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1 **N₂ fixation and cycling in *Alnus glutinosa*,**
2 ***Betula pendula* and *Fagus sylvatica* woodland**
3 **exposed to free air CO₂ enrichment.**

4
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25 Author contributions: JM conceived and designed the study, carried out sample
26 collection, analysed data and wrote the manuscript. DG conceived, designed and set
27 up BangorFACE. ARS carried out soil sampling and analysis. HG carried out stable
28 isotope analysis. DG, ARS and HG provided comments on the manuscript. The
29 authors declare that they have no conflicts of interest.

31 **Abstract**

32 We measured the effect of elevated atmospheric CO₂ on atmospheric nitrogen (N₂) fixation
33 for the tree species *Alnus glutinosa* growing in monoculture or in mixture with the non-N₂-
34 fixing tree species *Betula pendula* and *Fagus sylvatica*. We addressed the hypotheses that 1:
35 N₂ fixation in *A. glutinosa* will increase in response to increased atmospheric CO₂
36 concentrations, when growing in monoculture, 2: the impact of elevated CO₂ on N₂ fixation
37 in *A. glutinosa* is the same in mixture and in monoculture and 3: the impacts of elevated CO₂
38 on N cycling will be evident in a decrease in leaf δ¹⁵N and in the soil-leaf enrichment factor
39 (EF), and that these impacts will not differ between mixed and single species stands. Trees
40 were grown in a forest plantation on former agricultural fields for 4 growing seasons, after
41 which the trees were on average 3.8 m tall and canopy closure had occurred. Atmospheric
42 CO₂ concentrations were maintained at either ambient or elevated (by 200 ppm)
43 concentrations using a free-air CO₂ enrichment (FACE) system. Leaf δ¹⁵N was measured and
44 used to estimate the amount (N_{dfa}) and proportion (%N_{dfa}) of N derived from atmospheric
45 fixation. On average 62% of the N in *A. glutinosa* leaves was from fixation. %N_{dfa} and N_{dfa}
46 for *A. glutinosa* trees in monoculture did not increase under elevated CO₂, despite higher
47 growth rates. However, N₂ fixation did increase for trees growing in mixture, despite the
48 absence of significant growth stimulation. There was evidence that fixed N₂ was transferred
49 from *A. glutinosa* to *F. sylvatica* and *B. pendula*, but no evidence that this affected their CO₂
50 response. This study shows that N₂ fixation in *A. glutinosa* may be higher in a future elevated
51 CO₂ world, but that this effect will only occur where the trees are growing in mixed species
52 stands.

53 **Key words:** FACE; ¹⁵N natural abundance; greenhouse gasses; forest ecology; plant
54 interactions.

55 **Introduction**

56 Human manipulation of the carbon (C) cycle has increased the concentration of Carbon
57 Dioxide (CO₂) in the atmosphere, with future increases expected to have large environmental
58 impacts (Soloman et al. 2007). Forest ecosystems play an important role in the global C cycle
59 because they contain almost 60% of global terrestrial C (Grace 2004) and contribute approx.
60 50-60% of terrestrial net primary productivity (Saugier et al. 2001). As a result they exchange
61 large amounts of CO₂ with the atmosphere and are important sinks for anthropogenic CO₂
62 emissions (Pacala et al. 2001; Saugier et al. 2001; Janssens et al. 2003).

63 Tree growth is limited by present atmospheric CO₂ concentrations (Long et al. 2004) and so
64 is predicted to be stimulated by elevated atmospheric CO₂ (Norby et al. 2005). However, tree
65 growth in natural systems is also regularly limited by nitrogen (N) availability (Körner 2003;
66 Millard et al. 2007). Furthermore, trees may become increasingly N-limited as atmospheric
67 CO₂ concentrations rise, because increased growth is accompanied by increased N
68 requirement which may not be met by increased root N uptake (Luo et al. 2004). As a result
69 the ‘fertilisation’ effect of elevated CO₂ may be reduced (Oren et al. 2001; Ainsworth and
70 Long 2005; Reich et al. 2006b). However, elevated CO₂ might also stimulate increased N
71 uptake (Finzi et al. 2007), through increased plant investment in N capture to support
72 increased growth. This strong interdependence between N and C use means that
73 understanding the interactions between elevated atmospheric CO₂ and N use and cycling in
74 forests is essential, for the accurate prediction of future global C dynamics (Reich et al.
75 2006a). In particular the role of atmospheric N₂ fixation in plant and ecosystem responses to
76 elevated CO₂ has been relatively little studied in forest ecosystems.

77 By directly accessing N fixed from the atmosphere by symbiotic bacteria, N₂-fixing plants are
78 able to reduce their reliance on root-derived N to some extent (Postgate 1998; Vessey et al.
79 2005). Furthermore, N₂ fixation is an important source of N for forest ecosystems, providing
80 on average between 1.8 – 25.4 kg N ha⁻¹ globally, and up to 150 kg N ha⁻¹ in temperate

81 forests (Cleveland et al. 1999). N₂ fixation in trees may be stimulated by elevated CO₂
82 (Hungate et al. 1999; Temperton et al. 2003; Feng et al. 2004) due to increased carbon supply
83 to root nodules (Tissue et al. 1997). However, this effect may disappear in the long term due
84 to changes in light availability and/or reduced supply of other nutrients (e.g phosphorous,
85 iron and molybdenum) (Hungate et al. 2004). Therefore, the growth of N₂-fixing plants may
86 show a different response to elevated CO₂ than non-N₂-fixing plants, at least when N
87 availability is limiting (Bucher et al. 1998; Poorter and Navas 2003). For example, in the only
88 FACE (free-air CO₂ enrichment) experiment to-date to have included an N₂-fixing tree
89 species, Eguchi et al. (2008) found that the photosynthetic response of alder saplings was
90 different to that of birch saplings. Down regulation of photosynthesis occurred in birch under
91 elevated CO₂; for alder down regulation of photosynthesis occurred in fertile soil, but not in
92 infertile soil.

93 Plants rarely grow in isolation and their response to elevated CO₂ can be affected by the
94 extent and type of plant-plant interactions they experience (Poorter and Navas 2003). Plant
95 responses to elevated CO₂ when growing with other plants are poorly predicted by
96 performance in isolation (Poorter and Navas 2003). Additionally, the impact of elevated CO₂
97 on plant performance in mixture can differ from the impact on plant performance in
98 monoculture. Therefore, it is important to measure plant responses to elevated CO₂ when
99 growing in different combinations of species. For example, N limitation in the entire plant
100 community can be reduced when N₂-fixing plants are present (Roggy et al. 2004; Daudin and
101 Sierra 2008), which might influence the response of the community to elevated CO₂. FACE
102 studies in grassland systems have shown that the CO₂ effect on legume N₂ fixation is similar
103 in mixed and single species communities (Lee et al. 2003). The presence of N₂-fixing plants
104 in these communities enhanced leaf N content and photosynthesis in co-occurring non-N₂-
105 fixing plants, but did not affect the CO₂ response of these plants. No FACE studies in forest

106 systems have included mixed species stands containing N₂-fixing tree species. Therefore, it is
107 not clear how N₂-fixing and their non-N₂-fixing neighbours and will respond in mixed
108 species stands.

109 When growing with N₂-fixing plants, non-N₂-fixing plants may be able to access some fixed
110 N through direct transfer by release from nodulated roots, along common mycorrhizal
111 networks or indirectly through decomposition of nodules, roots or aboveground litter (He et
112 al. 2003; Roggy et al. 2004; Daudin and Sierra 2008). This facilitative plant-plant interaction
113 can provide a significant proportion of the total N requirements of non-N₂-fixing plants. For
114 example, significant amounts of the N in non-N₂-fixing species (*Pinus contorta* and
115 *Dichanthium aristatum*) has been shown to originate from atmospheric fixation by their N₂-
116 fixing neighbours (*Alnus glutinosa* and *Gliricidia sepium*) (Arnebrant et al. 1993; Daudin and
117 Sierra 2008). Nonetheless, as far as we are aware no study has considered the impact of
118 elevated atmospheric CO₂ on the transfer of fixed N between N₂-fixing and non-N₂-fixing
119 trees.

120 The measurement of the relative abundance of the two most abundant stable isotopes of N
121 (¹⁴N, which constitutes approximately 99.6% of all N and ¹⁵N, which constitutes
122 approximately 0.4% of all N), provides a useful tool for investigating the N cycle. Some
123 processes result in fractionation (i.e. the preferential movement or uptake of the heavier or
124 lighter isotope) resulting in relative ¹⁵N enrichment (i.e. an increase in the proportion of ¹⁵N
125 and therefore δ¹⁵N) or ¹⁵N depletion (i.e. a decrease in the amount of ¹⁵N and therefore δ¹⁵N).
126 Thus, the δ¹⁵N of a tree reflects the δ¹⁵N of the N source(s) subject to any fractionation that
127 occurs during movement from or to the tree, gains and losses of N and N pool mixing
128 (Robinson 2001). As such changes in δ¹⁵N can indicate changes in these components of forest
129 N cycling (Emmett et al. 1998; Robinson 2001; BassiriRad et al. 2003). While these changes
130 cannot necessarily be used to quantify specific differences in the N cycle, they can be used to

131 identify areas that might be affected by any impacts on the N cycle. However, where two
132 sources of N contribute to a pool, and the $\delta^{15}\text{N}$ of each is distinctly different, the $\delta^{15}\text{N}$ of the
133 sources and pool can be used to estimate the relative contribution of each source. This
134 method is well established for measuring the contribution of atmospherically fixed N to the
135 total N content of plants (Boddey et al. 2000; Unkovich et al. 2008).

136 In this study we measured the proportion of N that was derived from atmospheric fixation
137 (%Ndfa) for the N_2 -fixing tree *A. glutinosa* growing in monoculture or in a mixture with
138 *Betula pendula* and *Fagus sylvatica* in a FACE study (BangorFACE). Previous monitoring
139 showed no significant effect of CO_2 on biomass except for an increase in the biomass of *A.*
140 *glutiosa* growing in monoculture (Smith 2010). Specifically, we aimed to address the
141 hypotheses that 1: N_2 fixation in *A. glutinosa* will increase in response to increased
142 atmospheric CO_2 concentrations, when growing in monoculture, 2: the impact of elevated
143 CO_2 on N_2 fixation in *A. glutinosa* is the same in mixture and in monoculture and 3: the
144 impacts of elevated CO_2 on N cycling will be evident in a decrease in leaf $\delta^{15}\text{N}$ and in the
145 soil-leaf enrichment factor (EF), and that these impacts will not differ between mixed and
146 single species stands.

147 **Materials and Methods**

148 *Site description and sampling methods*

149 The BangorFACE site is located on a north west facing shallow slope of approximately $1\text{-}2^\circ$
150 on a deltaic fan at 13-18 m a.s.l. at the Henfaes research station of the University of Wales,
151 Bangor (UK Grid ref: SH655730; Lat. 53.23, Long. -4.02). The climate is hyperoceanic, with
152 annual rainfall of about 1000 mm. Soils are fine loamy brown earth over gravel (Rheidol
153 series) and are 63% sand, 28% silt and 9% clay (Teklehaimanot and Sinclair 1993). Water

154 table depth ranges between 1-6 m. Total wet and dry N deposition is estimated to be 27.9 kg
155 N ha⁻¹ year⁻¹ (3 year mean for 2006-2009, APIS 2010)

156 The FACE plots are located within a wider forest plantation, which is continuous over a total
157 area of 2.36 ha and is spread over two fields that are within 20 m of each other. This
158 plantation was established at the same time as the FACE plots in March 2004 and was
159 planted with a mixture of tree species (*Anus glutinosa* (L.) Gaertn., *Betula pendula* Roth.,
160 *Fagus sylvatica* L., *Fraxinus excelsior*, *Acer pseudoplatinus*, *Castanea satvia* and *Quercus*
161 *robur*) and has been subject to no human disturbance since planting. Four FACE and four
162 ambient plots were randomly located within this plantation, evenly split between the two
163 fields, in a complete replicated block design. These experimental plots were 8 m in diameter,
164 and planted at 80 cm spacing in a hexagonal design (approx. 18000 stems ha⁻¹) with 2 year
165 old *B. pendula*, *A. glutinosa* and *F. sylvatica*. These species are native to the UK, cover a
166 range of ecological and life history traits, and can grow together in semi-natural systems. At
167 planting the trees were approximately 60 cm in height, when the CO₂ system was turned on
168 in 2005 they were respectively 140.71±8.1, 116.82±6.3 and 51.17±2.63 cm in height, at the
169 time of leaf collection in 2008 canopy closure had occurred and the trees were on average
170 463.21±10.8, 487.83±9.7 and 196.25±7.2 cm in height respectively. The plots are surrounded
171 by a 10 m buffer strip of *B. pendula*, *A. glutinosa* and *F. sylvatica* planted at the same
172 density. The planting pattern within these plots created seven sub-plots with mixtures
173 containing one, two or three species. For the purpose of this study, trees in four of these sub-
174 plots were measured: three single species sub-plots and the sub-plot containing a mixture of
175 all three species. These three treatments (CO₂, mixture/monoculture and species) are
176 combined in a 2×2×3 full factorial design resulting in 12 treatment combinations.

177 Carbon dioxide enrichment was achieved using pure CO₂ from natural gas injected through
178 laser-drilled holes in tubing mounted on eight masts (Miglietta et al. 2001). The elevated CO₂

179 concentrations were measured at 1 minute intervals and were within 30% deviation from the
180 pre-set target concentration of 200 ppm above ambient (ambient=380 ppm, elevated=580
181 ppm) CO₂ for 75-79% of the time during the photosynthetically active part of 2005-2008 (i.e.
182 from spring bud-burst until autumn leaf abscission).

183 Total tree biomass (aboveground + belowground) in the plots was monitored over the course
184 of the experiment using stem diameters and site specific allometric equations and is reported
185 in Smith (2011). At the conclusion of the experiment in 2008 the only statistically significant
186 impact of elevated CO₂ was a 32% increase in total *A. glutinosa* biomass under elevated CO₂
187 when growing in monoculture. There was no significant impact of elevated CO₂ on any of the
188 other species growing in monoculture or any of the species growing in mixture. *Alnus*
189 *glutiosa* and *B. pendula* growing in mixture were significantly larger than when growing in
190 monoculture, whereas *F. sylvatica* were smaller when growing in mixture (Smith 2011).

191 Measurements and leaf samples were taken in 2008, when the trees were approximately 6.5
192 years old, after 4 growing seasons of the CO₂ treatment. Three individual trees were sampled
193 from each species growing in monoculture and in mixture (i.e. $n=3$ trees per species per sub-
194 plot, 18 trees per ring), in each of the 4 ambient and elevated FACE rings (total $n=144$ trees).

195 The trees to be sampled were chosen from those in the centre of each sub-plot (i.e.
196 monoculture or mixture), from where they were selected at random. For trees growing in
197 monoculture all 6 nearest neighbours (accounting for the hexagonal planting design) were the
198 same species. For trees growing in mixture the 6 nearest neighbours contained at least one
199 individual from each of the three species. For each tree, diameter of the main stem (stem
200 diameter at 22.5 cm height) and height were measured. Additionally, leaf samples ($n=5$ per
201 tree) were taken. A stratified random sample of leaves was taken from the canopy of each
202 tree to ensure that the leaf sample was representative. This is because $\delta^{15}\text{N}$ of tree leaves may
203 be dependent on their position in the canopy (Domenach et al. 1989). The vertical extent of

204 the canopy was measured using a telescopic height pole. One leaf was removed from each of
205 five equal size vertical strata within the canopy, covering the entire depth of the canopy. Leaf
206 samples and tree measurements were made in late summer (16- to 20-Aug-2008) when N
207 content was assumed to be at its peak. Soil samples were obtained from each of the four
208 stands in each ring during root coring in Jan-2008. An eight cm auger corer was used to
209 collect samples at three depths: 0-10, 10-20 and 20-30 cm.

210 The leaves were scanned into a computer using a flatbed scanner and the area was measured
211 using ImageJ image analysis software (Abramoff et al. 2004). The leaves were then dried at
212 80°C for 72 hours and weighed. They were then milled to a fine powder in a ball mill and the
213 $\delta^{15}\text{N}$ was analysed using a Carlo-Erba elemental analyser linked to a Dennis Leigh
214 Technologies IRMS. Leaf N concentration was then calculated on an area (N_{AREA}) and mass
215 (N_{MASS}) basis. Soil cores were coarse sieved (8 mm) to remove roots and large stones. A sub-
216 sample of the soil from each depth was taken, dried at 80°C overnight, sieved <2 mm and
217 ground to a fine powder. $\delta^{15}\text{N}$ was analysed using a Finnigan MAT Delta Plus XL continuous
218 flow mass spectrometer. The relative abundance of ^{14}N and ^{15}N is expressed using the
219 standard delta (δ) notation for stable isotopes. δ is the relative difference in the ratio of the
220 two forms of N in comparison to that of air and is expressed on a per mil basis (‰) ($\delta^{15}\text{N}$ of
221 air is therefore by definition 0‰). As such, $\delta = (R_{\text{sample}}/R_{\text{reference}}) - 1 \times 1000$, and $R = ^{15}\text{N}:^{14}\text{N}$.
222 Data are reported with respect to N in air.

223 *Natural abundance stable isotope method*

224 We measured the contribution of N derived from the atmosphere (N_{dfa}) to the N budget of the
225 *A. glutinosa* trees using the natural abundance stable isotope method (after Shearer and Kohl
226 1986). This method was used because it was not possible to add labelled N to the site due to
227 the potential for disturbing the N cycle and because the site is used for ongoing long-term

228 studies. The contribution of N_{dfa} to the N budget of N_2 -fixing plants can be estimated by
229 comparing $\delta^{15}N$ of the N_2 -fixing plant with non- N_2 -fixing reference plants (representing $\delta^{15}N$
230 of the N_2 -fixing species when obtaining all N from the soil) and N_2 -fixing species grown with
231 no root N addition (Boddey et al. 2000). In this study we compared $\delta^{15}N$ of *A. glutinosa* with
232 that of *B. pendula* and *F. sylvatica* growing in monoculture.

233 The ^{15}N natural abundance method provides quantification of N_2 fixation when rates of N_2
234 fixation are high and when the plants are demonstrably fixing N_2 (Unkovich et al. 2008).
235 Consistently reduced $\delta^{15}N$ and root nodulation observed in roots excised for other studies
236 (Smith 2011) demonstrates N_2 fixation of *A. glutinosa* in this study. $\delta^{15}N$ depletion in *A.*
237 *glutinosa* compared to the reference plants indicates high N_2 fixation rates. The value of B
238 ($\delta^{15}N$ of *A. glutinosa* trees with access to atmospheric N only) used was 4.5‰ lower than the
239 mean for the reference plants. While below the minimum value of 5‰ recommended by
240 Högberg (1997), there is clear and consistent separation between $\delta^{15}N$ of the reference trees
241 and *A. glutinosa*.

242 Boddey et al. (2000) and Unkovich et al. (2008) suggest that more than one reference species
243 should be used and that they should be of a similar life form, size, duration of growth and that
244 they should have no access to fixed N_2 . We used two reference species, and both were trees
245 planted at the same time at the *A. glutinosa* with similar rooting depths, though *F. sylvatica*
246 roots tend to be shallower (Atkinson 1992; Claessens et al. 2010; Bakker et al. 2008). In
247 addition, reference plants growing in ambient CO_2 concentrations were used to calculate
248 $\%N_{dfa}$ and N_{dfa} for *A. glutinosa* growing in ambient CO_2 concentrations. Reference plants
249 growing in elevated CO_2 concentrations were used to calculate $\%N_{dfa}$ and N_{dfa} of *A. glutinosa*
250 growing in elevated CO_2 . Furthermore, the calculations of N_{dfa} and $\%N_{dfa}$ using each
251 reference species are similar. There is good evidence that no fixed N is incorporated into the
252 reference trees. The $\delta^{15}N$ of *B. pendula* leaves from a larger (20×20 m) single species stand

253 in the same plantation was identical (2.2‰) to that of *B. pendula* in monoculture in the
254 closest study ring 30 m away.

255 Similarity in $\delta^{15}\text{N}$ of the sources of all three species and in fractionation within the trees is
256 assumed. The broad similarities of $\delta^{15}\text{N}$ in *F. sylvatica* and *B. pendula* leaves suggests that
257 this assumption holds (the difference in $\delta^{15}\text{N}$ between *F. sylvatica* and *B. pendula* is very
258 small (0.5‰) compared to the mean difference between these reference plants and *A.*
259 *glutinosa* (2.7‰)). Leaf $\delta^{15}\text{N}$ did not differ from weighted whole tree $\delta^{15}\text{N}$ for any of the
260 three species (details in supplementary information). Thus, the use of leaf samples as
261 representative of whole tree $\delta^{15}\text{N}$ is supported. We therefore consider the quantification to
262 remain robust.

263 *Data analysis*

264 The proportion of plant N derived from N_2 fixation ($\%N_{\text{dfa}}$) was calculated from the $\delta^{15}\text{N}$ of
265 the leaves using a simple one-isotope, two-source, end-member mixing model as follows
266 (after Shearer and Kohl 1986):

$$267 \text{ Equation 1: } \%N_{\text{dfa}} = \frac{(\delta^{15}\text{N}_{\text{REF}} - \delta^{15}\text{N}_{\text{TREE}})}{(\delta^{15}\text{N}_{\text{REF}} - B)} \times 100$$

268 where $\%N_{\text{dfa}}$ is the percentage of leaf-N fixed from the atmosphere, $\delta^{15}\text{N}_{\text{REF}}$ is the $\delta^{15}\text{N}$ of
269 trees for which the only source of N is through soil uptake (in this study the mean $\delta^{15}\text{N}$ of
270 leaves on *F. sylvatica* and *B. pendula* growing in monoculture in the same ring), $\delta^{15}\text{N}_{\text{TREE}}$ is
271 the $\delta^{15}\text{N}$ of the tree of interest and B is the $\delta^{15}\text{N}$ of trees for which the only source of N is
272 derived from atmospheric fixation, B of -1.9‰ was used, based on nodulated *A. glutinosa*
273 plants growing in an N-free medium, as determined by Domenach et al. (1989). $\%N_{\text{dfa}}$ and
274 N_{dfa} were calculated separately using *F. sylvatica* or *B. pendula* as reference plants and using
275 the mean value for the two species.

276 To isolate leaf $\delta^{15}\text{N}$ from differences in bulk soil $\delta^{15}\text{N}$, a soil-leaf N enrichment factor (EF)
277 was calculated for the two non- N_2 -fixing trees. The soil-leaf EF measures the relative ^{15}N
278 depletion/enrichment from bulk soil to leaf. Thus it provides a sensitive qualitative measure
279 of changes in N cycling in the plant-soil system where patterns in leaf $\delta^{15}\text{N}$ might be less
280 sensitive due to changes in bulk soil $\delta^{15}\text{N}$ (Amundson et al. 2003; Kahmen et al. 2008). EF
281 was calculated as follows for each tree (after Garten et al. 2007):

282 Equation 2: $EF = \delta^{15}\text{N}_{\text{SOIL}} - \delta^{15}\text{N}_{\text{LEAF}}$

283 where $\delta^{15}\text{N}_{\text{SOIL}}$ is the overall mean $\delta^{15}\text{N}$ of soil from 0-10, 10-20 and 20-30 cm depth and
284 $\delta^{15}\text{N}_{\text{LEAF}}$ is the overall mean $\delta^{15}\text{N}$ of all leaves taken from throughout the canopy.

285 Stem diameter at 22.5 cm of each tree was used to estimate total leaf mass using allometric
286 equations based on trees harvested in 2006 from the buffer zone around the FACE and
287 ambient rings (details in supplementary information). Estimates of total leaf mass were
288 combined with measurements of leaf N to calculate the total amount of leaf N (N_{TOTAL}), the
289 total amount of leaf N derived from the atmosphere (N_{dfa}) and the soil (N_{dfs}) on a per tree
290 basis.

291 The measurements for the five leaf samples per tree were averaged over the whole tree to
292 give one mean value per tree. These tree level data were analysed as a split-split-plot design
293 ANOVA in SPSS (SPSS Inc., 2008) using the general linear model (GLM). Individual rings
294 (Ring) were treated as 'plots' and were nested within CO_2 (CO_2) treatments.

295 Mixture/monoculture (MixMon) was treated as a sub-plot within ring and species was nested
296 within mixture/monoculture. The model used was: $\text{CO}_2 + \text{Ring}(\text{CO}_2) + \text{MixMon} + \text{Species} +$
297 $\text{MixMon} \times \text{Ring}(\text{CO}_2) + \text{Species} \times \text{Ring}(\text{CO}_2) + \text{CO}_2 \times \text{Species} + \text{CO}_2 \times \text{MixMon} + \text{Species}$
298 $\times \text{MixMon} + \text{Species} \times \text{MixMon} \times \text{Ring}(\text{CO}_2) + \text{CO}_2 \times \text{Species} \times \text{MixMon}$. N_{dfa} and $\% \text{N}_{\text{dfa}}$
299 were only analysed for *A. glutinosa*, using the same model but with the terms containing

300 ‘Species’ omitted. Soil $\delta^{15}\text{N}$ data were analysed using a repeated measures GLM. Where the
301 F-test was significant, Fisher’s protected LSD was used for post-hoc multiple comparisons.
302 *Betula pendula* and *F. sylvatica* trees had different numbers of *A. glutinosa* neighbours when
303 growing in mixture (between 1-4). The impact of the number of *A. glutinosa* neighbours on
304 $\delta^{15}\text{N}$ of leaves of *B. pendula* and *F. sylvatica* leaves was tested using a Kruskal-Wallis test,
305 because it was difficult to ascertain compliance with the assumptions of ANOVA due to the
306 uneven sample sizes. *Betula pendula* and *F. sylvatica* in monoculture were included as a
307 ‘zero *A. glutinosa* neighbours’ group. Where appropriate data were Log_{10} transformed to
308 conform to the assumptions of normality and heteroscedacity. The small number of replicates
309 for CO_2 treatment increases the risk of a type II error so α of 0.1 was used. While this
310 increases the risk of a type I error this was considered an acceptable trade-off.

311 **Results**

312 Leaf $\delta^{15}\text{N}$ differed significantly between species when growing in monoculture with *A.*
313 *glutinosa* considerably lower than *B. pendula* which was slightly lower than *F. sylvatica*
314 (Table 1, Fig. 1a). When compared with *A. glutinosa* across both CO_2 treatments, *B. pendula*
315 and *F. sylvatica* were relatively ^{15}N enriched, by 2.5‰ and 2.9‰ respectively. The leaves of
316 all species were ^{15}N depleted under elevated CO_2 , by on average 0.4‰ compared to those in
317 ambient CO_2 , but this effect was only statistically significant for *F. sylvatica* (*A. glutinosa* =
318 0.3‰, *B. pendula* = 0.1‰, *F. sylvatica* = 0.8‰; Fig 1a, Table 1, CO_2 effect and $\text{CO}_2 \times \text{Species}$
319 interaction). Species composition had a significant impact on $\delta^{15}\text{N}$ values of trees grown in
320 mixture, which were significantly ^{15}N depleted compared to those in monoculture (Fig. 1a,
321 Table1). Furthermore, the leaves of the non- N_2 -fixing species became less ^{15}N enriched with
322 increasing numbers of *A. glutinosa* neighbours (Fig. 2). Though when considering the two
323 species separately this effect was less clear.

324 Soil was consistently ^{15}N enriched under elevated CO_2 across stands, by on average 0.4‰,
325 but became significantly less ^{15}N enriched with increasing depth (Fig. 3). However, soil $\delta^{15}\text{N}$
326 did not differ significantly between stands (data not shown). Overall the soil-leaf ^{15}N
327 enrichment factor (EF) for trees growing in elevated CO_2 was more negative than those in
328 ambient CO_2 by 0.8‰, reflecting increased soil-leaf ^{15}N depletion, though this CO_2 effect
329 was largest and only statistically significant for *F. sylvatica* (Table 1, Fig. 1b). Overall, there
330 was no significant difference in EF between *F. sylvatica* and *B. pendula* (Fisher's LSD,
331 $P>0.05$).

332 The total amount of leaf N in the trees was calculated by multiplying leaf N concentration
333 (N_{MASS}) by estimated leaf mass (from site specific allometric equations). Total leaf N differed
334 between species and followed the pattern of tree biomass (measured in the same study by
335 Smith, 2011). *Alnus glutinosa* and *B. pendula* contained the same amount of N, with both of
336 these species containing a far greater amount of N than *F. sylvatica*. Elevated CO_2 increased
337 the total amount of leaf N in all trees in all treatments, by an average of 14% (Table 1, Fig.
338 4a), but this CO_2 effect was not statistically significant. Furthermore, total leaf N differed for
339 trees growing in mixture or monoculture, due to a large, significant difference between total
340 leaf N of *A. glutinosa* in mixture and in monoculture (mixture= 20.0 ± 1.6 g. tree $^{-1}$,
341 monoculture= 12.8 ± 1.6 g. tree $^{-1}$, Fisher's LSD $P<0.05$). There was no difference between the
342 other two species growing in mixture and monoculture. The source of this leaf N varied
343 between species. There was significantly less soil-derived N in the leaves of *A. glutinosa* than
344 those of *B. pendula*, with that of *F. sylvatica* being considerably lower than both (Fig. 4a,
345 Table 2). The high total leaf N in *A. glutinosa* was due to the contribution of fixed N.

346 Patterns of N_{AREA} and N_{MASS} were broadly similar (Fig. 4b, 4c; Table 1). For both of these
347 measures of leaf N concentration there were differences between species, with leaf N
348 concentration of *A. glutinosa* and *B. pendula* showing no significant difference and both these

349 species having higher leaf N concentrations than did *F. sylvatica*. The differences were
350 greater when trees were growing in mixture compared to when species differences were
351 compared for trees growing in monoculture. However, when considering responses to
352 elevated CO₂, N_{AREA} and N_{MASS} were affected differently. There was no impact of elevated
353 CO₂ on N_{MASS}. However, elevated CO₂ reduced N_{AREA} by an average of 5.3%. This reduction
354 was consistent for all species.

355 When δ¹⁵N was used to estimate the amount of fixed N in *A. glutinosa* the trees gained on
356 average 10.5±0.9 g. tree⁻¹ of N from fixation. For trees growing in mixture there was a trend
357 towards increased N_{dfa} under elevated CO₂, with *A. glutinosa* trees obtaining 46% more N
358 from fixation than under ambient atmospheric CO₂ (Fig. 4a, Table 2, CO₂×‘MixMon’,
359 P=0.15). While this effect is not statistically significant, the magnitude of the effect is likely
360 to be biologically significant. As a result of this increase in mixture there was a significant
361 effect of species composition on N_{dfa} but no overall effect of CO₂ treatment (Table 2). This
362 fixed N contributed on average 62.1±0.1 % of the total N in *A. glutinosa* leaves. As a result of
363 the increased N₂ fixation under elevated CO₂ for trees in mixture, the percentage contribution
364 of fixed N increased by 6.9% for these trees compared to those in ambient CO₂ (68.3%
365 compared to 61.4%, Fig. 5). This effect resulted in a significant impact of species
366 composition on %N_{dfa} and a trend towards an interaction (though not statistically significant)
367 between species composition and the impact of elevated CO₂, but no significant effect of CO₂
368 overall (Table 2).

369 **Discussion**

370 Our study is the first to measure N₂ fixation in a tree species in FACE conditions. The
371 observed increased growth of *A. glutinosa* in monoculture under elevated CO₂ was not
372 supported by increased N₂ fixation, either on an absolute (N_{dfa}) or relative (%N_{dfa}) basis. Thus

373 we cannot support our first hypotheses, that N₂ fixation in *A. glutinosa* will increase in
374 response to increased atmospheric CO₂ concentrations, when growing in monoculture.
375 Instead elevated CO₂ resulted in a slight (but not statistically significant) increase in root N
376 uptake and decrease in leaf N concentration (thought this was only statistically significant on
377 an area basis). Previous studies have shown that in some circumstances N₂ fixation increases
378 to support higher growth rates under elevated CO₂. Norby (1987) and Vogel et al. (1997)
379 found that *A. glutinosa* trees growing in elevated CO₂ were larger and fixed more N, but that
380 this was due to their larger size rather than an increase in the rate of N₂ fixation per se.
381 However, Temperton et al. (2003) grew *A. glutinosa* trees in more realistic field conditions
382 and found that elevated CO₂ had no statistically significant impact on N₂ fixation. Our study,
383 with the findings of Temperton et al. (2003) suggests that when growing in single species
384 stands, in 'real world' conditions *A. glutinosa* does not support CO₂ induced growth increase
385 with N₂ fixation, but rather with an increase in root N uptake and nitrogen use efficiency.
386 However, it is possible that over longer periods of time this might change.

387 Our study suggests fundamental differences in forest ecosystem function in mixed stands
388 compared to single species stands. These differences have impacted on the response of N₂
389 fixation to elevated CO₂. Thus we cannot support our second hypothesis, that the impact of
390 elevated CO₂ on N₂ fixation in *A. glutinosa* is the same in mixture and in monoculture. As
391 such, our findings differ from patterns found in other systems. For example, Lee et al. (2003)
392 found that N₂ fixation in *Lupinus* sp. was increased by elevated atmospheric CO₂ in both
393 monoculture and in a mixed grassland system. We provide some evidence that N₂ fixation
394 might have been stimulated by elevated CO₂ for *A. glutinosa* trees growing in mixture,
395 despite there being no statistically significant impact of CO₂ on tree biomass. There were
396 large differences in growth rate, N uptake and N₂ fixation for *A. glutinosa* trees growing in
397 mixture, compared to those growing in monoculture, which might account for the difference

398 in response. Biomass of *A. glutinosa* trees in mixture was approximately 50% greater than
399 that of those in monoculture (the same trees measured by Smith 2011), with a commensurate
400 56% increase in total leaf N and nearly double the amount of fixed N. Decreased $\delta^{15}\text{N}$ of the
401 trees when species are growing in mixture also suggests that N cycling is different in mixture
402 than in monoculture. This might be due to increased ecosystem resource utilisation when
403 more trees species are present, for example through niche differentiation. These differences
404 may result from impacts on any part of the N-cycle, for example, inputs of fixed N_2 ,
405 mycorrhizae (e.g. Hobbie et al. 2000) or litter inputs and decomposition (e.g. Zak et al. 2003)
406 all of which might be affected by changes in atmospheric CO_2 .

407 When growing in mixture with *A. glutinosa*, *F. sylvatica* and *B. pendula* leaves were less
408 enriched in ^{15}N compared to the leaves of these species growing in monoculture.

409 Furthermore, leaves of *F. sylvatica* and *B. pendula* with greater numbers of *A. glutinosa* trees
410 as direct neighbours were significantly depleted in ^{15}N compared to those with fewer. It
411 seems likely that these changes in $\delta^{15}\text{N}$ are explained by the incorporation of fixed N_2 into
412 these tissues. This is consistent with other studies where $\delta^{15}\text{N}$ of N_2 -fixing trees has been
413 compared with co-occurring non- N_2 -fixing species (e.g. Daudin and Sierra 2008) and where
414 the transfer of fixed N_2 specifically has been measured. For example the contribution of
415 transferred N to total N was on average 5-15% (Arnebrant et al. 1993) and 1.3-3% (Ekblad
416 and Huss-Danell 1995) between *A. glutinosa* and *P. contorta* and *A. incana* and *P. sylvestris*
417 respectively. These inputs of fixed N_2 do not translate into differences in $\delta^{15}\text{N}$ of the soil in
418 stands containing *A. glutinosa*. This suggests that inputs of fixed N_2 are small relative to the
419 ecosystem N pool, or that little fixed N_2 makes its way into the soil N pool, possibly due to a
420 tightly coupled leaf-soil-plant N cycle. Additionally, the clear impact of *A. glutinosa* on $\delta^{15}\text{N}$
421 of these species in mixture highlights the importance of choosing reference plants that are not
422 growing in close proximity to N_2 -fixing plants.

423 There is clear evidence that the *A. glutinosa* trees in this study obtained a significant
424 proportion of their N from biological fixation. The leaves of *A. glutinosa* trees were ^{15}N
425 depleted relative to those of *F. sylvatica* or *B. pendula* in the same plots. This suggests that a
426 large proportion (approximately 62%) of the N contained in the trees was fixed from the
427 atmosphere. This is consistent with previous studies of *Alnus*. For example, (Sanborn et al.
428 2002) found that *A. viridis* fixed 10-15 kg N ha⁻¹ year⁻¹ and that this contributed >90% of the
429 total N content of these trees. Ekblad and Huss-Danell (1995) found that for *A. incana* fixed
430 N₂ contributed between 45% and 90% of total N. As a result of this uptake of fixed N₂, *A.*
431 *glutinosa* in our study relied on root derived N to a far smaller extent than did the non-N₂-
432 fixing species.

433 Ecosystem C and N pools are tightly linked (Chen and Coops 2009). Therefore, forest
434 responses to elevated atmospheric CO₂ are linked to ecosystem N availability and cycling
435 (Oren et al. 2001; Ainsworth and Long 2005; Norby and Iversen, 2006; Reich et al. 2006b;
436 Zak et al. 2006; Finzi et al. 2007). For non-N₂-fixing trees leaf $\delta^{15}\text{N}$ is determined by source
437 (i.e. soil) $\delta^{15}\text{N}$ subject to any fractionation that occurs during uptake or within the tree. Thus,
438 changes in leaf $\delta^{15}\text{N}$ might reflect changes in bulk soil $\delta^{15}\text{N}$, differential uptake of different
439 forms of N (with different $\delta^{15}\text{N}$ signatures) or changes in fractionation during uptake. The
440 impact of elevated CO₂ on N cycling can therefore be reflected in leaf $\delta^{15}\text{N}$, with a tendency
441 towards a decrease in $\delta^{15}\text{N}$ when CO₂ is elevated for both woody and herbaceous plants
442 (BassiriRad et al. 2003).

443 The relative ^{15}N depletion by 0.4‰ of tree leaves under elevated CO₂ in our study was
444 matched by relative enrichment of soil by 0.4‰. Thus the $\delta^{15}\text{N}$ of the plant-soil system
445 appears to have remained constant, but elevated CO₂ appears to have resulted in a change in
446 distribution of ^{15}N from plant to soil. The use of a soil-leaf enrichment factor (EF) quantifies
447 this change in ^{15}N distribution. The EF for the trees in our study was consistently lower by on

448 average 0.8‰ under elevated CO₂ indicating a consistent change in the distribution of ¹⁵N
449 between soil and leaf. The relative leaf ¹⁵N depletion and associated changes in the soil-plant
450 ¹⁵N enrichment factor (EF) for trees growing under elevated CO₂ follow the trend for
451 identified by Bassirirad et al. (2003). The opposing response of soil and leaves suggests that
452 changes in leaf δ¹⁵N are not due to changes in bulk soil δ¹⁵N, or internal fractionation.
453 Furthermore, the largest ¹⁵N depletion was in one of the non-N₂-fixing trees suggesting that
454 the effect is not due to atmospheric N₂ fixation. This is good evidence to support our third
455 hypothesis, that the impacts of elevated CO₂ on N cycling will be evident in a decrease in leaf
456 δ¹⁵N and in the soil-leaf enrichment factor (EF), and that these impacts will not differ
457 between mixed and single species stands. A strong candidate for the observed ¹⁵N depletion
458 is increased reliance on mycorrhizal derived N, which tends to be ¹⁵N depleted (Hobbie et al.
459 2000; Mayor et al. 2008). Increased mycorrhizal infection under elevated CO₂ is regularly
460 observed due to increased C supply to roots (e.g. Norby et al. 1987; Drigo et al. 2008).
461 Alternatively, this relative depletion might be due to changes in uptake of relatively ¹⁵N
462 enriched NH₄⁺ or relatively ¹⁵N depleted NO₃⁻ (Högberg 1997). This may be due to changes
463 in the availability of these sources of N in the soil, or changes in uptake due to increasing N
464 demand. More comprehensive and detailed measurement of the size and δ¹⁵N of the various
465 N pools would be required to better resolve this.

466 In conclusion, we found no evidence that increased growth of *A. glutionsa* when atmospheric
467 CO₂ was elevated was supported by increased N₂ fixation. We found some evidence of
468 biologically significant CO₂ stimulation of N₂ fixation in mixed stands, despite there being no
469 statistically significant increase in growth. We found evidence of significant impacts of
470 elevated CO₂ on aspects of the N cycle, shown through differences in N₂ fixation and δ¹⁵N.
471 These impacts are dependent on the species composition of the forest. This study shows clear
472 evidence that the N-cycle in mixed species stands functions differently to that in single

473 species stands. This is suggested by higher rates of N₂ fixation in *A. glutinosa*, transfer of
474 fixed N₂ to non-N₂-fixing species, changes in leaf δ¹⁵N and large differences in tree N
475 content. These different impacts have important consequences for how we consider the
476 impacts of global environmental change and interactions with ecosystem function. Changes
477 in atmospheric CO₂ will occur concurrently with changes in plant community species
478 composition due to this and other drivers of global environmental change (Badeck et al.
479 2001). Thus forest species compositions that exist when the atmospheric CO₂ concentrations
480 used in this and other studies are reached will be different to those at present. Our study
481 shows that these changes can result in very real effects on forest N budgets and in the impact
482 of elevated CO₂ on these N budgets.

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491 **References**

492 Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics*
493 *International* 11:36-42

494 Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO₂
495 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy
496 properties and plant production to rising CO₂. *New Phytol.* 165:351-71

497 Amundson R, Austin AT, Schuur EAG, Yoo K, Matzek V, Kendall C, Uebersax A, Brenner
498 D, Baisden WT (2003) Global patterns of the isotopic composition of soil and plant nitrogen.
499 *Global Biogeochem. Cycles* 17:1031

500 APIS (2010) *Air Pollution Information System*. [online] available at: <<http://www.apis.ac.uk>>
501 [accessed 21st October 2011].

502 Arnebrant K, Ek H, Finlay RD, Söderström B (1993) Nitrogen translocation between *Alnus*
503 *glutinosa* (L.) Gaertn. seedlings inoculated with *Frankia* sp. and *Pinus contorta* (Doug.) ex
504 Loud seedlings connected by a common mycelium. *New Phytol.* 124:231-242

505 Atkinson MD (1992) *Betula pendula* Roth. (*B. verrucosa* Ehrh.) and *B. pubescens* Ehrh. J.
506 *Ecol.* 80:837–870

507 Bakker MR, Turpault MP, Huet S, Nys C (2008) Root distribution of *Fagus sylvatica* in a
508 chronosequence in western France. *J. For. Res-Jpn* 13:176-184

509 BassiriRad H, Constable JV, Lussenhop J, Kimball BA, Norby RJ, Oechel WC, Reich PB,
510 Schlesinger WH, Zitzer S, Sehtiya HL, Silim S (2003) Widespread foliage $\delta^{15}\text{N}$ depletion
511 under elevated CO_2 : inferences for the nitrogen cycle. *Glob. Change Bio.* 9:1582-1590

512 Boddey RM, Peoples MB, Palmer B, Dart PJ (2000) Use of the ^{15}N natural abundance
513 technique to quantify biological nitrogen fixation by woody perennials. *Nutr. Cycl.*
514 *Agroecosys.* 57:235-270

515 Bucher JB, Tarjan DP, Siegwolf RT, Saurer M, Blum H, Hendrey GR (1998) Growth of a
516 tree seedling community in response to elevated CO_2 and nutrient supply. *Chemosphere*
517 36:777-782

518 Chen B, Coops, NC (2009) Understanding of coupled terrestrial carbon, nitrogen and water
519 dynamics – an overview. *Sensors* 9:8624-8657

520 Classens H, Oosterbaan A, Savil P, Rondeux J (2010) A review of the characteristics of black
521 alder (*Alnus glutinosa* (L.) (Gaertn.) and their implications for silvicultural practices.
522 *Forestry*, doi: 10.1093/forestry/cpp038

523 Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth, RW, Hedin LO, Perakis SS,
524 Latty EF, Von Fischer JC, Elseroad A, Wsson MF (1999) Global patterns of terrestrial
525 biological nitrogen (N₂) fixation in natural ecosystems. *Global Biogeochem. Cy.* 13:623-645

526 Daudin D, Sierra J (2008) Spatial and temporal variation of below-ground N transfer from a
527 leguminous tree to an associated grass in an agroforestry system. *Agr. Ecosyst. Environ.*
528 126:275-280

529 Domenach AM, Kurdali F, Bardin R (1989) Estimation of symbiotic dinitrogen fixation in
530 alder forest by the method based on natural ¹⁵N abundance. *Plant Soil* 118:51-59

531 Drigo B, Kowalchuk GA, van Veen JA (2008) Climate change goes underground: effects of
532 elevated atmospheric CO₂ on microbial community structure and activities in the
533 rhizosphere. *Biol. Fert. Soils* 44: 667-679

534 Ekblad A, Huss-Danell K (1995) Nitrogen fixation by *Alnus incana* and nitrogen transfer
535 from *A. incana* to *Pinus sylvestris* influenced by macronutrients and ectomycorrhiza. *New*
536 *Phytol.* 131:453-459

537 Emmett BA, Kjønnaas OJ, Gundersen P, Koopmans C, Tietema A, Sleep D (1998) Natural
538 abundance of ¹⁵N in forests across a nitrogen deposition gradient. *Forest Ecol. Manag.* 101:9-
539 18

540 Feng Z, Dyckmans J, Flessa H (2004) Effects of elevated carbon dioxide concentration on
541 growth and N₂ fixation of young *Robinia pseudocacia*. *Tree Physiol.* 24:323-330

542 Finzi AC, Norby RJ, Calfapietra C, Gallet-Budynek A, Gielen B, Holmes WE, Hoosbeek
543 MR, Iversen CM, Jackson RB, Kubiske ME, Ledford J, Liberloo M, Oren R, Polle A,
544 Pritchard S, Zak DR, Shlesinger WH, Ceulemans R (2007) Increases in nitrogen uptake
545 rather than nitrogen-use efficiency support higher rates of temperate forest productivity under
546 elevated CO₂. *P. Natl. Acad. Sci. USA* 104:14014-14019

547 Garten CT, Hanson PJ, Todd DE, Lu BB, Brice BJ (2007) Natural ¹⁵N- and ¹³C-abundance as
548 indicators of forest nitrogen status and soil carbon dynamics. In: Michener R, Lajtha K (eds)
549 *Stable Isotopes in Ecology and Environmental Science*. Blackwell Publishing Ltd, Oxford,
550 UK, pp 61-82

551 Grace J (2004) Understanding and managing the global carbon cycle. *J. Ecol.* 92:189-202

552 He X, Critchley C, Bledsoe C (2003) Nitrogen Transfer Within and Between Plants Through
553 Common Mycorrhizal Networks (CMNs). *Crit. Rev. Plant Sci.* 22:531-567

554 Hobbie EA, Macko SA, Williams M (2000) Correlations between foliar δ¹⁵N and nitrogen
555 concentrations may indicate plant mycorrhizal interactions. *Oecologia* 122:273-283.

556 Högberg P (1997) ¹⁵N natural abundance in soil-plant systems. *New Phytol.* 137:179-203

557 Hungate BA, Dijkstra P, Johnson DW, Hinkle CR, Drake BG (1999) Elevated CO₂ increases
558 nitrogen fixation and decreases soil nitrogen mineralization in Florida scrub oak. *Global*
559 *Change Biol.* 5:781-789

560 Hungate BA, Stiling PD, Dijkstra P, Johnson DW, Ketterer ME, Hymus GJ, Hinkle CR,
561 Drake BG (2004) CO₂ elicits long-term decline in nitrogen fixation. *Science* 304:1291

562 Janssens IA, Freibauer A, Ciais P, Smith P, Nabuurs GJ, Folberth G, Schlamadinger B,
563 Hutjes RWA, Ceulemans R, Schulze ED, Valentini R, Dolman AJ (2003) Europe's terrestrial
564 biosphere absorbs 7 to 12% of European anthropogenic CO₂ emissions. *Science* 300:1538-
565 1542

566 Kahmen A, Wanek W, Buchmann N (2008) Foliar $\delta^{15}\text{N}$ values characterize soil N cycling
567 and reflect nitrate or ammonium preference of plants along a temperate grassland gradient.
568 *Oecologia* 156:861-870

569 Körner C (2003) Carbon limitation in trees. *J. Ecol.* 91:4-17

570 Lee TD, Reich PB, Tjoelker MG (2003) Legume presence increases photosynthesis and N
571 concentrations of co-occurring non-fixers but does not modulate their responsiveness to
572 carbon dioxide enrichment. *Oecologia* 137:22-31

573 Long SP, Ainsworth EA, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide: plants
574 FACE the future. *Annu. Rev. Plant Biol.* 55:591-628

575 Luo Y, Su B, Currie WS, Dukes JS, Finzi A, Hartwig U, Hungate B, McMurtrie RE, Oren R,
576 Parton WJ, Pataki DE, Shaw MR, Zak RD, Field C (2004) Progressive nitrogen limitation of
577 ecosystem responses to rising atmospheric carbon dioxide. *BioScience* 54:731-739.

578 Mayor JR, Schuur EA, Henkel TW (2008) Elucidating the nutritional dynamics of fungi
579 using stable isotopes. *Ecol. Lett.* 12:171-183

580 Miglietta F, Peressotti A, Vaccari FP, Zalder A, DeAngelis P, Scarascia P (2001) Free-air
581 CO₂ enrichment (FACE) of a poplar plantation: the POPFACE fumigation system. *New*
582 *Phytol.* 150:465-476

583 Millard P, Sommerkorn M, Grelet G (2007) Environmental change and carbon limitation in
584 trees: a biochemical, ecophysiological and ecosystem appraisal. *New Phytol.* 175:11-28

585 Norby RJ (1987) Nodulation and nitrogenase activity in nitrogen fixing woody plants
586 stimulated by CO₂ enrichment of the atmosphere. *Physiol. Plantarum* 71: 77-82

587 Norby R, O'Neill EG, Gregory Hood W, Luxmoore RJ. (1987) Carbon allocation, root
588 exudation and mycorrhizal colonization of *Pinus echinata* seedlings grown under CO₂
589 enrichment. *Tree Physiol.* 3: 203-210

590 Norby RJ, Delucia EH, Gielen B, Calfapietra C, Giardina CP, King JS, Ledford J, McCarthy
591 HR, Moore DJP, Caulemans R, De Angelis P, Finzi AC, Karnosky DF, Kubiske ME, Lukac
592 M, Pregitzer KS, Scarascia-Mugnozza GE, Schlesinger WH, Oren R (2005). Forest response
593 to elevated CO₂ is conserved across a broad range of productivity. *P. Natl. Acad. Sci. USA*
594 102:18052-18056

595 Norby RJ, Iversen CM (2006) Nitrogen uptake, distribution, turnover, and efficiency of use
596 in a CO₂-enriched sweetgum forest. *Ecology* 87:5-14

597 Oren R, Ellsworth DS, Johnsen KH, Phillips N, Ewers BE, Maier C, Schäfer KVR, McCarthy
598 H, Hendrey G, McNulty SG, Katul GG (2001) Soil fertility limits carbon sequestration by
599 forest ecosystems in a CO₂-enriched atmosphere. *Nature* 411:469-72

600 Pacala SW, Hurtt GC, Baker D, Peylin P, Houghton RA, Birdsey RA, Heath L, Sundquist
601 ET, Stallard RF, Ciais P, Moorcroft P, Caspersen JP, Shevliakova E, Moore B, Kohlmaier G,
602 Holland E, Gloor M, Harmon ME, Fan S-M, Sarmiento JL, Goodle CL, Schimel D, Field CB.
603 (2001) Consistent land- and atmosphere-based U.S. carbon sink estimates. *Science* 292:2316-
604 2320

605 Poorter H, Navas M (2003) Plant growth and competition at elevated CO₂: on winners, losers
606 and functional groups. *New Phytol.* 157:175-198

607 Postgate JR (1998) Nitrogen fixation. Cambridge University Press, Cambridge, UK

608 Reich PB, Hobbie SE, Lee T, Ellsworth DS, West JB, Tilman D, Knops JMH, Naeem S,
609 Trost J (2006a) Nitrogen limitation constrains sustainability of ecosystem response to CO₂.
610 *Nature* 440:922-925

611 Reich PB, Hungate BA, Luo Y (2006b) Carbon-Nitrogen interactions in terrestrial
612 ecosystems in response to rising atmospheric carbon dioxide. *Annu. Rev. Ecol. Syst.* 37:611-
613 636

614 Robinson D (2001) $\delta^{15}\text{N}$ as an integrator of the nitrogen cycle. *Trends Ecol. Evol.* 16:153-162

615 Roggy JC, Moiroud A, Lensi R, Domenach AM (2004) Estimating N transfers between N₂ -
616 fixing actinorhizal species and the non-N₂ -fixing *Prunus avium* under partially controlled
617 conditions. *Biol. Fert. Soils* 39:312-319

618 Sanborn P, Preston C, Brockley R (2002) N₂-fixation by Sitka alder in a young lodgepole
619 pine stand in central interior British Columbia, Canada. *Forest Ecol. Manag.* 167:223-231

620 Saugier B, Roy J, Mooney HA (2001) Estimations of global terrestrial productivity:
621 converging toward a single number?. In: Roy J, Saugier B, Mooney HA (eds) *Terrestrial*
622 *Global Productivity*. Academic Press, San diego, pp 543-557

623 Shearer GB, Kohl DH (1986) N₂ - fixation in field settings: estimations based on natural ¹⁵N
624 abundance. *Aust. J. Plant Physiol.* 13:699-756

625 Smith A (2011) The effect of atmospheric CO₂ enrichment on biogeochemical cycling of
626 temperate forest ecosystems. PhD dissertation, University of Wales, Bangor

627 Soloman S, Qin D, Manning M, Alley RB, Berntsen T, Bindoff NL, Chen Z, Chidthaisong A,
628 Gregory JM, Hegerl GC, Heimann M, Hewitson B, Hoskins BJ, Joos F, Jouzel J, Kattsov V,
629 Lohmann U, Matsuno T, Molina M, Nicholls N, Overpack J, Raga G, Ramaswamy V, Ren J,
630 Rusticucci M, Somerville R, Stocker TF, Whetton P, Wood RA, Wratt D (2007) Technical
631 Summary. In: Soloman S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M,
632 Miller HL (eds) The Physical Science Basis. Contribution of Working Group I to the Fourth
633 Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge
634 University Press, Cambridge, UK, 19-92

635 SPSS for Windows, rel. 17.0.0 2008. Chicago: SPSS inc.

636 Teklehaimanot Z, Sinclair FL (1993) Establishment of the silvopastoral network experiment
637 site, Henfaes, Bangor. *Agroforestry Forum* 4:18-21

638 Temperton VM, Grayston SJ, Jackson G, Millard P, Jarvis PG (2003) Effects of elevated
639 carbon dioxide concentration on growth and nitrogen fixation in *Alnus glutinosa*. *Tree*
640 *Physiol.* 23:1051-1059

641 Tissue DT, Megonigal JP, Thomas RB (1997) Nitrogenase activity and N₂ fixation are
642 stimulated by elevated CO₂ in a tropical N₂-fixing tree. *Oecologia* 109: 28-33

643 Unkovich M, Herridge DF, Peoples MB, Boddey RM, Cadisch G, Giller K, Alves B, Chalk
644 PM (2008) Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR
645 Monograph No.136. Canberra: Australian Centre for International Agricultural Research.

646 Vessey JK, Pawlowski K, Bergman B (2005) Root-based N₂-fixing Symbioses: Legumes,
647 Actinorhizal Plants, *Parasponia* sp. and Cycads. *Plant Soil* 274:51-78

648 Vogel CS, Curtis PS, Thomas RB (1997) Growth and nitrogen accretion of dinitrogen-fixing
649 *Alnus glutinosa* (L.) Gaertn. Under elevated carbon dioxide. *Plant Ecol.* 130:63-70

650 Zak DR, Holms WE, Finzi AC, Norby RJ, Schlesinger WH (2003) Soil nitrogen cycling
651 under elevated CO₂: a synthesis of forest FACE experiments. *Ecol. Appl.* 13:1508-1514

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653 **Table 1** Results of univariate GLM for characteristics of trees of three species (*Alnus glutinosa*, *Betula. pendula* and
654 *Fagus sylvatica*) growing in monoculture or mixture (Mix/Mon) at ambient or elevated (ambient + 200 ppm) CO₂ growing in
655 the BangorFACE experiment. Presented are *P*-values from the analyses of δ¹⁵N, soil-to-leaf nitrogen enrichment factor
656 (EF), total leaf N per tree (N_{TOTAL}), leaf N per unit area (N_{AREA}), N per unit mass (N_{MASS}) and N derived from soil (N_{dfs}).
657 Significant (*P*<0.1) effects are in bold.

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Effect	d.f.	δ ¹⁵ N	EF	N _{TOTAL}	N _{AREA} ^a	N _{MASS} ^a	N _{dfs}
CO ₂	1, 6	0.05	0.09	0.43	0.04	0.96	0.928
Species	2, 12	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Mix/Mon	1, 6	<0.001	0.25	0.16	0.75	0.09	0.571
CO ₂ x Species	2, 12	0.05	0.04	0.95	0.49	0.24	0.815
CO ₂ x Mix/Mon	1, 6	0.51	0.59	0.86	0.15	0.34	0.590
Species x Mix/Mon	2, 12	0.39	0.78	0.001	0.002	0.01	0.098
CO ₂ x Species x Mix/Mon	2, 12	0.21	0.20	0.76	0.21	0.44	0.585

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668 ^aData were Log₁₀ transformed before analysis

669 **Table 2** Results of univariate GLM for impacts on N₂ fixation in *Alnus glutinosa* growing
 670 in monoculture or in mixture with *Betula pendula* and *Fagus sylvatica* (Mix/Mon) at
 671 ambient or elevated (ambient + 200 ppm) CO₂ growing in the BangorFACE experiment.
 672 Presented are the F and P-values from the analyses of %N_{dfa} and N_{dfa}. Significant
 673 (P<0.1) effects are in bold. Values are calculated based on the mean obtained from
 674 using both *B. pendula* and *F. sylvatica* as reference plants.

Effect	d.f.	N _{dfa}		%N _{dfa}	
		F	P	F	P
CO ₂	1, 6	1.35	0.29	0.87	0.39
Mix/Mon	1, 6	5.55	0.057	5.21	0.06
CO ₂ x Mix/mon	1, 6	1.71	0.15	2.64	0.16

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678 **Figure legends**

679 **Fig. 1** Difference in a) $\delta^{15}\text{N}$ and b) soil-leaf N enrichment factor (EF) of leaves of *Alnus*
680 *glutinosa*, *Betula pendula* and *Fagus sylvatica* growing in the BangorFACE experiment.
681 Presented are mean \pm SE of trees growing in monoculture (Mon) or in a mixture (Mix) of
682 all three species at ambient (filled bars) or elevated (ambient + 200 ppm, open bars)
683 atmospheric CO₂. Note that the x-axis minimum is -1.9. This is the expected $\delta^{15}\text{N}$ for
684 *Alnus glutinosa* growing with no root N. Statistics results in Table 1

685 **Fig. 2** $\delta^{15}\text{N}$ of leaves of *B. pendula* and *F. sylvatica* trees growing with different numbers
686 of *A. glutinosa* neighbours in the BangorFACE experiment. Box-plots show the median
687 and 25th and 75th percentile; whiskers show the minimum and maximum. Values for
688 zero (0) neighbours are from trees growing in monoculture; the remaining data are for
689 trees growing in a mixture of all three species. Numbers of individual trees are shown
690 for each group. Kruskal-Wallis results: both species together: d.f. = 4, χ^2 = 12.94,
691 $P=0.01$; *B. pendula*: χ^2 = 7.78, $P=0.1$; *F. sylvatica*: χ^2 = 5.57, $P=0.135$)

692 **Fig. 3** $\delta^{15}\text{N}$ (mean \pm SE) of soil in the BangorFACE experiment at three depths at
693 ambient (filled bars) or elevated (ambient + 200 ppm, open bars) atmospheric CO₂.
694 Pooled data from three different stand types (*A. glutinosa*, *B. pendula* and *F. sylvatica*
695 monoculture or in a mixture of all three species) are presented because there were no
696 significant differences between stands. Bars with different letters are significantly
697 different from each other (Fisher's protected LSD, $P<0.05$). Repeated Measures GLM
698 results: Depth - $P<0.001$, CO₂ - $P=0.034$; Stand - $P=0.69$, Depth \times CO₂ - $P=0.32$,
699 Depth \times Stand $P<0.001$, CO₂ \times Stand - $P=0.98$, Depth \times CO₂ \times Stand - $P=0.50$

700 **Fig. 4** Characteristics of three tree species growing in the BangorFACE experiment in
701 monoculture (Mon) or in a mixture (Mix) of all three species at ambient (filled bars) or
702 elevated (ambient + 200 ppm, open bars) atmospheric CO₂. a) total leaf N content per
703 tree (upper parts of bars for *A. glutinosa* indicate N from atmospheric fixation (N_{dfa}), all
704 other bars are N from soil (N_{dfs})); b) leaf N concentration on an area basis (N_{AREA}); c)
705 leaf N concentration on a mass basis (N_{MASS}). Data for a are mean±SE, for b and c
706 geometric mean±SE (note log y axis). Statistics results are in Table 1, results for N_{dfa}
707 are in Table 2

708 **Fig. 5** The percent of N derived from atmospheric fixation (N_{dfa}) in *A. glutinosa* grown in
709 mixture (with *B. pendula* and *F. Sylvatica*) and in monoculture, under ambient CO₂
710 (filled bars) and elevated CO₂ (open bars). Presented are the mean±SE. Statistics
711 results are in Table 2

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Figure 1

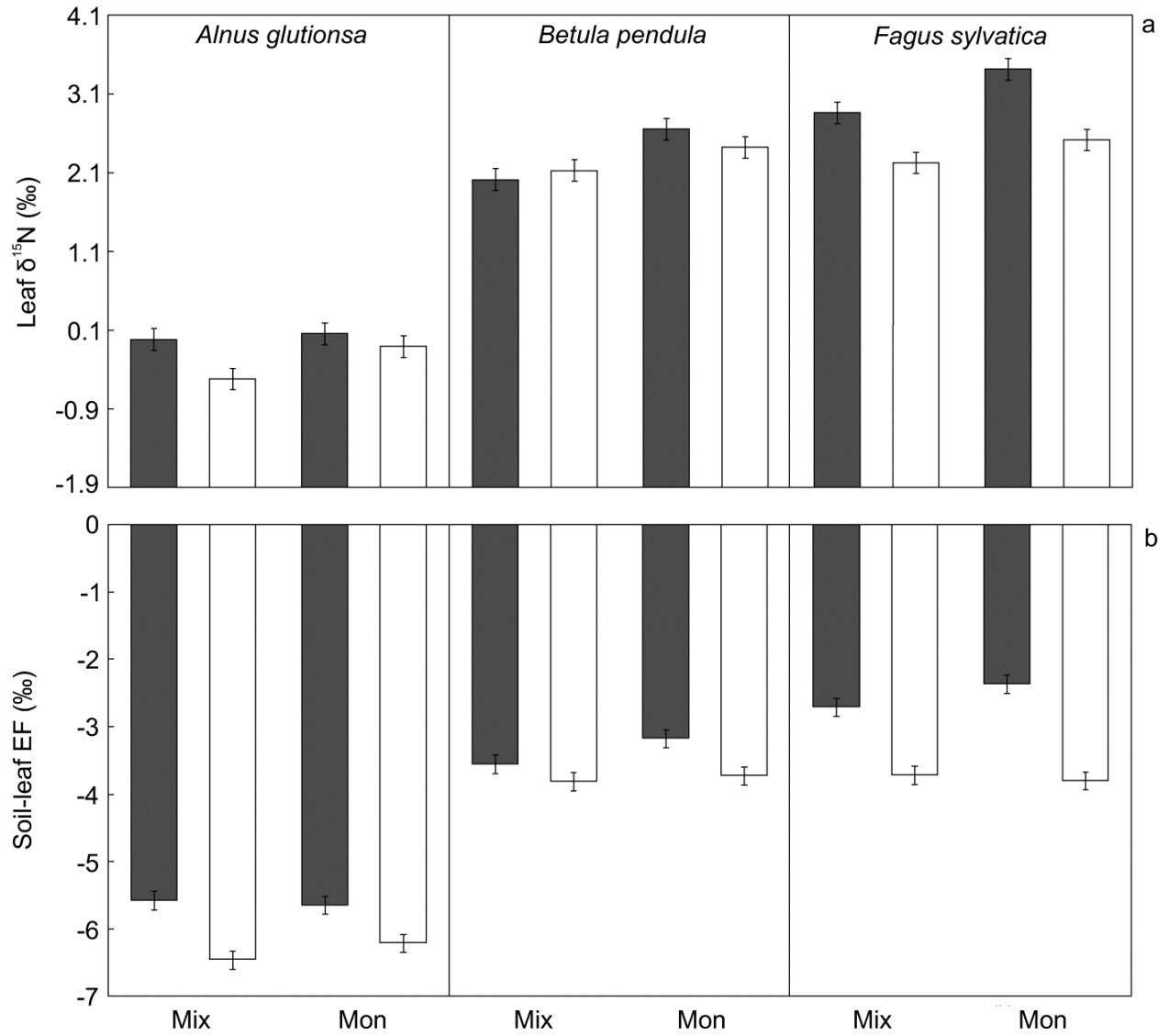


Figure 2.

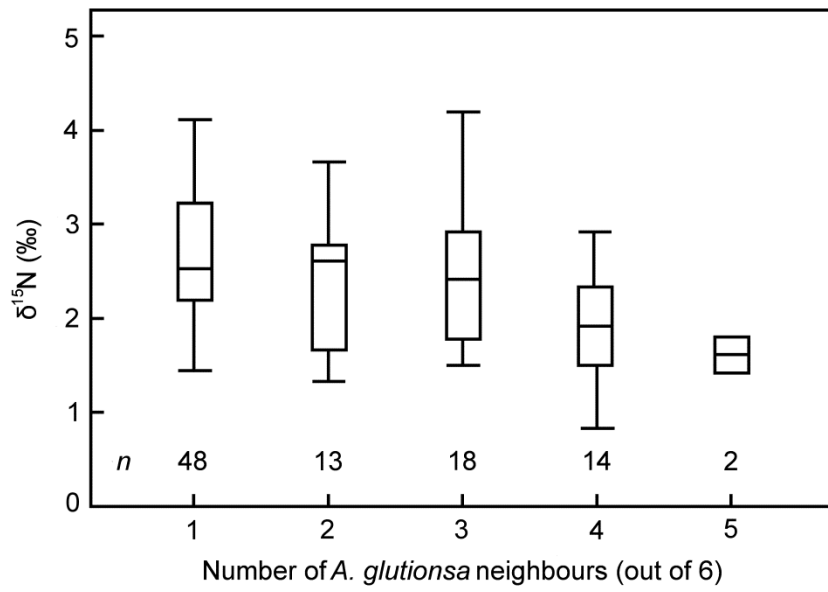


Figure 3.

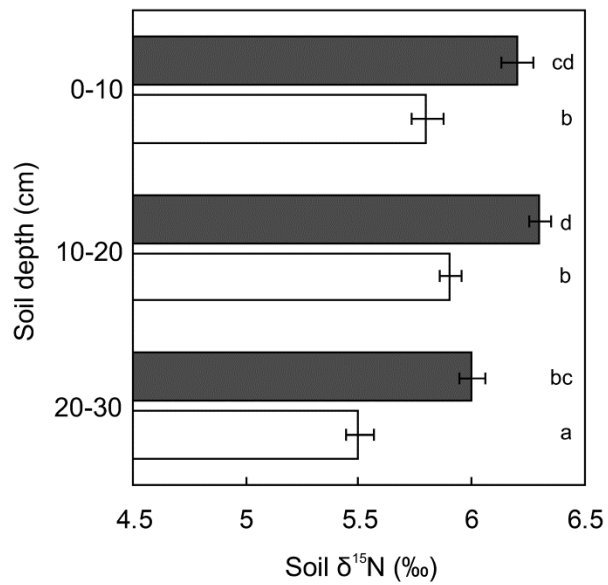


Figure 4.

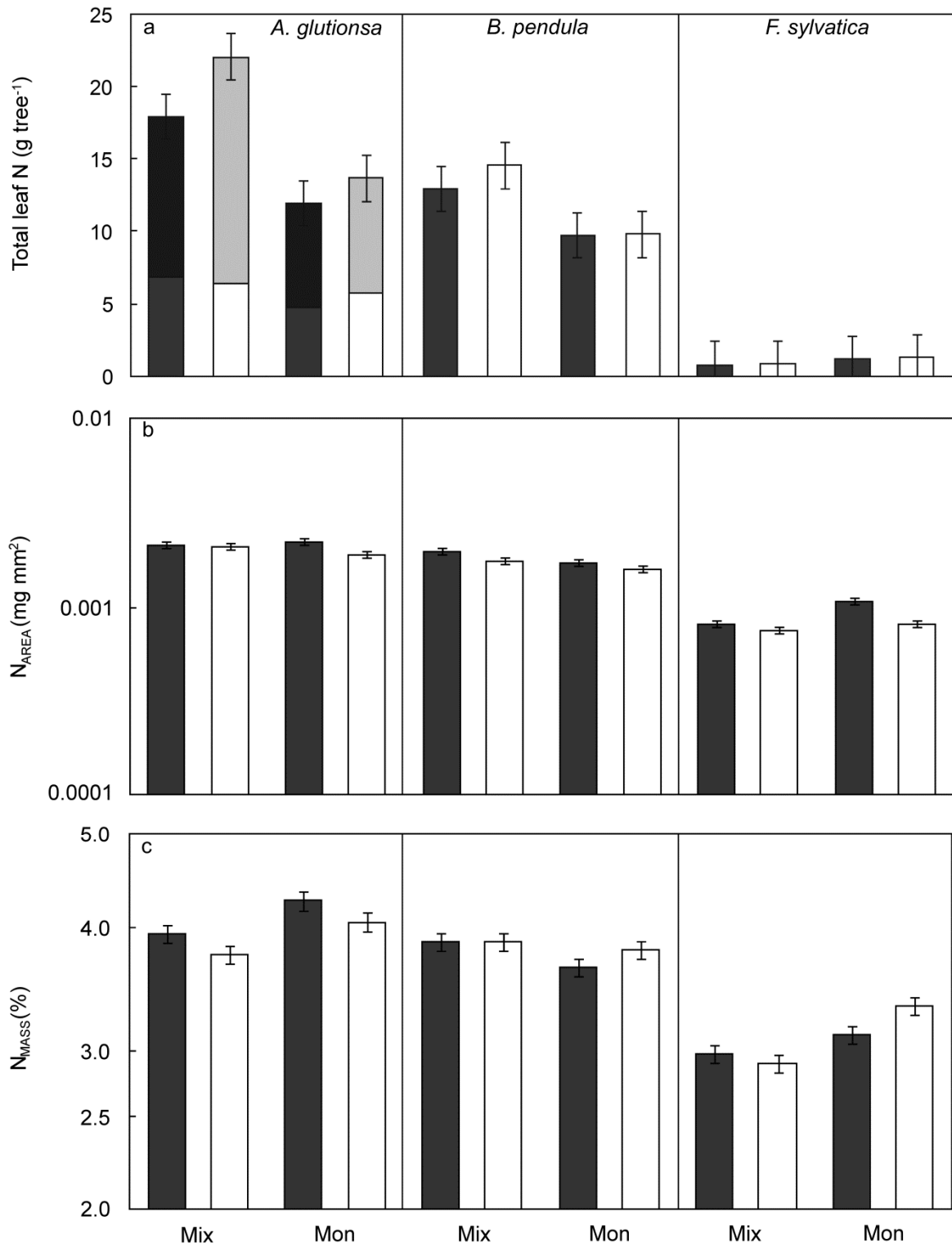


Figure 5.

