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26 **Abstract (206 words)**

27 **Background and Aims:** Rhizodeposition plays an important role in mediating soil nutrient availability in
28 ecosystems. However, owing to methodological difficulties (i.e. narrow zone of soil around roots, rapid
29 assimilation by soil microbes) fertility-induced changes in rhizodeposition remain mostly unknown.

30 **Methods:** We developed a novel long-term continuous ^{13}C labelling method to address the effects of two levels
31 of nitrogen (N) fertilization on rhizodeposited carbon (C) by species with different nutrient acquisition strategies.

32 **Results:** Fertility-induced changes in rhizodeposition were modulated by root responses to N availability rather
33 than by changes in soil microbial biomass. Differences among species were mostly related to plant biomass:
34 species with higher total leaf and root biomass also had higher total rhizodeposited C, whereas species with
35 lower root biomass had higher specific rhizodeposited C (per gram root mass). Experimental controls
36 demonstrated that most of the biases commonly associated with this type of experiment (i.e., long-term steady-
37 state labelling) were avoided using our methodological approach.

38 **Conclusions:** These results suggest that the amount of rhizodeposited C from plants grown under different levels
39 of N were driven mainly by plant biomass and root morphology rather than microbial biomass. They also
40 underline the importance of plant characteristics (i.e. biomass allocation) as opposed to traits associated with
41 plant resource acquisition strategies in predicting total C rhizodeposition.

42

43

44 **Key words:** carbon, exudation, leaf traits, root traits, nitrogen, plant nutrient use strategies.

45

46 **Introduction**

47 The availability and management of nitrogen (N) is a major constraint on plant productivity in many
48 agro-ecosystems worldwide (Passioura 2002; Goll et al. 2012). In most natural ecosystems and croplands that are
49 not amended with inorganic fertilizers, nutrient availability to plants is largely determined by the activity of
50 saprotrophic soil microbes that decompose soil organic matter (SOM). The resulting products, carbon dioxide
51 (CO₂) and inorganic nutrients, are in turn readily taken up by plants and associated mycorrhizas (Molina and
52 Smith 1998; Valé et al. 2005). In parallel, nutrient cycling in the rhizosphere can also be regulated by the
53 provision of plant carbon (C) to saprotrophic microbiota (Paterson 2003; Bahn et al. 2013). Rhizodeposition,
54 inputs of labile organic solutes and sloughed-off cell tissues from plant roots, can represent around 11% of net
55 fixed C and up to 30% of the C allocated to roots (Jones et al. 2009). This process is believed to constitute a
56 strategy whereby growing plants improve soil nutrient availability by supplying additional energy-rich C (i.e.,
57 carbohydrates) to soil microbes capable of decomposing SOM (Bardgett et al. 1998; 2008). Soluble compounds
58 exuded by roots - mainly sugars, carboxylic acids and amino acids - may account for 1 to 10% of rhizodeposition
59 (Paterson 2003; Jones et al. 2004) and together with other rhizodeposits, they provide energy to soil microbiota
60 for the mineralization of organic N and other nutrients from SOM (Paterson 2003; Jones et al., 2004; Kuzyakov
61 et Xu 2013). Although mineralized N is initially incorporated into the microbial biomass, the rapid turnover of
62 microbial cells relative to roots, ultimately makes it available to plants (Schmidt et al. 2007). Rhizodeposition
63 may thus play an important role in accelerating nutrient cycling in the rhizosphere, which facilitates plant growth
64 (Lynch 1990; Bardgett et al. 1998). Among other factors, the amount of C allocation by plants into the soil can
65 be affected by nutrient availability (Merckx et al. 1987).

66 The role of rhizodeposition in soil nutrient availability has previously been addressed in studies
67 comparing plants grown under different fertility conditions (Paterson and Sim 1999; 2000; Deneff et al. 2009).
68 Nevertheless, the results of these studies are highly variable, which leads to substantial discrepancies in the
69 subsequent conclusions. For instance, studies with plants grown in artificial substrates (such as hydroponics and
70 axenic sand culture systems) demonstrated that the loss of C per unit root mass increased under low N supply
71 compared to high N inputs (Paterson and Sim 1999; 2000). In contrast, the results of studies in which plants are
72 grown in soil are inconclusive, with both positive and negative effects of low N supply on rhizodeposition (Jones
73 et al. 2004; Deneff et al. 2009). These contradictory findings may be partly due to methodological constraints.
74 Rhizodeposited compounds are rapidly assimilated by soil microbes, which limits accurate estimates of total
75 plant rhizodeposit-C (Paterson et al. 2003; 2005; Dilkes et al. 2004). Furthermore, most studies investigating

76 rhizosphere effects on SOM cycling have used pulse labelling or natural tracer techniques, using ^{13}C or ^{14}C
77 isotopes to differentiate plant-derived $\text{CO}_2\text{-C}$ from SOM-derived $\text{CO}_2\text{-C}$ fluxes (Meharg 1994; Andrews et al.
78 1999; Nguyen et al. 1999). On the one hand, continuous ^{14}C labelling is hazardous and requires special facilities
79 that are not readily available to many researchers. On the other hand, pulse-labelling precludes the separation of
80 total plant-derived $\text{CO}_2\text{-C}$ from SOM derived $\text{CO}_2\text{-C}$ because it does not label all plant C pools uniformly
81 (Kuzyakov and Cheng 2004) and consequently, the estimated release of C from roots is biased towards those
82 pools receiving the greatest contribution from recent photoassimilates (Meharg 1994; Paterson et al. 2009).

83 A further possible source of the discrepancies among studies of rhizodeposition could be the use of
84 plant species with distinct nutrient acquisition strategies. Two of these strategies have been described in detail
85 and are strongly linked to soil nutrient availability (Grime 1979; Tilman 1980; Wright et al. 2004). Nutrient-poor
86 ecosystems are dominated by species with low relative growth rates (RGR), coupled with low nutrient
87 concentrations in tissues, such as root nitrogen concentration (RNC) and leaf nitrogen concentration (LNC)
88 (Lavorel and Garnier 2002; Roumet et al. 2006). Conversely, nutrient-rich ecosystems are dominated by species
89 with high RGR and high LNC and RNC (Craine et al. 2001). These contrasting strategies may result in highly
90 variable fertility-induced changes in root exudation among different plant species (Rovira 1969; Dakora and
91 Phillips 2002). Plant species in nutrient-rich ecosystems are most likely to have high C rhizodeposition (Van der
92 Krift et al. 2001) because (i) rapid plant growth is linked to large quantities of photoassimilates, which can be
93 allocated to roots and (ii) root morphologies with high biomass (Craine et al. 2001) and low levels of structural C
94 allow passive diffusion of exudates through root tissue (Valé et al. 2005). In contrast, plant species in nutrient-
95 poor ecosystems have low relative growth rates, which may result in lower rates of C rhizodeposition in soil.
96 Although these species may increase exudation to stimulate soil N mineralization (Van der Krift et al. 2001), its
97 subsequent uptake by plants could be limited by competition with active microbes, which can reduce N-
98 acquisition by plants in the short term (Jones et al. 2004; Kuzyakov and Xu 2013).

99 In this study, we aimed to elucidate the effects of two levels of N availability on rhizodeposited C and
100 the subsequent impact on soil microbiota. To distinguish the effects of N availability from plant nutrient
101 acquisition strategies, we used four grassland plant species with contrasting strategies: exploitative species were
102 represented by *Dactylis glomerata* L. and *Lolium perenne* L.; conservative species were represented by
103 *Anthoxantum odoratum* L. and *Briza media* L. (Quétier et al. 2007; Harrison and Bardgett, 2010). We
104 hypothesised that (i) plants with exploitative strategies would have higher rhizodeposition rates, which would in
105 turn stimulate microbial activity; and that (ii) the changes in rhizodeposition rates of plant species in response to

106 N fertilization would be dependent on plant nutrient acquisition strategies. To achieve this, plants were subjected
107 to continuous ^{13}C labelling in growth chambers for almost three months. Additional soil and plant analyses
108 (NH_4^+ and NO_3^- analysis, soil pH, $^{13}\text{CO}_2$ respired by the roots) were performed in order to identify underlying
109 processes of rhizodeposition patterns among species and fertilization treatments

110 **Material and methods**

111 **Plant cultures**

112 Two exploitative species (*Dactylis glomerata*, DG and *Lolium perenne*, LP), and two conservative species
113 (*Anthoxantum odoratum*, AO and *Briza media*, BM) were selected for this study, all of which belong to the
114 *Poaceae* family. Ramets of *Dactylis glomerata* and *Briza media* were collected from adult tussocks in an alpine
115 grassland (French Alps, 2000 m a.s.l., 45° 4' N - 6°34' E) and ramets of *Lolium perenne* and *Anthoxantum*
116 *odoratum* were collected from adult tussocks in an English grassland (54°18' N - 2°5' W, Yorkshire Dales,
117 United Kingdom) during July 2009.

118 For each species, Roots and leaves were cut 5-cm from the base of each ramet and planted individually in the
119 pot. Each pot was filled with 220 g d.w. grassland topsoil (0–30-cm) collected at the Helmholtz Zentrum
120 experimental station in Scheyern (Germany, 479 m a.s.l., 48°30' N, 11°28' E). The soil was sandy (7.6% clay,
121 10.3% of silt and 82.1% sand) with total soil organic C and N concentration of 0.19g kg⁻¹ and 0.01g kg⁻¹
122 respectively and a soil pH_{H2O} of 6.5.

123 A total of 22 pots per species were prepared and an additional 18 pots without plants ('bare soil pots') were
124 included as controls. In order to inhibit algal growth on the soil surface, the soil in each pot was covered by a
125 circle of dark paper with a hole cut in the center. All pots were distributed in two environment-controlled growth
126 chambers (Conviron E15, Controlled Environments Ltd., Winnipeg, Manitoba, Canada). In both growth
127 chambers, the photoperiod was 10 h day with a photosynthetic photon flux density (PPFD) of *c.* 400 ± 30 μmol
128 m⁻² s⁻¹, mean air temperatures were 22°C during the day and 18°C at night, and relative air humidity was
129 maintained constant at 46%. The soil moisture content in the pots was maintained constant at 70% soil water
130 holding capacity (WHC) by counterbalancing evapotranspiration losses by adding the corresponding amount of
131 water to the pots each every day.

132 **Fertilization treatment**

133 The fertilization treatment aimed to simulate the fertility of the grassland soils where the plant species were
134 collected. The fertilizer was dissolved in irrigating solution containing macronutrients (0.7 mM K, 3.18 mM Ca,
135 0.55 mM P) and micronutrients (6 μM Cl, 14 μM B, 3 μM Zn, 0.7 μM Cu, 0.7 μM Mo, 0.1 μM Co and 200 μM
136 Fe as EDTA) to avoid limitation by nutrients other than N. Half of the replicate pots of each plant species and
137 half of the bare soil controls were fertilized with ammonium nitrate to provide 100 kg N ha⁻¹, whereas the other
138 half received the same irrigating solution without nitrogen. Irrigating solution (with and without nitrogen) was
139 applied twice during the experiment, on the 30/10/09 and on the 06/11/09; for N-fertilized plants, the first and

140 second applications corresponded to a supply of 40 kg N ha⁻¹ and 60 kg N ha⁻¹, respectively.

141 **Labelling procedure**

142 In order to estimate rhizodeposited C, we conducted steady state labelling in a ¹³CO₂ enriched atmosphere. The
143 labelling procedure lasted 81 days in total, from the 02/11/09 until the 21/01/10. Sixteen replicates per species (*n*
144 = 8 per fertilization level) and twelve bare soil pots (*n* = 6 per fertilization level) were placed in a growth
145 chamber with a ¹³C-labelled atmosphere (76 pots in total). The remaining six replicates per species (*n* = 3 per
146 fertilization level) and six bare soil pots (*n* = 3 per fertility level) were kept as controls in the second growth
147 chamber with an unlabelled atmosphere (30 pots total). The design of the experiment is described in detail in S1
148 and S2.

149 In the ¹³C-labelled growth chamber, a 50 ml syringe (SGE, Ringwood, Australia) and needle (model microlance
150 3, BD, Plymouth, UK) were filled with 99.9% ¹³C-CO₂ each day and placed on a syringe pump, which delivered
151 6 ml h⁻¹. This system allowed homogeneous labelling throughout the day. Calculations based on the CO₂ injected
152 by the syringe pump and the volume of the growth chamber gave a total concentration of 410 ppm CO₂ in the
153 growth chamber without taking plant gas fluxes into account. IRGA measurements showed that CO₂
154 concentration varied from 300 to 500 ppm during the day depending on plant gas exchange activity (see S3).
155 Diurnal isotopic composition of atmospheric CO₂ in the growth chamber reached 200 to 300 ‰ (see S3). No
156 ¹³C-CO₂ was injected at night.

157 **Plant and soil sampling**

158 After 71 days, two harvests of pots in the ¹³CO₂-enriched atmosphere were performed 7 days apart to estimate
159 ¹³C flows and mass-balance ¹³C of the soil-plant systems (*t_i* and *t_f*, see S2). In the growth chamber with the ¹³C-
160 labelled atmosphere, 8 replicates per species (*n* = 4 per fertilization level) and 6 'bare soil' pots (*n* = 3 per
161 fertilization level) were harvested on 11/01/10 (*t_i*), and the remaining pots were harvested on 18/01/10 (*t_f*). Thus,
162 plants harvested at *t_f* were grown in the enriched atmosphere for an additional seven days.

163 In the growth chamber with the unlabelled atmosphere, all pots (with and without plants) were sampled in a
164 single day on 21/01/10 (see S2).

165 All pots and treatments were processed following the same procedure: when plants were present, the whole plant
166 was removed from the soil and was shaken by hand to recover soil adhering to the roots; roots and leaves were
167 then separated. Fresh soil from the pots was homogenized by sieving (2-mm mesh) and any remaining root
168 fragments were removed and pooled with the respective root samples. Soil CO₂ efflux and its isotopic signature
169 were analysed as described below. Leaf samples were dried at 60°C for 48h, weighed and ground. Root samples

170 were washed, gently dried with tissue paper and immediately used for measuring root CO₂ efflux and its isotopic
171 signature ($\delta^{13}\text{C}$; see subsequent section), after which, the samples were dried at 60°C for 48h and weighed. The
172 analyses of root CO₂ efflux were used for the interpretation of processes underlying rhizodeposition patterns
173 among species and fertilization treatments but did not allow estimates of ¹³C rhizodeposition (see section on
174 'Isotopic calculations and mass-balance ¹³C budget calculations').

175 **CO₂ gas exchange and isotopic composition measurements**

176 On both sampling dates (t_i and t_f), empty 0.4L Perspex™ dark chambers were previously flushed for 30 min with
177 CO₂-free air passing through the soda-lime column (CO₂ absorbent) of an infra-red gas analyser (Li-6200, LI-
178 COR, Inc., Lincoln, NE, USA). Each root and soil sample was then enclosed separately in one of the chambers.
179 Root and SOM derived CO₂-C effluxes were estimated every minute for 10–15 min. Air temperature (*c.* 20°C)
180 and relative air humidity were recorded during all measurements. The dark chambers were then closed
181 hermetically and CO₂ concentrations were allowed to rise between 600-1000 ppm, after which air samples were
182 collected using the same 50 ml syringe and needle described above (Nogués et al. 2004). The gas samples were
183 passed through a magnesium perchlorate column (water vapour trap) and then immediately injected into a 10 ml
184 vacutainer (BD vacutainer, Plymouth, UK). To avoid contamination, the vacutainers were overpressurized with
185 N₂ and the syringe and needle were also flushed with N₂ before taking each sample.

186 The isotopic composition of root and SOM derived CO₂-C efflux was analysed by gas chromatography-
187 combustion-isotope ratio mass spectrometry (GC-C-IRMS) as described by Nogués et al. (2008). After gas
188 sampling, isotope composition was determined in roots using an Elemental Analyser Flash 1112 (Carlo Erba,
189 Milan) coupled to isotope ratio mass spectrometry IRMS Delta C through a Conflo III Interface (Thermo-
190 Finnigan, Germany).

191 **Soil analysis**

192 Sieved, root-free fresh soil samples were used for all subsequent analyses; soil samples of 18 g dry-weight (DW)
193 equivalent were used to estimate the amount and isotopic signature ($\delta^{13}\text{C}$) of SOM-derived CO₂-C efflux,
194 following the same procedure described for roots above. Additional subsamples were used to determine
195 gravimetric soil water content by drying at 105 °C for 5 h and soil pH in water (1:5 ratio of soil to deionised
196 water). Soil microbial and extractable C and N fractions were determined by the fumigation-extraction method
197 (Vance et al. 1987) using 8 g soil (DW equivalent). In brief, control (unfumigated) subsamples were immediately
198 extracted in 20 ml 0.3 M K₂SO₄, then shaken for 60 minutes, centrifuged and filtered. Fumigated subsamples
199 were exposed to ethanol-free CHCl₃ inside a dark vacuum chamber for 24 h prior to K₂SO₄-extraction. The

200 content and the isotopic composition of the soil C in both the fumigated and unfumigated soil extracts were
 201 estimated by injecting 1 ml of extract into a Surveyor MS HPLC Pump plus (Thermo-Finnigan) coupled to an
 202 IRMS (Delta V Advantage, Thermo-Finnigan, Germany) via an LC Isolink interface (Thermo-Finnigan). The
 203 mobile phase used was a degassed MilliQ water constantly set to 400 µl/min. The quantitative chemical
 204 oxidation of compounds was performed in the LC Isolink interface with sodium peroxodisulfate (Na₂S₂O₈)
 205 solution (100 g/l) and orthophosphoric acid (1.5M H₃PO₄) within an oxidation reactor kept at 99.9°C.
 206 Soil ammonium (NH₄⁺) and nitrate (NO₃⁻) concentrations were analysed by spectrophotometry using 1 to 3 ml of
 207 extract, following the protocols described in Baethgen and Alley (1989) and Cataldo et al. (1975) respectively.
 208 Soil microbial C and N fractions were subsequently calculated as the difference between fumigated and non-
 209 fumigated extractions without correction for extraction efficiency (Vance et al. 1987).

210 **Isotopic calculations and mass-balance ¹³C budget calculations**

211 To estimate ¹³C enrichment in leaf, root and soil samples, the Atom ¹³C % excess for each type of sample was
 212 calculated as difference in the Atom %¹³C between labelled and unlabelled samples (see S1 and S2):

$$\begin{aligned}
 \text{Atom}\%^{13}\text{C}_{\text{excess}} &= \text{Atom}\%^{13}\text{C}_{\text{labelled}} - \text{Atom}\%^{13}\text{C}_{\text{unlabelled}} \\
 &= \left[100 \cdot \left(\frac{R_{\text{labelled}}}{R_{\text{labelled}} + 1} \right) \right]_{\text{labelled}} - \left[100 \cdot \left(\frac{R_{\text{unlabelled}}}{R_{\text{unlabelled}} + 1} \right) \right]_{\text{unlabelled}}
 \end{aligned} \tag{1}$$

213 Where *R* is the absolute isotope ¹³C/¹²C ratio for labelled and unlabelled samples.

214 The labelling-derived ¹³C content (γ¹³C, in µg ¹³C) in leaf, root and soil samples was then calculated as:

$$\gamma^{13}\text{C} = \frac{\text{Atom}\%^{13}\text{C}_{\text{excess}}}{100} \cdot \text{massC}_{\text{sample}} \tag{2}$$

215 where massC_{sample} represents the C content of the sample (in µg).

216 Root- and SOM-derived ¹³CO₂-C (γ¹³R_C, in µg ¹³C g⁻¹ DW h⁻¹) were calculated as:

$$\gamma^{13}\text{Resp} = \frac{\text{Atom}\%^{13}\text{C}_{\text{excess}}}{100} \cdot \text{Resp}_{\text{sample}} \cdot \text{mass}_{\text{sample}} \tag{3}$$

217 where mass_{sample} is the mass of root or soil sample (g DW) and Resp_{sample} is the CO₂-C efflux from root or
 218 microbial biomass (µg C g⁻¹ DW h⁻¹).

219 Cumulative rhizodeposited ¹³C was calculated as the sum of (1) the cumulative labelling-derived ¹³C content in
 220 extractable soil organic C between t_i and t_f, (2) the cumulative labelling-derived ¹³C content immobilized in
 221 microbial biomass between t_i and t_f, and (3) the cumulative labelling-derived ¹³C respired by microbial biomass
 222 between t_i and t_f. As the air temperature during soil CO₂ efflux measurements was similar to temperature inside

223 the growth chamber, cumulative labelling-derived ^{13}C respired by microbial biomass was estimated by
224 integrating soil CO_2 efflux measurements over seven days.

225 Hence, cumulative rhizodeposited ^{13}C ($\mu\text{g}^{13}\text{C}$) over seven days was thus equal to:

$$\int_{t_i}^{t_f} {}^{13}\text{C}_{\text{rhizodeposited}} = \int_{t_i}^{t_f} \gamma {}^{13}\text{C}_{\text{labile soil}} + \int_{t_i}^{t_f} \gamma {}^{13}\text{C}_{\text{microbial biomass}} + \int_{t_i}^{t_f} \gamma {}^{13}\text{Resp}_{\text{microbial respired}} \quad (4)$$

226

227 Rhizodeposited ^{13}C ($\mu\text{g}^{13}\text{C d}^{-1}$) was calculated as cumulative rhizodeposited ^{13}C divided by the number of days
228 between t_i and t_f (i.e. seven days). Finally, specific rhizodeposited ^{13}C ($\mu\text{g}^{13}\text{C g}^{-1}$ root DW d^{-1}) was calculated as
229 rhizodeposited ^{13}C divided by root dry weight.

230 **Statistical analyses**

231 Differences between parameters at times t_i and t_f were calculated as the mean value at t_f subtracted from the
232 replicate i value. Then the mean of the i replicates was determined. For all data, a three-way ANOVA was
233 performed in order to test for the effects of N fertility, species and time (t_i and t_f) effects. Regression analyses
234 were used to explore the relationships between rhizodeposition and plant parameters (plant biomass, root
235 biomass and leaf and root nitrogen concentration). Where main treatment effects were significant, *a posteriori*
236 differences among species were determined by Tukey's HSD (Honestly Significant Differences) test. All
237 analyses were performed with Jump software (V7.0 for window, SAS Institute Inc., Cary, NC, USA).

238

239 **Results**

240 *Plant characteristics*

241 Total plant biomass, leaf and root biomass differed significantly among species and the observed ranking,
242 DG>LP>AO=BM, mainly reflected variations in root biomass (Tables 1 and 2). LP exhibited a significantly
243 higher root:shoot ratio than the others species (1.53 vs. 0.88 on average for the other three species). There were
244 also slight but significant variations in leaf- and root N concentrations among species, where BM had the highest
245 values (Table 1 and 2).

246 Fertilization with N led to a 22% increase in total biomass and a 12% increase in leaf biomass across all species
247 but had no consistent effect on leaf and root N concentration (Table 1 and 2). Root biomass was strongly
248 inversely related to root N concentration (S4, $R^2=0.62$, $F=99.7$, $P<0.0001$) but there was no relationship between
249 leaf biomass and leaf N concentration (S4).

250 The species \times fertility interaction was significant for all biomass parameters ($P<0.001$; Table 2). Leaf and root
251 biomass increased in response to N fertilization in DG and AO, but decreased in BM and LP displayed an
252 increase in leaf biomass but a decrease in root biomass in response to N fertilization. However, root:shoot ratios
253 did not change in response to fertilization (data not shown) but differed among species as DG and LP had higher
254 root:shoot ratios than AO and BM (data not shown, $F=9.23$, $P<0.001$).

255 *Steady-state ^{13}C labelling*

256 The isotopic composition of C in leaves differed significantly between t_i and t_f but the isotopic composition of
257 root C was similar between the two sampling dates (S6). The isotopic composition of root C was lower in AO
258 (mean 238‰) compared to the others species (mean 276‰; S5 and S6) but this pattern was not apparent in
259 isotopic composition of leaf C (overall means for LP, BM and DG: 322‰ compared to 324‰ for AO; S5 and
260 S6).

261 The isotopic composition of extractable soil C and microbial biomass in bare soil pots was similar in growth
262 chambers with labelled and unlabelled atmosphere (S7). The $^{13}\text{CO}_2$ respired by the microorganisms was strongly
263 correlated with ^{13}C microbial immobilization ($R^2=0.56$, $F=36.28$, $P<0.0001$; S8).

264 *Estimation of total rhizodeposited ^{13}C*

265 When all species and fertility treatments were considered, cumulative labelling-derived ^{13}C content in extractable
266 soil C was very low ($-0.0068 \pm 0.0012 \mu\text{g } ^{13}\text{C}$). In contrast, the mean total labelling-derived ^{13}C immobilized in
267 the microbial biomass was high ($48.1 \pm 8.0 \mu\text{g } ^{13}\text{C}$). The mean CO_2 respired by microbial biomass was $0.58 \pm$
268 $3.31 \text{ ng } ^{13}\text{C.g}^{-1} \text{ soil DW min}^{-1}$ over the 7 days; the mean of the $^{13}\text{CO}_2$ respired by microbial biomass efflux was

269 $0.014 \pm 0.001 \text{ ng } ^{13}\text{C.g}^{-1} \text{ soil DW min}^{-1}$. Mean cumulative labelling-derived ^{13}C respired by microbial biomass
270 over the 7 day-period was $30.0 \pm 3.7 \mu\text{g } ^{13}\text{C}$. Total rhizodeposited ^{13}C varied between 5 and $18 \mu\text{g } ^{13}\text{C d}^{-1}$
271 depending on species and fertilization treatment (Fig. 1a).

272 ***Effect of species and fertility on rhizodeposited ^{13}C***

273 More than 65% of the rhizodeposited ^{13}C was immobilized in the microbial biomass and the rest was respired
274 (Fig. 2a and 2b). Accordingly, the immobilization of ^{13}C in the K_2SO_4 -extractable soil organic C fraction
275 represented less than 0.001% of total rhizodeposited ^{13}C (data not shown).

276 When both fertilization treatments were considered together, there was a threefold difference in total
277 rhizodeposited ^{13}C and specific rhizodeposited ^{13}C among species (Table 3, Fig. 1a and 1b). Total rhizodeposited
278 ^{13}C was significantly higher in DG and significantly lower in BM compared to all other species but there was no
279 difference between AO and LP mainly due to the strong effect of fertilization (Fig. 1a). In contrast, specific
280 rhizodeposited ^{13}C was highest in AO, followed by BM, DG and LP and all species differences were significant
281 (Fig. 1b).

282 Fertilization significantly increased total rhizodeposited ^{13}C by 13% across all species (Fig. 1a; Table 3), which
283 was mainly explained by increases in ^{13}C microbial immobilization and ^{13}C respired by microbial biomass in DG
284 and AO pots (Fig. 2a and 2b). In contrast, there was no consistent effect of fertilization on specific
285 rhizodeposited ^{13}C (Fig. 1b; Table 3).

286 The species \times fertility interaction was significant for both total rhizodeposited ^{13}C and specific rhizodeposited
287 ^{13}C (Table 3). Whereas total ^{13}C rhizodeposition in DG and AO was higher with fertilization, the opposite was
288 observed for LP and BM. Specific ^{13}C rhizodeposition was lower with fertilization in DG, LP and BM but
289 increased in AO.

290 Total rhizodeposited ^{13}C was significantly correlated to leaf biomass and leaf N concentration (Fig. 3a, b) and to
291 root biomass and root N concentration (Fig. 3c, d). A significant negative relationship was observed between
292 specific rhizodeposited ^{13}C and root biomass (Fig. 4c) but there was no relationship between specific
293 rhizodeposited ^{13}C and leaf biomass (Fig. 4a), leaf N concentration (Fig.4b) or root N concentration (Fig. 4d).

294 **Impact of species and fertility on soil properties**

295 Soil nitrate concentrations were extremely low in all treatments (*i.e.* below detection limit; data not presented).
296 Concentrations of soil NH_4^+ and microbial biomass N were influenced by plant species, fertilization and
297 sampling date (Table 4 and 5). Soil NH_4^+ concentrations were significantly higher for LP than for AO, DG and
298 BM, whereas microbial biomass N was lower in LP compared to the other species. Soil NH_4^+ content was

299 significantly lower in fertilized pots for all species except BM, whereas microbial biomass N was significantly
300 higher (Table 4 and 5). Soil NH_4^+ concentrations and microbial biomass N were consistently higher at t_i
301 compared to t_f . Total microbial biomass C was not affected by plant species or fertilization (Table 4 and 5) but
302 microbial biomass C was higher at t_f than at t_i across all treatments.

303 Soil pH was significantly affected by species and fertilization (Table 5): soil pH in bare soil pots was 0.30 to
304 0.50 units lower than in pots with plants. Furthermore, soil pH was lowest in BM pots, followed by AO, LP and
305 DG and the difference between the lowest and the highest values was 0.12 units. Finally, fertilization
306 significantly decreased soil pH by a mean of 0.06 units.

307 **Discussion**

308 The aim of this study was to elucidate the impact of two levels of fertilization on the rhizodeposition of grassland
309 species with different nutrient acquisition strategies. Our experimental design avoided several methodological
310 biases reported in the literature (Paterson et al. 2005; 2009), which limit the comparison of rhizodeposited C
311 between species or under different treatments such as fertility levels. Previous techniques based on pulse-
312 labelling were rarely sufficient to uniformly label plant inputs into soil and consequently, only the contribution of
313 recently labelled assimilates could be estimated (Paterson et al. 2005). In our study, the isotopic composition of
314 roots remained constant from t_i to t_f for each species, which indicates that a steady-state was reached and plant
315 inputs to the soil were uniformly labelled. Additionally, a further bias in ^{13}C labelling studies can be introduced
316 by direct fixation of ^{13}C in the microbial biomass by phosphoenolpyruvate carboxylation. In our experiment, the
317 isotopic composition of extractable soil C and microbial biomass in bare soil pots were highly similar in growth
318 chambers with labelled and unlabelled atmosphere and hence direct ^{13}C carboxylation by soil microbes was
319 negligible. Collectively, these results demonstrate that the common biases related to the nature of experiment
320 (i.e. steady-state long-term labelling) were avoided. Although we did not measure soil and root respiration *in*
321 *situ*, our estimates of microbial- and root CO_2 effluxes were similar to those reported in the literature (Tjoelker et
322 al, 2005; Baptist et al. 2009). Hence, although our study precludes estimates of-rhizodeposited ^{13}C fluxes in
323 natural conditions, our experimental design provides accurate comparative data to assess differences among
324 species and fertilization treatments.

325 We observed large differences in the total amount of rhizodeposited ^{13}C as well as specific ^{13}C rhizodeposition,
326 among the four species. Contrary to our expectations, the conservative species displayed higher rhizodeposition
327 fluxes per unit dry weight of root than the two exploitative species. Fertilization consistently affected total
328 rhizodeposited ^{13}C but not specific rhizodeposited ^{13}C . The rate of total rhizodeposited ^{13}C was higher in DG and
329 AO with fertilization whereas the opposite was observed in LP and BM. Hence, the two exploitative species
330 studied, as well as the two conservative species, showed respectively an opposite response to fertilization. These
331 findings suggest that plant rhizodeposition responses to the fertilization were not directly related to nutrient
332 acquisition strategies as we had anticipated, but were species dependent. Therefore, rhizodeposition patterns
333 could not be associated with the leaf economic spectrum (Freschet et al. 2010). Various underlying processes can
334 explain these results including differences in sink strength applied by rhizospheric microbial biomass changes
335 (Jones et al., 2009; Bahn et al., 2013), differences in plant biomass allocation and root morphology (Van der
336 Krift et al. 2001; Warembourg et al. 2003; Darwent et al. 2003) or in shoot architecture which might induce

337 changes in light interception and therefore in whole plant carbon partitioning (Hodge et al. 1997, Paterson et al.
338 1999).

339 Although we observed changes in rhizodeposition patterns among plant species and fertility treatments, we
340 found no differences in soil C microbial biomass; this suggests that, in contrast to other studies, rhizospheric
341 microbial biomass was not the main driver of rhizodeposition process (Jones et al. 2009; Bahn et al. 2013).
342 Indeed, in accordance with the study of Henry et al. (2005), no differences in soil C microbial biomass were
343 found between plant species and fertility treatment whereas changes in rhizodeposition patterns were observed.
344 Furthermore, there was no correlation between total or specific rhizodeposited ^{13}C and soil microbial biomass
345 variations between t_i and t_f (data not shown). The stability of microbial biomass C between sampling dates could
346 be explained by the fact that soil microorganisms were not limited by C availability in any of the treatments
347 (Paterson et al. 2009) but might have suffered from limitation by another resource, such as nitrate. Indeed, nitrate
348 concentrations in the soil in all treatments were below detection limits. It is conceivable that N availability to the
349 soil microbial community may have been limited by competition with plants (Legay et al. 2013) or by exuded
350 plant compounds inhibiting nitrification (Subbarao et al. 2009), resulting in a reduction in microbial growth.

351 Although microbial biomass did not change between sampling dates, we cannot exclude a change in community
352 composition in response to fertilization. Although analyses of microbial community structure was beyond the
353 scope of this work, fertilization with N can favour the development of bacteria at the expense of the fungal
354 community (Denef et al. 2009), which would modify the strength of the C sink in rhizospheric microbial biomass
355 (Jones et al., 2009; Bahn et al., 2013) and affect plant rhizodeposition rates.

356 Microbial biomass did not change in response to different rhizodeposition patterns of plant species, which lends
357 further support the hypothesis that microbial biomass was not a driving force for rhizodeposition rate in our
358 study. In contrast, we observed strong relationships between total rhizodeposited ^{13}C and leaf or root biomass,
359 which supports previous assumptions that exudation patterns are affected by species identity in general and root
360 biomass in particular (Van der Krift et al. 2001; Blagodatskaya et al. 2014). Collectively, these results suggest
361 that changes in rhizodeposition rates following fertilization were not driven by the soil microbial community but
362 rather indicate a substantial influence of plant characteristics on rhizodeposition processes (Farrar et al., 2003;
363 Warembourg et al., 2003; Kuzyakov and Cheng, 2004; Badri and Vivanco, 2009), as demonstrated by previous
364 studies on grassland (Van der Krift et al. 2001; Pausch et al. 2013) and forest species (Bowden et al. 2004).

365 Interestingly, total and specific rhizodeposited ^{13}C were both strongly related to total root biomass. Total
366 rhizodeposited ^{13}C was positively correlated to root biomass and leaf biomass, which indicates that

367 rhizodeposition increases with plant growth. Conversely, specific rhizodeposited ^{13}C (i.e., ^{13}C per g root dry
368 weight) decreased with increasing root biomass, indicating a loss in rhizodeposition efficiency when
369 belowground biomass increases (Henry et al. 2005) but there was no relationship between specific
370 rhizodeposited ^{13}C and leaf biomass, or leaf- and root N concentrations. This loss of rhizodeposition efficiency
371 has previously been observed in two grasses with contrasting nutrient acquisition strategies, *L. perenne*
372 (exploitative) and *Festuca rubra* (conservative), which both displayed a decrease in rhizodeposition rate in
373 response to fertilisation, despite larger root biomass (Paterson and Sim, 1999, 2000). This was attributed to
374 greater specific root length (SRL) at low N, whereby longer but finer roots would increase soil exploration and
375 exudation efficiency, allowing greater nutrient acquisition and stimulation of microbial mineralization processes
376 (Paterson and Sim, 1999, 2000). Moreover, high SRL is associated with a greater number of root tips (Arredondo
377 and Johnson 1999), which have the highest rhizodeposition rate compared to other parts of the root system
378 (Darwent et al. 2003). Taken together, these findings suggest that changes in the amount of rhizodeposited C per
379 unit dry weight root were mainly driven by belowground biomass and root morphology rather than traits
380 associated to plant resource acquisition strategies such as leaf and root N concentration.

381

382 **Conclusions**

383 The new continuous labelling method developed in this study permitted the separation of plant-derived $\text{CO}_2\text{-C}$
384 from microbial-derived $\text{CO}_2\text{-C}$ and avoided most of the biases related to this type of experiment (i.e. steady state
385 long term labelling). It provides a useful approach in order to assess mechanisms involved in patterns of plant
386 rhizodeposition under different soil fertility levels. We suggest that the observed changes in the amount of
387 rhizodeposited C under different levels of N availability were driven mainly by plant biomass rather than soil
388 microbes. Finally, our results underline the potential importance of plant characteristics (i.e; root biomass) as
389 opposed to traits associated with plant resource acquisition strategies in predicting total C rhizodeposition.
390 However, further studies with more species will be necessary in order to confirm these findings.

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Table 1. Responses of plant traits to fertilization treatments without (-N) or with nitrogen addition (+N); as there was no effect of sampling date (see Table 2), data from all pots per treatment and date were pooled to give means and standard errors for $n=8$ per treatment.

Species	Abbr.	Total biomass (g DW)		Leaf biomass (g DW)		Root biomass (g DW)		Leaf N concentration (% DW)		Root N concentration (% DW)	
		-N	+N	-N	+N	-N	+N	-N	+N	-N	+N
Fertility level											
<i>Dactylis glomerata</i>	DG	0.92 (0.07)	1.16 (0.07)	0.56 (0.05)	0.82 (0.04)	0.47 (0.06)	0.89 (0.09)	1.63 (0.14)	1.51 (0.04)	0.76 (0.03)	0.62 (0.03)
<i>Lolium perenne</i>	LP	0.60 (0.03)	0.84 (0.03)	0.29 (0.02)	0.41 (0.04)	0.56 (0.04)	0.47 (0.07)	1.70 (0.05)	2.00 (0.10)	0.65 (0.03)	0.78 (0.03)
<i>Anthoxantum odoratum</i>	AO	0.54 (0.04)	0.83 (0.10)	0.15 (0.02)	0.40 (0.03)	0.16 (0.02)	0.31 (0.03)	1.71 (0.17)	1.51 (0.20)	0.86 (0.04)	0.80 (0.03)
<i>Briza media</i>	BM	0.54 (0.05)	0.49 (0.12)	0.42 (0.04)	0.33 (0.08)	0.27 (0.05)	0.19 (0.05)	1.71 (0.12)	2.35 (0.25)	0.92 (0.09)	1.21 (0.18)

Table 2. Results of analysis of variance (ANOVA) investigating the effects of plant species, fertilization, and time (sampling date) on plant biomass and plant functional traits; F statistics and degrees of freedom (*df*) are given, and significant results at $P < 0.05$ are show in bold.

Variable	Effect	<i>F</i> (<i>df</i>)	<i>P</i>
Total biomass (g DW)	<i>Species</i>	37.47 (3,47)	<0.0001
	<i>Time</i>	2.00 (1,47)	0.16
	<i>Fertility</i>	16.16 (1,47)	0.0002
	<i>Species</i> × <i>Time</i>	1.64 (3,47)	0.19
	<i>Species</i> × <i>Fertility</i>	5.88 (3,47)	0.001
	<i>Time</i> × <i>Fertility</i>	0.10 (1,47)	0.77
Leaf biomass (g DW)	<i>Species</i>	34.15 (3,47)	<0.0001
	<i>Time</i>	1.01 (1,47)	0.32
	<i>Fertility</i>	17.38 (1,47)	<0.0001
	<i>Species</i> × <i>Time</i>	0.97 (3,47)	0.41
	<i>Species</i> × <i>Fertility</i>	6.28 (3,47)	0.001
	<i>Time</i> × <i>Fertility</i>	0.002 (1,47)	0.96
Root biomass (g DW)	<i>Species</i>	32.82 (3,47)	<0.0001
	<i>Time</i>	2.60 (1,47)	0.11
	<i>Fertility</i>	7.04 (1,47)	0.01
	<i>Species</i> × <i>Time</i>	2.16 (3,47)	0.10
	<i>Species</i> × <i>Fertility</i>	9.77 (3,47)	<0.0001
	<i>Time</i> × <i>Fertility</i>	0.20 (1,47)	0.65
Leaf N concentration (% DW)	<i>Species</i>	3.32 (3,44)	0.03
	<i>Time</i>	0.56 (1,44)	0.45
	<i>Fertility</i>	1.96 (1,44)	0.16
	<i>Species</i> × <i>Time</i>	0.18 (3,44)	0.90
	<i>Species</i> × <i>Fertility</i>	2.66 (3,44)	0.06
	<i>Time</i> × <i>Fertility</i>	0.12 (1,44)	0.73
Root N concentration (% DW)	<i>Species</i>	16.93 (3,59)	<0.0001
	<i>Time</i>	1.07 (1,59)	0.30
	<i>Fertility</i>	3.65 (1,59)	0.06
	<i>Species</i> × <i>Time</i>	2.11 (3,59)	0.11
	<i>Species</i> × <i>Fertility</i>	6.94 (3,59)	0.0006
	<i>Time</i> × <i>Fertility</i>	1.54 (1,59)	0.22

Table 3. Results of analysis of variance (ANOVA) investigating the effects of species and fertilization on total rhizodeposited ^{13}C , specific rhizodeposited ^{13}C , microbially immobilized ^{13}C , microbially respired $^{13}\text{CO}_2$ and K_2SO_4 -extractable soil organic ^{13}C ; F statistics and degrees of freedom (df) are given, and significant results at $P < 0.05$ are show in bold.

Variable	Effect	F (df)	P
Total rhizodeposited ^{13}C ($\mu\text{g } ^{13}\text{C d}^{-1}$)	<i>Species</i>	66.4 (3,22)	<0.0001
	<i>Fertility</i>	10.9 (1,22)	0.0032
	<i>Species</i> \times <i>Fertility</i>	83.2 (3,22)	<0.0001
Specific rhizodeposited ^{13}C ($\mu\text{g } ^{13}\text{C g}^{-1}$ root DW d^{-1})	<i>Species</i>	55.3 (3,22)	<0.0001
	<i>Fertility</i>	0.14 (1,22)	0.70
	<i>Species</i> \times <i>Fertility</i>	27.1 (3,22)	<0.0001
^{13}C microbially immobilized ($\mu\text{g } ^{13}\text{C d}^{-1}$)	<i>Species</i>	66.0 (3,22)	<0.0001
	<i>Fertility</i>	15.9 (1,22)	0.006
	<i>Species</i> \times <i>Fertility</i>	150.1 (3,22)	<0.0001
$^{13}\text{CO}_2$ respired by microbial biomass ($\mu\text{g } ^{13}\text{C d}^{-1}$)	<i>Species</i>	12.7 (3,22)	<0.0001
	<i>Fertility</i>	1.38 (1,22)	0.25
	<i>Species</i> \times <i>Fertility</i>	17.1 (3,22)	<0.0001
Extractable soil ^{13}C ($\mu\text{g } ^{13}\text{C}$)	<i>Species</i>	2.55 (3,22)	0.08
	<i>Fertility</i>	5.72 (1,22)	0.03
	<i>Species</i> \times <i>Fertility</i>	1.58 (3,22)	0.22

Table 4. Soil properties without (-N) or with nitrogen addition (+N); soil nitrate concentrations are not given as they were below analytical detection limits; as there was no effect of sampling date (see Table 5), data from all pots per treatment and date were pooled to give means and standard errors for $n=4$ per plant species and $n=3$ for bare soil pots; NA = not measured.

Species / Bare soil plots	Time	Soil NH ₄ ⁺ (µg N-NH ₄ ⁺ kg ⁻¹ soil DW)		Immobilized NH ₄ ⁺ N microbial biomass (µg N kg ⁻¹ soil DW)		C microbial biomass (µg C g ⁻¹ soil DW)		pH	
		-N	+N	-N	+N	-N	+N	-N	+N
<i>Dactylis glomerata</i>	t _i	0.56 (0.08)	0.46 (0.001)	3.05 (0.06)	3.44 (0.12)	312.6 (125.5)	115.0 (6.6)	6.87 (0.04)	6.83 (0.04)
	t _f	0.38 (0.02)	0.40 (0.03)	2.44 (0.21)	3.05 (0.27)	399.9 (72.2)	539.7 (65.5)	6.88 (0.02)	6.80 (0.02)
<i>Lolium perenne</i>	t _i	0.73 (0.04)	0.67 (0.03)	2.68 (0.09)	2.87 (0.11)	330.9 (156.5)	141.0 (67.8)	6.82 (0.01)	6.79 (0.06)
	t _f	0.75 (0.04)	0.63 (0.04)	2.46 (0.12)	2.52 (0.02)	462.0 (119.7)	382.7 (142.7)	6.85 (0.02)	6.77 (0.03)
<i>Anthoxantum odoratum</i>	t _i	0.63 (0.001)	0.63 (0.03)	2.95 (0.09)	3.67 (0.16)	158.0 (37.1)	58.4 (25.4)	6.78 (0.02)	6.75 (0.03)
	t _f	0.63 (0.07)	0.34 (0.06)	2.85 (0.13)	2.71 (0.10)	329.6 (30.0)	520.8 (47.2)	6.80 (0.01)	6.75 (0.01)
<i>Briza media</i>	t _i	0.46 (0.05)	1.92 (0.80)	2.94 (0.06)	3.17 (0.05)	73.5 (40.1)	68.9 (20.4)	6.76 (0.04)	6.69 (0.03)
	t _f	0.30 (0.05)	10.21 (5.70)	2.84 (0.13)	2.84 (0.11)	434.0 (84.1)	479.4 (46.6)	6.76 (0.02)	6.69 (0.03)
Bare soil	-	0.46 (0.02)	0.42 (0.001)	1.80 (0.09)	1.98 (0.35)	NA	NA	6.54 (0.04)	6.29 (0.01)

Table 5. Results of analysis of variance (ANOVA) investigating the effects of species, fertilization and time on soil properties and microbial biomass; F statistics and degrees of freedom (df) are given, and significant results at $P < 0.05$ are show in bold.

Variable	Effect	F (df)	P
Soil NH₄⁺ (µg N-NH₄⁺ kg⁻¹ soil DW)	<i>Species</i>	41.4 (3,46)	<0.0001
	<i>Time</i>	22.8 (1,46)	<0.0001
	<i>Fertility</i>	12.7 (1,46)	0.0009
	<i>Species × Time</i>	2.16 (3,46)	0.10
	<i>Species × Fertility</i>	0.96 (3,46)	0.41
	<i>Time × Fertility</i>	1.50 (1,46)	0.23
N microbial biomass (µg N kg⁻¹ soil DW)	<i>Species</i>	8.37 (3,46)	0.0002
	<i>Time</i>	34.6 (1,46)	<0.0001
	<i>Fertility</i>	15.5 (1,46)	0.0003
	<i>Species × Time</i>	1.44 (3,46)	0.24
	<i>Species × Fertility</i>	1.91 (3,46)	0.14
	<i>Time × Fertility</i>	3.65 (1,46)	0.06
C microbial biomass (µg C g⁻¹ soil DW)	<i>Species</i>	0.98 (3,45)	0.41
	<i>Time</i>	48.3 (1,45)	<0.0001
	<i>Fertility</i>	0.34 (1,45)	0.56
	<i>Species × Time</i>	1.05 (3,45)	0.38
	<i>Species × Fertility</i>	0.98 (3,45)	0.41
	<i>Time × Fertility</i>	5.73 (1,45)	0.02
pH	<i>Species</i>	11.8 (3,47)	<0.0001
	<i>Time</i>	0.03 (1,47)	0.86
	<i>Fertility</i>	14.9 (1,47)	0.0004
	<i>Species × Time</i>	0.07 (3,47)	0.97
	<i>Species × Fertility</i>	0.24 (3,47)	0.86
	<i>Time × Fertility</i>	1.03 (1,47)	0.31

Figure legends

Figure 1. Total rhizodeposited ^{13}C (a) and specific rhizodeposited ^{13}C (b) measured in a stable isotope labelling experiment with (+N) or without (-N) nitrogen addition in pots with planted with *Dactylis glomerata* (DG), *Lolium perenne* (LP), *Anthoxantum odoratum* (AO), or *Briza media* (BM); significant differences among species at $P < 0.05$ (for both fertilization treatments pooled) are indicated by different letters above bars; standard error bars are shown for $n = 4$.

Figure 2. Microbially immobilized ^{13}C (a) and $^{13}\text{CO}_2$ respired by microbial biomass measured in a stable isotope labelling experiment with (+N) or without (-N) nitrogen addition in pots with planted with *Dactylis glomerata* (DG), *Lolium perenne* (LP), *Anthoxantum odoratum* (AO), *Briza media* (BM); significant differences among species at $P < 0.05$ (for both fertilization treatments pooled) are indicated by different letters above bars; standard error bars are shown for $n = 4$.

Figure 3. Relationships between total rhizodeposited ^{13}C and (a) leaf biomass, (b) leaf N concentration, (c) root biomass and (d) root N concentration in a stable isotope labelling experiment with (+N) or without (-N) nitrogen addition in pots with planted with *Dactylis glomerata* (DG), *Lolium perenne* (LP), *Anthoxantum odoratum* (AO), *Briza media* (BM).

Figure 4. Relationships between specific rhizodeposited ^{13}C and (a) leaf biomass, (b) leaf N concentration, (c) root biomass and (d) root N concentration in a stable isotope labelling experiment with (+N) or without (-N) nitrogen addition in pots with planted with *Dactylis glomerata* (DG), *Lolium perenne* (LP), *Anthoxantum odoratum* (AO), *Briza media* (BM).

Figure 1

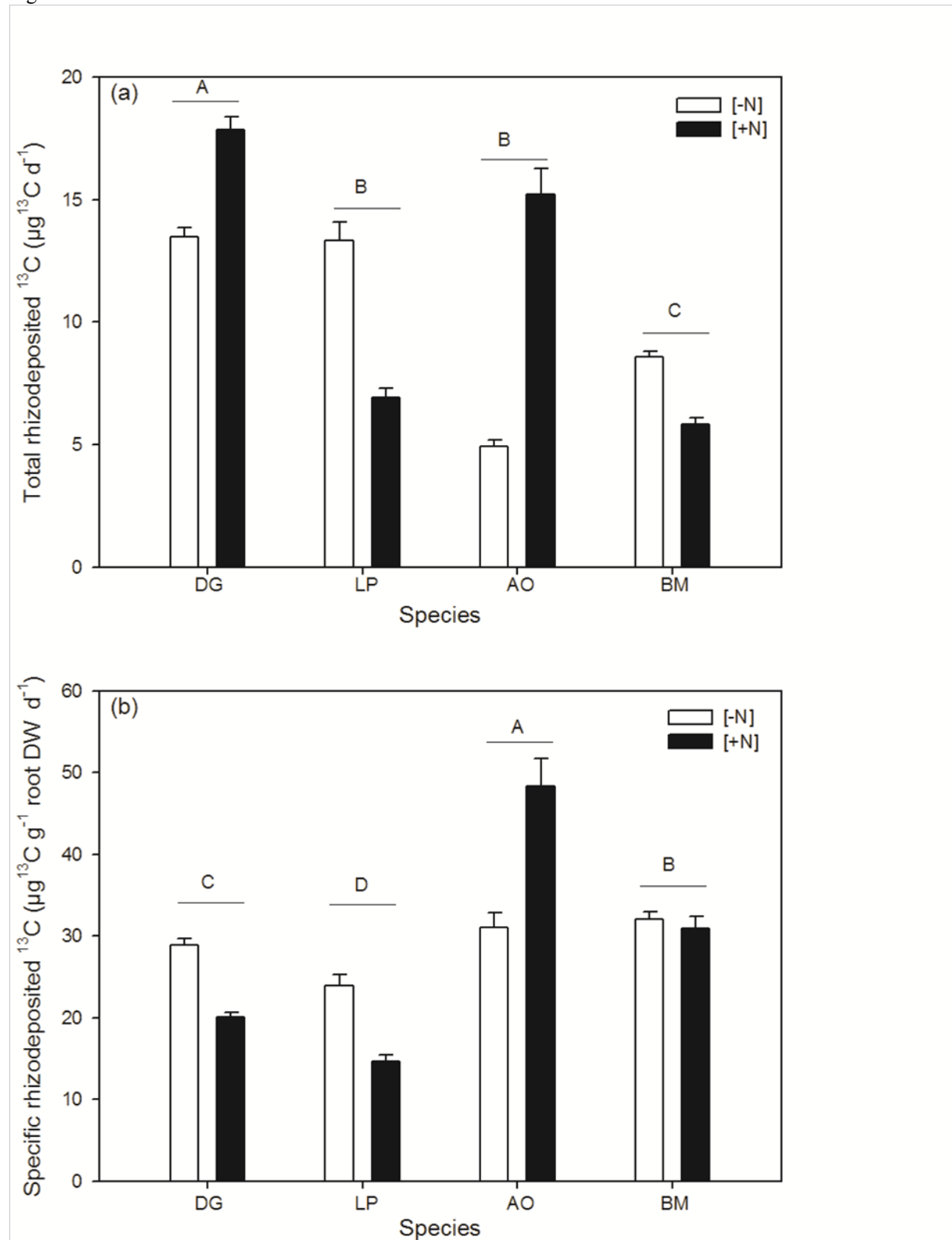


Figure 2

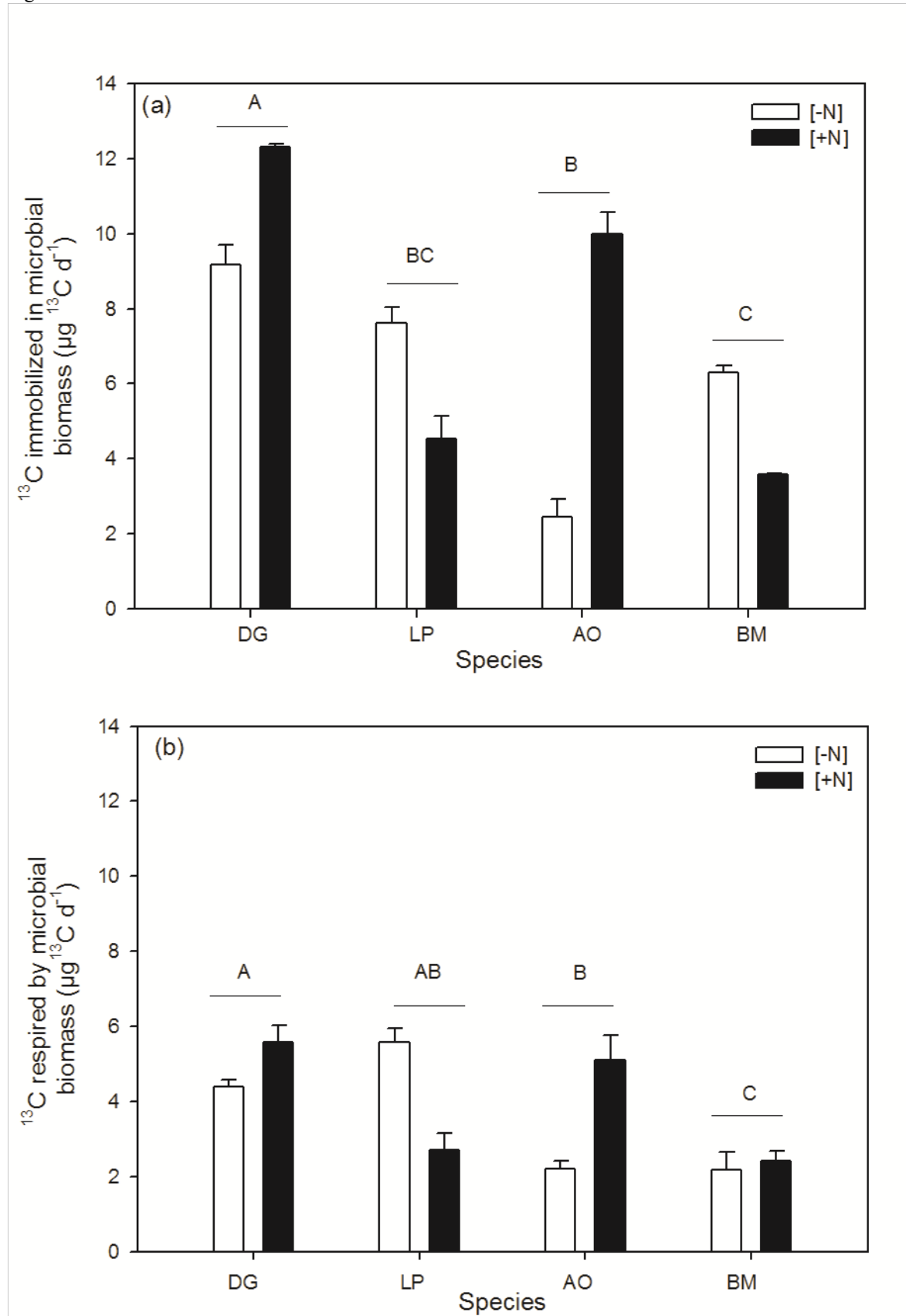


Figure 3

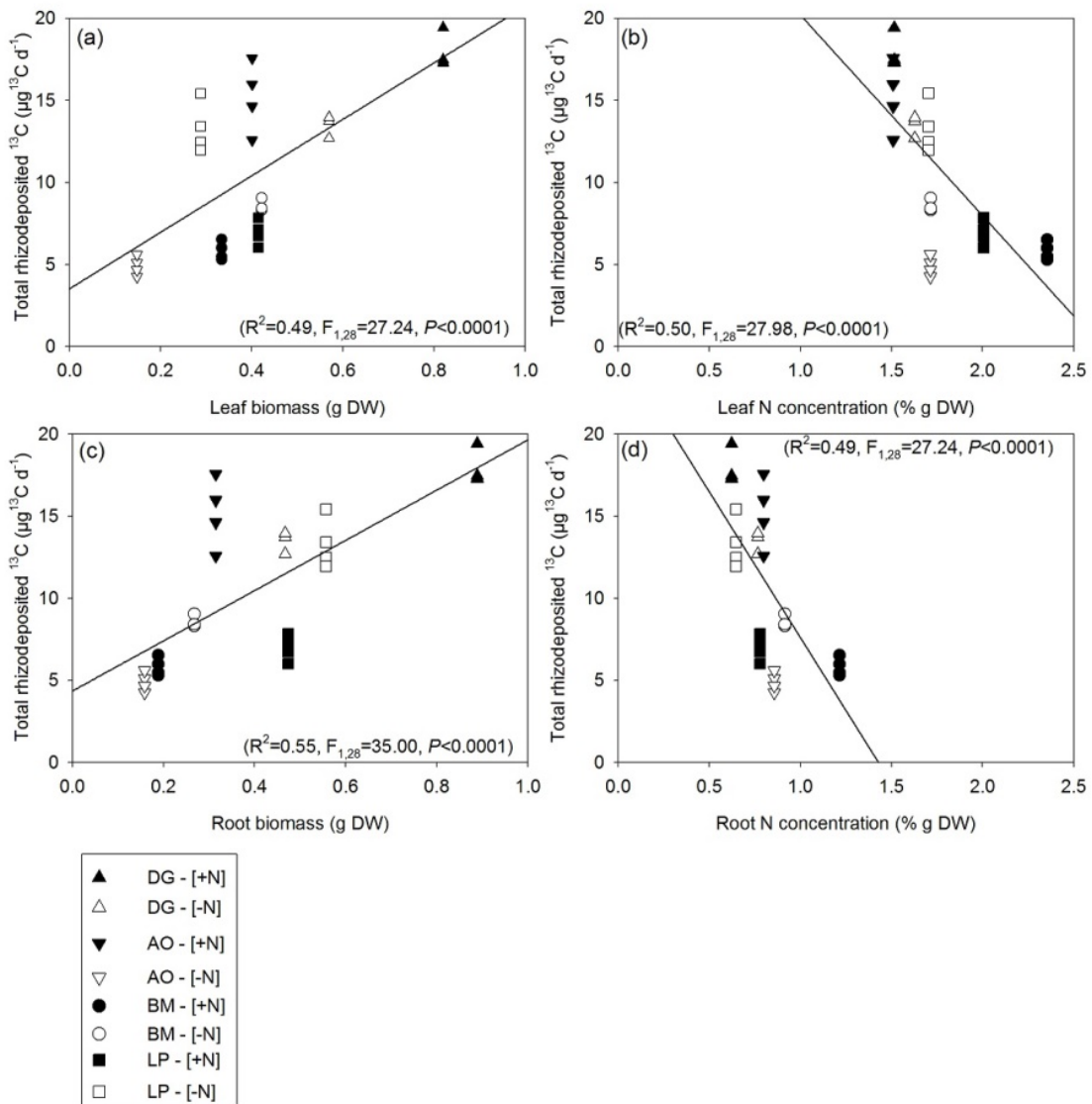


Figure 4

