned yellowish.

418 Soil inorganic N content tended to be higher at 26 and 36 weeks after emergence
419 than at 16 weeks (Figure 6), which was probably due to increased mineralisation of clover
420 tissues combined with decreased clover N uptake. In addition to this, the elevated soil
421 water content during the labelling at 26 weeks (Figure 5 B) may have caused the
422 remarkably high soil inorganic N content in the FIX pots. During the following $N_2O$
423 measurements, the soil was allowed to dry slightly, which enabled nitrification in aerobic
424 microsites. In conclusion, at 26 and 36 weeks after plant emergence $N_2O$ loss via
425 denitrification was favoured by high availability of inorganic N, labile carbon compounds
426 and anaerobic microsites.

427 Soil water content is often found to be a key factor influencing $N_2O$ emissions (e.g.
428 Carran et al. 1995; Ruz-Jerez et al. 1994). Comparing $N_2O$ emission from grass and grass-
429 clover pots (Figure 5 A) having the same soil water content (Figure 5 B – grass-clover at
430 16 weeks, grass at 36 weeks) reveals a tendency for higher $N_2O$ emission from the grass-
431 clover pots. This conforms with results obtained by Duxbury et al. (1982) indicating that

432 legumes can increase $N_2O$ emissions by factor two to three compared to unfertilised grass
433 swards.
434

435 *Transfer of fixed N*

436 It is generally acknowledged that transfer of N from white clover to companion grass
437 mainly involves the long-term mineralisation of dead clover tissues taking place on a scale
438 of months (e.g. Goodman 1988; Ledgard 1991). In addition to the long-term N release, a
439 pool of relatively easily degradable clover residues (e.g. exudates, lysates, secretion and
440 decaying fine roots) may contribute to soil inorganic N on a short-term scale, *viz.* within
441 days or weeks after elimination from the clover plant. Consistent with this view, Laidlaw et
442 al. (1996) observed high release of inorganic N from clover indicating a total turnover of
443 clover root N within three months. In the present study, release of recently fixed N into the
444 soil probably took place through this latter pathway.

445 Transfer of recently fixed N from clover to companion grass was observed in the
446 FIX pots at 26 and 36 weeks after emergence only. However, at 16 weeks recently fixed N
447 was emitted as $N_2O$, demonstrating that recently fixed N was released from the clover
448 component into the soil at that time. In line with this, an increase in $^{15}N$ abundance of grass
449 shoots was detected eight days later in the EMI pots, indicating a slower transfer rate for
450 16 week old mixtures. The reason could be the high competition for light at 16 weeks
451 (clover content 82 %), which seemed to suppress ryegrass growth and thereby N uptake
452 (Table 2 A). On the other hand, the observed transfer at 26 and 36 weeks may be explained
453 by improved light conditions (clover content 65 and 51 %), which tended to stimulate
454 ryegrass growth and N uptake (Table 2 B, C). This conclusion is consistent with results
455 attained by Høgh-Jensen and Schjoerring (2000), showing highest N transfer in spring and
456 autumn, where white clover growth is low and the growth of ryegrass is high.

457 Fixed N transferred from clover to grass constituted 1.7 ± 0.3 % of the N
458 accumulated in grass shoots during the labelling period. In contrast, two other $^{15}N_2$ studies
459 showed no transfer of fixed N from white clover to companion ryegrass in a 19 and 129
460 day experiment (McNeill et al. 1994; McNeill and Wood 1990). However, long-term field
461 studies using the $^{15}N$ dilution technique have reported apparent transfer of fixed N from
462 white clover to companion ryegrass in the range 0 to 80 % of the grass N content (Boller
463 and Nösberger 1987; Ledgard 1991), with the percentage increasing over time after
464 labelling. The transfer of fixed N found in the present study is low compared to the
465 mentioned studies, which supports the general view that short-term N transfer via easily
466 degradable clover residues is less important than the long-term transfer through decay of
467 more recalcitrant clover tissues.
468 It is striking that recently fixed N was transferred to companion grass at 26 and 36
469 weeks after emergence, but was not detected in the emitted $N_2O$. This could indicate that
470 ryegrass and the $N_2O$ producing bacteria utilized different pools of labile N in the soil. One
471 reason may be differences in the spatial distribution of grass roots and $N_2O$ producing
472 bacteria in relation to the zones of clover residue release. Another explanation may be that
473 the fixed N was less available for the nitrifiers and denitrifiers, either due to amino acid
474 uptake by the grass (Falkengren-Grerup et al. 2000) or because N was mostly transferred
475 directly through common mycorrhizal mycelium (Frey and Schüepp 1992).
476
477 *$N_2O$-N derived from fixed $N_2$*
478 Recently fixed N released via easily degradable clover residues may be important in the
479 flow from $N_2$ fixation to $N_2O$ emission. However, the present study revealed that only 3.2
480 ± 0.5 ppm of the recently fixed N was emitted as $N_2O$ on a daily basis. Furthermore,
481 recently fixed N accounted for 2.1 ± 0.5 % of the emitted $N_2O$-N only. A large part of the

482 remaining $N_2O$-N was most likely derived from previously fixed $N_2$, which indicates that
483 long-term N release through decay of more recalcitrant clover tissues probably contributes
484 considerably to the flow from $N_2$ fixation to $N_2O$ emission.

485 The standard IPCC $N_2O$ emission factor of 1.25 % is criticised by some authors for
486 overestimating the $N_2O$ emission from mineral fertilizer (e.g. Lægreid and Aastveit 2002)
487 and by others for underestimating the long-term effect of manure and mineral fertilizer
488 application (e.g. Schmid et al. 2001). According to the methodology currently
489 recommended by IPCC, the national $N_2O$ inventories should not include the contribution
490 from biological $N_2$ fixation in grasslands (IPCC 1997). Some countries, e.g. Denmark and
491 Switzerland, nonetheless include quantitative estimates of biological $N_2$ fixation in
492 grasslands in their $N_2O$ inventory, using an emission factor of 1.25 % as for other N inputs
493 (Schmid et al. 2001; Mikkelsen et al. 2005).

494 However, the standard $N_2O$ emission factor of 1.25 % might be considerably
495 unrepresentative for biologically fixed $N_2$ as only a part of the fixed N is mineralised
496 during the lifetime of the crop (Petersen and Olesen 2002), and because the mineralisation
497 occurs slowly (Velthof et al. 1998). The extent to which clover N released through
498 mineralisation will give rise to $N_2O$ emission from the sward depends on several factors.
499 First, it depends on the sinks for inorganic N, e.g. uptake by grass and clover,
500 immobilisation in microbial biomass and loss by leaching. Second, it depends on whether
501 the abiotic conditions favour $N_2O$ production, e.g. temperature, carbon source and $O_2$ level,
502 mainly regulated by the soil water content. Goodman (1991) showed that white clover
503 primarily contributes to soil organic matter in autumn. In line with this, Garret et al. (1992)
504 found that under mild conditions, 70 % of the annual $N_2O$ emission from a white clover-
505 ryegrass pasture occurred during autumn and winter. In the present study, the $N_2O$
506 emission was measured under temperature and soil water regimes representative of

507 summer conditions. However, even under conditions more favourable for $N_2O$ emission,

508 the contribution of recently fixed N to the $N_2O$ emission would still be minor.

509 Via modelling, Schmid et al. (2001) estimated the $N_2O$ emission factor for

510 biologically fixed $N_2$ in permanent grasslands to be 0.22 %. However, after steady state in

511 soil carbon and nitrogen was reached, the emission factor increased to 0.56 %. Obviously,

512 these emission factors are associated with extremely large uncertainties. Ruz-Jerez et al.

513 (1994) found that the annual $N_2O$ emission represented about 1 % of the N input by

514 legume fixation in grazed grass-clover swards. The $N_2O$ loss often increased following a

515 grazing period, mainly because of N return in animal excreta. Thus, the estimated emission

516 factor includes the contribution from $N_2$ fixation as well as the effect of grazing. In

517 contrast, the effect of grazing animals is accounted for separately in the IPCC

518 methodology.

519 Biological $N_2$ fixation in grass-legume swards should not be neglected as a source

520 of $N_2O$ in the national greenhouse gas inventories, especially not when considering the

521 large area of Europe covered by managed grasslands. However, based on the present study

522 and data from the literature we find it unlikely that the $N_2O$ emission factor for biologically

523 fixed $N_2$ in grass-clover swards would reach the standard emission factor of 1.25 %.

524

525 **Conclusions**

526 Our results support the general view that recently fixed N contributes little to the N transfer

527 from white clover to companion grass. Moreover, only a tiny fraction of the biologically

528 fixed $N_2$ was lost as $N_2O$ over the course of a few weeks, and this fraction represented

529 about 2 % of the total $N_2O$-N emission. Thus, the long-term mineralisation of dead clover

530 tissues is probably more important than recently fixed N for the flow from $N_2$ fixation to

531 $N_2O$ emission.

532

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539

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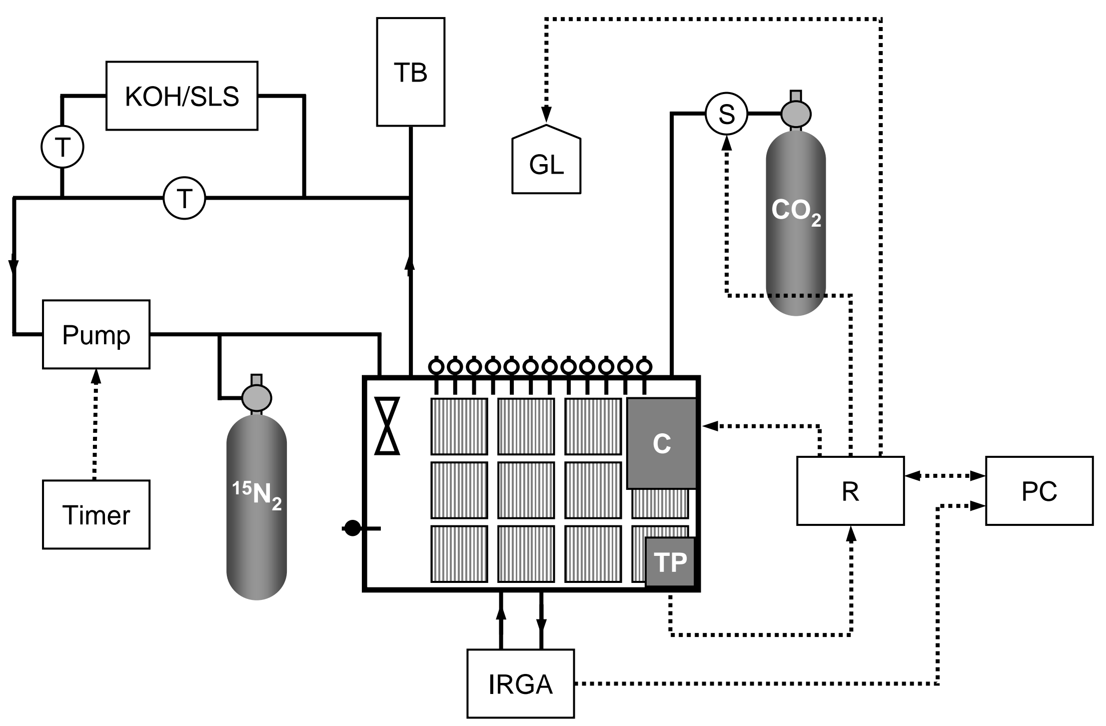
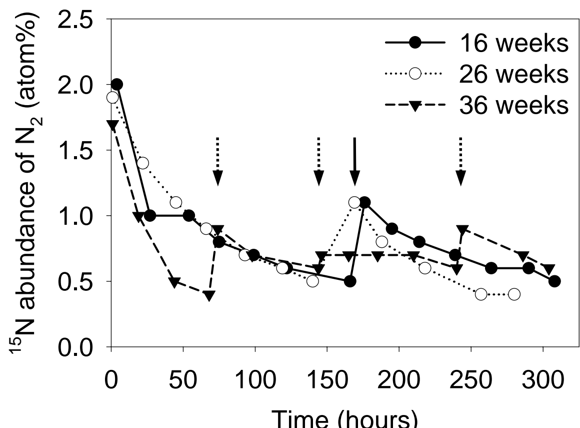
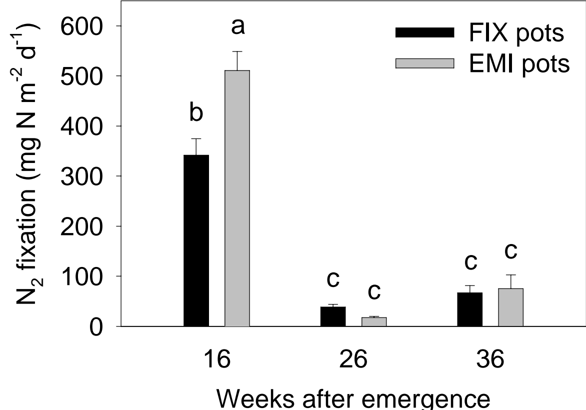


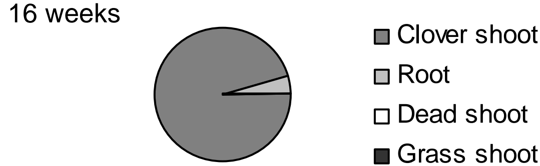

| | |
|---|---|
| C | Compressor |
| GL | Growth lamps |
| IRGA | Infra-red gas analyser |
| KOH | Potassium hydroxide scrub |
| PC | Computer |
| R | Relays |
| S | Solenoid valve |
| SLS | Soda lime scrub |
| T | Toggle valve |
| TP | Temperature probe |
| TB | Tedlar bag to prevent over-pressure |
| | Fan |
| | Tube for irrigation |
| | Tube for collection of condensate |
| | Pipe or tube |
| | Wiring and signal direction |

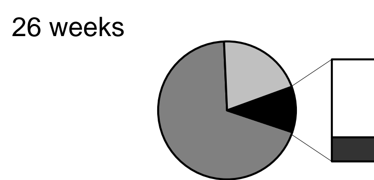


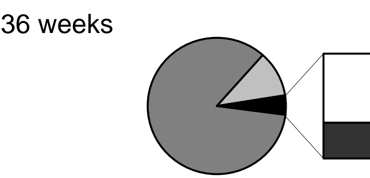
16 weeks
26 weeks
36 weeks
$^{15}$N abundance of N$_2$ (atom%)
Time (hours)
0.0
0.5
1.0
1.5
2.0
2.5
0
50
100
150
200
250
300

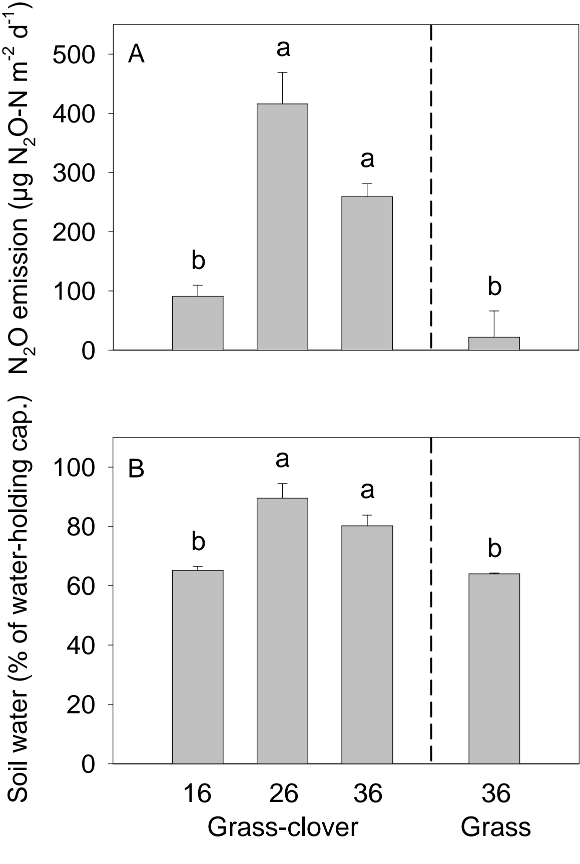


$N_2$ fixation (mg N m$^{-2}$ d$^{-1}$)
600
500
400
300
200
100
0
FIX pots
EMI pots
a
b
c
c
c
c
16
26
36
Weeks after emergence

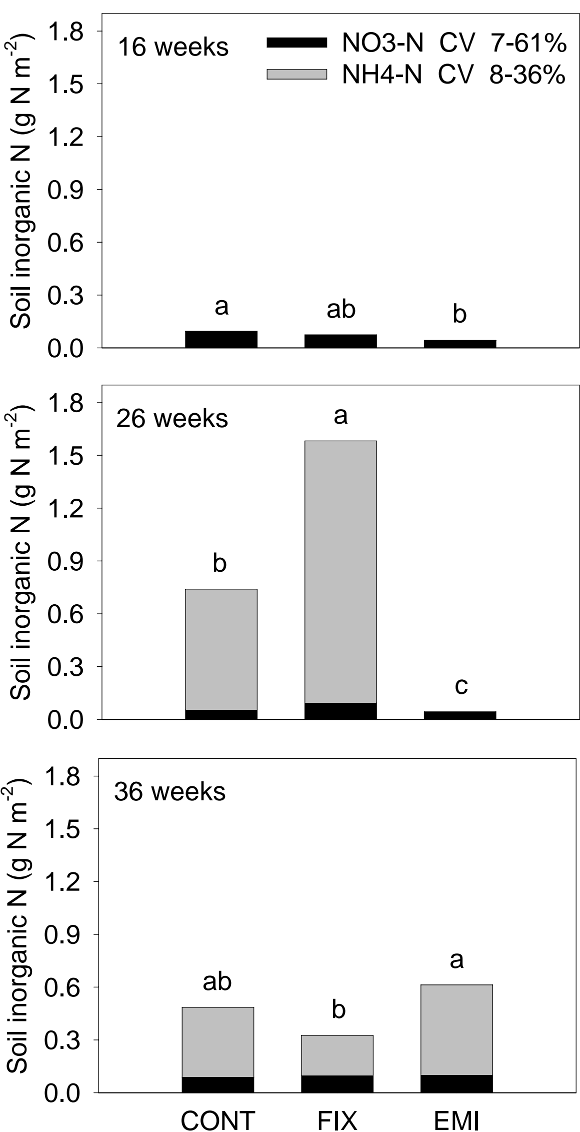

16 weeks
Clover shoot
Root
Dead shoot
Grass shoot


26 weeks


36 weeks



A
B
a
b
N$_2$O emission (µg N$_2$O-N m$^{-2}$ d$^{-1}$)
500
400
300
200
100
0
Soil water (% of water-holding cap.)
100
80
60
40
20
16
26
36
36
Grass-clover
Grass
Weeks after emergence



16 weeks
NO3-N CV 7-61%
NH4-N CV 8-36%
Soil inorganic N (g N m-2)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
a
ab
b
26 weeks
a
b
c
36 weeks
ab
b
a
CONT
FIX
EMI

*Table 1.* Biomass (g dry matter $m^{-2}$) of the fractions clover shoot, dead shoot, grass shoot and root in grass-clover and grass pots at the start (START), for control (CONT), for determination of $N_2$ fixation (FIX) and for determination of $N_2O$ emission (EMI), (A) 16 weeks, (B) 26 weeks and (C) 36 weeks after emergence; n = 4, means and SE (in brackets).

A. 16 weeks

| | Grass-clover pots | | | | Grass pots | | |
|---|---|---|---|---|---|---|---|
| Fraction | START | CONT | FIX | EMI | START | CONT | FIX |
| Clover shoot | 402 (42) | 548 (44) | 493 (34) | 645 (47) | – | – | – |
| Grass shoot | 124 (8) | 147 (10) | 109 (5) | 146 (4) | 190 (13) | 211 (8) | 207 (3) |
| Root | 431 (75) | 322 (38) | 221 (19) | 257 (40) | 235 (27) | 190 (20) | 218 (25) |
| Living biomass [a] | 957 (116) | 1017 (74) | 823 (52) | 1048 (78) | 424 (34) | 400 (23) | 425 (25) |

B. 26 weeks

| | Grass-clover pots | | | | Grass pots | | |
|---|---|---|---|---|---|---|---|
| Fraction | START | CONT | FIX | EMI | START | CONT | FIX |
| Clover shoot | 392 (47) | 341 (60) | 302 (34) | 271 (40) | – | – | – |
| Dead shoot | 177 (16) | 205 (25) | 198 (16) | 226 (20) | – | – | – |
| Grass shoot | 127 (3) | 183 (6) | 158 (8) | 193 (7) | 312 (22) | 293 (27) | 266 (6) |
| Root | 214 (11) | 277 (29) | 273 (13) | 276 (36) | 239 (29) | 226 (30) | 232 (22) |
| Living biomass [a] | 734 (55) | 802 (85) | 733 (36) | 740 (70) | 551 (36) | 518 (56) | 499 (20) |

C. 36 weeks

| | Grass-clover pots | | | | Grass pots | | |
|---|---|---|---|---|---|---|---|
| Fraction | START | CONT | FIX | EMI | START | CONT | FIX |
| Clover shoot | 274 (27) | 235 (62) | 260 (75) | 221 (55) | – | – | – |
| Dead shoot | 334 (35) | 327 (23) | 330 (21) | 424 (22) | – | – | – |
| Grass shoot | 245 (16) | 254 (5) | 224 (9) | 281 (14) | 309 (8) | 385 (12) | 312 (14) |
| Root | 238 (15) | 314 (31) | 275 (37) | 207 (34) | 194 (26) | 245 (36) | 184 (19) |
| Living biomass [a] | 756 (23) | 802 (86) | 759 (102) | 709 (100) | 504 (26) | 630 (40) | 497 (15) |

[a] Living biomass includes the fractions clover shoot, grass shoot and root.

*Table 2.* Amount of N (g N $m^{-2}$) in the fractions clover shoot, dead shoot, grass shoot and root in grass-clover and grass pots at the start (START), for control (CONT), for determination of $N_2$ fixation (FIX) and for determination of $N_2O$ emission (EMI), (A) 16 weeks, (B) 26 weeks and (C) 36 weeks after emergence; n = 4, means and SE (in brackets).

A. 16 weeks

| | Grass-clover pots | | | | Grass pots | | |
|---|---|---|---|---|---|---|---|
| Fraction | START | CONT | FIX | EMI | START | CONT | FIX |
| Clover shoot | 10.9 (0.6) | 16.0 (1.3) | 15.4 (0.4) | 21.7 (1.8) | – | – | – |
| Grass shoot | 1.58 (0.06) | 1.71 (0.06) | 1.57 (0.07) | 1.95 (0.09) | 1.69 (0.15) | 1.94 (0.10) | 1.85 (0.08) |
| Root | 4.60 (0.67) | 3.88 (0.68) | 2.25 (0.11) | 4.16 (0.46) | 1.49 (0.15) | 1.01 (0.06) | 1.21 (0.15) |
| Living biomass [a] | 17.1 (1.1) | 21.6 (1.9) | 19.3 (0.5) | 27.8 (2.3) | 3.18 (0.29) | 2.96 (0.14) | 3.06 (0.22) |

B. 26 weeks

| | Grass-clover pots | | | | Grass pots | | |
|---|---|---|---|---|---|---|---|
| Fraction | START | CONT | FIX | EMI | START | CONT | FIX |
| Clover shoot | 9.55 (1.50) | 6.76 (1.14) | 6.44 (0.81) | 5.57 (0.60) | – | – | – |
| Dead shoot | 3.81 (0.39) | 4.49 (0.57) | 3.96 (0.28) | 4.84 (0.44) | – | – | – |
| Grass shoot | 1.66 (0.05) | 2.02 (0.09) | 1.90 (0.08) | 2.29 (0.13) | 2.73 (0.16) | 2.64 (0.16) | 2.44 (0.04) |
| Root | 3.48 (0.28) | 4.95 (0.61) | 4.85 (0.49) | 4.47 (0.59) | 1.49 (0.16) | 1.43 (0.14) | 1.58 (0.20) |
| Living biomass [a] | 14.7 (1.7) | 13.7 (1.7) | 13.2 (1.0) | 12.3 (1.0) | 4.23 (0.21) | 4.07 (0.30) | 4.02 (0.18) |

C. 36 weeks

| | Grass-clover pots | | | | Grass pots | | |
|---|---|---|---|---|---|---|---|
| Fraction | START | CONT | FIX | EMI | START | CONT | FIX |
| Clover shoot | 5.09 (0.64) | 5.39 (1.42) | 6.16 (1.45) | 5.04 (1.25) | – | – | – |
| Dead shoot | 6.52 (0.68) | 7.12 (0.46) | 7.84 (0.59) | 9.56 (0.91) | – | – | – |
| Grass shoot | 2.15 (0.11) | 2.59 (0.10) | 2.72 (0.07) | 3.07 (0.09) | 2.90 (0.04) | 3.30 (0.13) | 3.13 (0.11) |
| Root | 3.52 (0.30) | 4.45 (0.64) | 3.50 (0.42) | 2.82 (0.51) | 1.19 (0.12) | 1.56 (0.14) | 1.39 (0.24) |
| Living biomass [a] | 10.8 (0.7) | 12.4 (2.1) | 12.4 (1.8) | 10.9 (1.8) | 4.09 (0.11) | 4.85 (0.27) | 4.51 (0.33) |

[a] Living biomass includes the fractions clover shoot, grass shoot and root.

*Table 3.* Nitrogen-15 abundance (atom%) of the fractions clover shoot, dead shoot, grass shoot, root and soil in pots for control (CONT), determination of $N_2$ fixation (FIX) and determination of $N_2O$ emission (EMI) at 16, 26 and 36 weeks after emergence, (A) grass-clover pots, (B) grass pots; n = 4, means.[a b]

A. Grass-clover pots

| | 16 weeks | | | 26 weeks | | | 36 weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| Fraction | CONT | FIX | EMI | CONT | FIX | EMI | CONT | FIX | EMI |
| Clover shoot | 0.3661 | **0.4787** | **0.4754** | 0.3659 | **0.3857** | **0.3783** | 0.3660 | **0.4077** | **0.4185** |
| Dead shoot | – | – | – | 0.3662 | **0.3697** | **0.3685** | 0.3661 | **0.3672** | **0.3669** |
| Grass shoot | 0.3669 | 0.3675 | **0.3689** | 0.3659 | **0.3683** | **0.3677** | 0.3660 | **0.3677** | **0.3672** |
| Root | 0.3667 | **0.3994** | **0.4054** | 0.3666 | **0.3742** | 0.3696 | 0.3666 | **0.3760** | **0.3771** |
| Soil | 0.3688 | 0.3692 | **0.3697** | 0.3692 | 0.3692 | 0.3689 | 0.3691 | 0.3691 | 0.3692 |

B. Grass pots

| | 16 weeks | | 26 weeks | | 36 weeks | |
|---|---|---|---|---|---|---|
| Fraction | CONT | FIX | CONT | FIX | CONT | FIX |
| Shoot | 0.3673 | **0.3680** | 0.3667 | **0.3675** | 0.3667 | **0.3671** |
| Root | 0.3677 | **0.3684** | 0.3676 | 0.3677 | 0.3675 | 0.3676 |
| Soil | 0.3694 | 0.3691 | 0.3693 | 0.3692 | 0.3692 | 0.3692 |

[a] Bold indicates significant increase in $^{15}N$ abundance from CONT pots to FIX and EMI pots.
[b] Coefficient of variance (CV) of $^{15}N$ abundance in the fractions clover shoot and root in grass-clover FIX and EMI pots was in the range 0.47-4.18 %. For all remaining fractions CV was in the range 0.01-0.36 %.