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N_2 fixation and cycling in *Alnus glutinosa*, *Betula pendula* and *Fagus sylvatica* woodland exposed to free air CO_2 enrichment.

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

Abstract

We measured the effect of elevated atmospheric CO_2 on atmospheric nitrogen (N_2) fixation for the tree species *Alnus glutinosa* growing in monoculture or in mixture with the non-N_2-fixing tree species *Betula pendula* and *Fagus sylvatica*. We addressed the hypotheses that 1: N_2 fixation in *A. glutinosa* will increase in response to increased atmospheric CO_2 concentrations, when growing in monoculture, 2: the impact of elevated CO_2 on N_2 fixation in *A. glutinosa* is the same in mixture and in monoculture and 3: the impacts of elevated CO_2 on N cycling will be evident in a decrease in leaf $\delta^{15}N$ and in the soil-leaf enrichment factor (EF), and that these impacts will not differ between mixed and single species stands. Trees were grown in a forest plantation on former agricultural fields for 4 growing seasons, after which the trees were on average 3.8 m tall and canopy closure had occurred. Atmospheric CO_2 concentrations were maintained at either ambient or elevated (by 200 ppm) concentrations using a free-air CO_2 enrichment (FACE) system. Leaf $\delta^{15}N$ was measured and used to estimate the amount (N_{dfa}) and proportion ($\%N_{dfa}$) of N derived from atmospheric fixation. On average 62% of the N in *A. glutinosa* leaves was from fixation. $\%N_{dfa}$ and N_{dfa} for *A. glutinosa* trees in monoculture did not increase under elevated CO_2, despite higher growth rates. However, N_2 fixation did increase for trees growing in mixture, despite the absence of significant growth stimulation. There was evidence that fixed N_2 was transferred from *A. glutinosa* to *F. sylvatica* and *B. pendula*, but no evidence that this affected their CO_2 response. This study shows that N_2 fixation in *A. glutinosa* may be higher in a future elevated CO_2 world, but that this effect will only occur where the trees are growing in mixed species stands.

Key words: FACE; ^{15}N natural abundance; greenhouse gasses; forest ecology; plant interactions.

Introduction

Human manipulation of the carbon (C) cycle has increased the concentration of Carbon Dioxide (CO_2) in the atmosphere, with future increases expected to have large environmental impacts (Soloman et al. 2007). Forest ecosystems play an important role in the global C cycle because they contain almost 60% of global terrestrial C (Grace 2004) and contribute approx. 50-60% of terrestrial net primary productivity (Saugier et al. 2001). As a result they exchange large amounts of CO_2 with the atmosphere and are important sinks for anthropogenic CO_2 emissions (Pacala et al. 2001; Saugier et al. 2001; Janssens et al. 2003).

Tree growth is limited by present atmospheric CO_2 concentrations (Long et al. 2004) and so is predicted to be stimulated by elevated atmospheric CO_2 (Norby et al. 2005). However, tree growth in natural systems is also regularly limited by nitrogen (N) availability (Körner 2003; Millard et al. 2007). Furthermore, trees may become increasingly N-limited as atmospheric CO_2 concentrations rise, because increased growth is accompanied by increased N requirement which may not be met by increased root N uptake (Luo et al. 2004). As a result the 'fertilisation' effect of elevated CO_2 may be reduced (Oren et al. 2001; Ainsworth and Long 2005; Reich et al. 2006b). However, elevated CO_2 might also stimulate increased N uptake (Finzi et al. 2007), through increased plant investment in N capture to support increased growth. This strong interdependence between N and C use means that understanding the interactions between elevated atmospheric CO_2 and N use and cycling in forests is essential, for the accurate prediction of future global C dynamics (Reich et al. 2006a). In particular the role of atmospheric N_2 fixation in plant and ecosystem responses to elevated CO_2 has been relatively little studied in forest ecosystems.

By directly accessing N fixed from the atmosphere by symbiotic bacteria, N_2-fixing plants are able to reduce their reliance on root-derived N to some extent (Postgate 1998; Vessey et al. 2005). Furthermore, N_2 fixation is an important source of N for forest ecosystems, providing 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). N_2 fixation in trees may be stimulated by elevated CO_2
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 N_2-fixing plants may
86 show a different response to elevated CO_2 than non-N_2-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 CO_2 enrichment) experiment to-date to have included an N_2-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 CO_2; 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 CO_2 can be affected by the
94 extent and type of plant-plant interactions they experience (Poorter and Navas 2003). Plant
95 responses to elevated CO_2 when growing with other plants are poorly predicted by
96 performance in isolation (Poorter and Navas 2003). Additionally, the impact of elevated CO_2
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 CO_2 when
99 growing in different combinations of species. For example, N limitation in the entire plant
100 community can be reduced when N_2-fixing plants are present (Roggy et al. 2004; Daudin and
101 Sierra 2008), which might influence the response of the community to elevated CO_2. FACE
102 studies in grassland systems have shown that the CO_2 effect on legume N_2 fixation is similar
103 in mixed and single species communities (Lee et al. 2003). The presence of N_2-fixing plants
104 in these communities enhanced leaf N content and photosynthesis in co-occurring non-N_2-
105 fixing plants, but did not affect the CO_2 response of these plants. No FACE studies in forest

106 systems have included mixed species stands containing N_2-fixing tree species. Therefore, it is
107 not clear how N_2-fixing and their non-N_2-fixing neighbours and will respond in mixed
108 species stands.

109 When growing with N_2-fixing plants, non-N_2-fixing plants may be able to access some fixed
110 N through direct transfer by release from nodulated roots, along common mychorrhizal
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-N_2-fixing plants. For
114 example, significant amounts of the N in non-N_2-fixing species (*Pinus contorta* and
115 *Dichanthium aristatum*) has been shown to originate from atmospheric fixation by their N_2-
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 CO_2 on the transfer of fixed N between N_2-fixing and non-N_2-fixing
119 trees.

120 The measurement of the relative abundance of the two most abundant stable isotopes of N
121 (^{14}N, which constitutes approximately 99.6% of all N and ^{15}N, 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 ^{15}N enrichment (i.e. an increase in the proportion of ^{15}N
125 and therefore $\delta^{15}N$) or ^{15}N depletion (i.e. a decrease in the amount of ^{15}N and therefore $\delta^{15}N$).
126 Thus, the $\delta^{15}N$ of a tree reflects the $\delta^{15}N$ 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 $\delta^{15}N$ 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

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

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

Materials and Methods

Site description and sampling methods

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

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

The FACE plots are located within a wider forest plantation, which is continuous over a total area of 2.36 ha and is spread over two fields that are within 20 m of each other. This plantation was established at the same time as the FACE plots in March 2004 and was planted with a mixture of tree species (*Anus glutinosa* (L.) Gaertn., *Betula pendula* Roth., *Fagus sylvatica* L., *Fraxinus excelsior*, *Acer pseudoplatinus*, *Castanea satvia* and *Quercus robur*) and has been subject to no human disturbance since planting. Four FACE and four ambient plots were randomly located within this plantation, evenly split between the two fields, in a complete replicated block design. These experimental plots were 8 m in diameter, and planted at 80 cm spacing in a hexagonal design (approx. 18000 stems ha^{-1}) with 2 year old *B. pendula*, *A. glutinosa* and *F. sylvatica*. These species are native to the UK, cover a range of ecological and life history traits, and can grow together in semi-natural systems. At planting the trees were approximately 60 cm in height, when the CO_2 system was turned on in 2005 they were respectively 140.71±8.1, 116.82±6.3 and 51.17±2.63 cm in height, at the time of leaf collection in 2008 canopy closure had occurred and the trees were on average 463.21±10.8, 487.83±9.7 and 196.25±7.2 cm in height respectively. The plots are surrounded by a 10 m buffer strip of *B. pendula*, *A. glutinosa* and *F. sylvatica* planted at the same density. The planting pattern within these plots created seven sub-plots with mixtures containing one, two or three species. For the purpose of this study, trees in four of these sub-plots were measured: three single species sub-plots and the sub-plot containing a mixture of all three species. These three treatments (CO_2, mixture/monoculture and species) are combined in a 2×2×3 full factorial design resulting in 12 treatment combinations.

Carbon dioxide enrichment was achieved using pure CO_2 from natural gas injected through laser-drilled holes in tubing mounted on eight masts (Miglietta et al. 2001). The elevated CO_2

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) CO_2 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 CO_2 was a 32% increase in total *A. glutinosa* biomass under elevated CO_2
187 when growing in monoculture. There was no significant impact of elevated CO_2 on any of the
188 other species growing in monoculture or any of the species growing in mixture. *Alnus*
189 *glutionsa* 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 CO_2 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 $\delta^{15}N$ of tree leaves may
203 be dependent on their position in the canopy (Domenach et al. 1989). The vertical extent of

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

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

Natural abundance stable isotope method

We measured the contribution of N derived from the atmosphere (N_{dfa}) to the N budget of the *A. glutinosa* trees using the natural abundance stable isotope method (after Shearer and Kohl 1986). This method was used because it was not possible to add labelled N to the site due to 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. glutionsa*.

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. glutionsa* 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 references 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 $\delta^{15}N$ of the sources of all three species and in fractionation within the trees is
256 assumed. The broad similarities of $\delta^{15}N$ in *F. sylvatica* and *B. pendula* leaves suggests that
257 this assumption holds (the difference in $\delta^{15}N$ 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 $\delta^{15}N$ did not differ from weighted whole tree $\delta^{15}N$ for any of the
260 three species (details in supplementary information). Thus, the use of leaf samples as
261 representative of whole tree $\delta^{15}N$ 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 ($\%N_{dfa}$) was calculated from the $\delta^{15}N$ of
265 the leaves using a simple one-isotope, two-source, end-member mixing model as follows
266 (after Shearer and Kohl 1986):

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

268 where %Ndfa is the percentage of leaf-N fixed from the atmosphere, $\delta^{15}N_{REF}$ is the $\delta^{15}N$ of
269 trees for which the only source of N is through soil uptake (in this study the mean $\delta^{15}N$ of
270 leaves on *F. sylvatica* and *B. pendula* growing in monoculture in the same ring), $\delta^{15}N_{TREE}$ is
271 the $\delta^{15}N$ of the tree of interest and B is the $\delta^{15}N$ 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). %Ndfa and
274 Ndfa 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 $\delta^{15}N$ from differences in bulk soil $\delta^{15}N$, 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 $\delta^{15}N$ might be less
280 sensitive due to changes in bulk soil $\delta^{15}N$ (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: $EF = \delta^{15}N_{SOIL} - \delta^{15}N_{LEAF}$

283 where $\delta^{15}N_{SOIL}$ is the overall mean $\delta^{15}N$ of soil from 0-10, 10-20 and 20-30 cm depth and
284 $\delta^{15}N_{LEAF}$ is the overall mean $\delta^{15}N$ 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 (CO2) 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: CO2 + Ring(CO2) + MixMon + Species +
297 MixMon × Ring(CO2) + Species × Ring(CO2) + CO2 × Species + CO2 × MixMon + Species
298 × MixMon + Species × MixMon × Ring(CO2) + CO2 × Species × MixMon. Ndfa and $\%N_{dfa}$
299 were only analysed for *A. glutinosa*, using the same model but with the terms containing

300 'Species' omitted. Soil $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 CO_2×Species
319 interaction). Species composition had a significant impact on $\delta^{15}N$ 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.

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

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

Patterns of N_{AREA} and N_{MASS} were broadly similar (Fig. 4b, 4c; Table 1). For both of these measures of leaf N concentration there were differences between species, with leaf N 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 CO_2, N_{AREA} and N_{MASS} were affected differently. There was no impact of elevated
353 CO_2 on N_{MASS}. However, elevated CO_2 reduced N_{AREA} by an average of 5.3%. This reduction
354 was consistent for all species.

355 When $\delta^{15}N$ 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 CO_2, with *A. glutinosa* trees obtaining 46% more N
358 from fixation than under ambient atmospheric CO_2 (Fig. 4a, Table 2, CO_2×'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 CO_2 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 N_2 fixation under elevated CO_2 for trees in mixture, the percentage contribution
364 of fixed N increased by 6.9% for these trees compared to those in ambient CO_2 (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 CO_2, but no significant effect of CO_2
368 overall (Table 2).

369 ## Discussion

370 Our study is the first to measure N_2 fixation in a tree species in FACE conditions. The
371 observed increased growth of *A. glutinosa* in monoculture under elevated CO_2 was not
372 supported by increased N_2 fixation, either on an absolute (N_{dfa}) or relative ($\%N_{dfa}$) basis. Thus

373 we cannot support our first hypotheses, that N_2 fixation in *A. glutinosa* will increase in
374 response to increased atmospheric CO_2 concentrations, when growing in monoculture.
375 Instead elevated CO_2 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 N_2 fixation increases
378 to support higher growth rates under elevated CO_2. Norby (1987) and Vogel et al. (1997)
379 found that *A. glutionsa* trees growing in elevated CO_2 were larger and fixed more N, but that
380 this was due to their larger size rather than an increase in the rate of N_2 fixation per se.
381 However, Temperton et al. (2003) grew *A. glutionsa* trees in more realistic field conditions
382 and found that elevated CO_2 had no statistically significant impact on N_2 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. glutionsa* does not support CO_2 induced growth increase
385 with N_2 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 N_2
389 fixation to elevated CO_2. Thus we cannot support our second hypothesis, that the impact of
390 elevated CO_2 on N_2 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 N_2 fixation in *Lupinus* sp. was increased by elevated atmospheric CO_2 in both
393 monoculture and in a mixed grassland system. We provide some evidence that N_2 fixation
394 might have been stimulated by elevated CO_2 for *A. glutionsa* trees growing in mixture,
395 despite there being no statistically significant impact of CO_2 on tree biomass. There were
396 large differences in growth rate, N uptake and N_2 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. glutionsa* 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 $\delta^{15}N$ 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 $\delta^{15}N$ are explained by the incorporation of fixed N_2 into
412 these tissues. This is consistent with other studies where $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$
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.

There is clear evidence that the *A. glutinosa* trees in this study obtained a significant proportion of their N from biological fixation. The leaves of *A. glutinosa* trees were ^{15}N depleted relative to those of *F. sylvatica* or *B. pendula* in the same plots. This suggests that a large proportion (approximately 62%) of the N contained in the trees was fixed from the atmosphere. This is consistent with previous studies of *Alnus*. For example, (Sanborn et al. 2002) found that *A. viridis* fixed 10-15 kg N ha^{-1} $year^{-1}$ and that this contributed >90% of the total N content of these trees. Ekblad and Huss-Danell (1995) found that for *A. incana* fixed N_2 contributed between 45% and 90% of total N. As a result of this uptake of fixed N_2, *A. glutinosa* in our study relied on root derived N to a far smaller extent than did the non-N_2–fixing species.

Ecosystem C and N pools are tightly linked (Chen and Coops 2009). Therefore, forest responses to elevated atmospheric CO_2 are linked to ecosystem N availability and cycling (Oren et al. 2001; Ainsworth and Long 2005; Norby and Iversen, 2006; Reich et al. 2006b; Zak et al. 2006; Finzi et al. 2007). For non-N_2-fixing trees leaf $\delta^{15}N$ is determined by source (i.e. soil) $\delta^{15}N$ subject to any fractionation that occurs during uptake or within the tree. Thus, changes in leaf $\delta^{15}N$ might reflect changes in bulk soil $\delta^{15}N$, differential uptake of different forms of N (with different $\delta^{15}N$ signatures) or changes in fractionation during uptake. The impact of elevated CO_2 on N cycling can therefore be reflected in leaf $\delta^{15}N$, with a tendency towards a decrease in $\delta^{15}N$ when CO_2 is elevated for both woody and herbaceous plants (BassiriRad et al. 2003).

The relative ^{15}N depletion by 0.4‰ of tree leaves under elevated CO_2 in our study was matched by relative enrichment of soil by 0.4‰. Thus the $\delta^{15}N$ of the plant-soil system appears to have remained constant, but elevated CO_2 appears to have resulted in a change in distribution of ^{15}N from plant to soil. The use of a soil-leaf enrichment factor (EF) quantifies 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 CO_2 indicating a consistent change in the distribution of ^{15}N
449 between soil and leaf. The relative leaf ^{15}N depletion and associated changes in the soil-plant
450 ^{15}N enrichment factor (EF) for trees growing under elevated CO_2 follow the trend for
451 identified by Bassirirad et al. (2003). The opposing response of soil and leaves suggests that
452 changes in leaf $\delta^{15}N$ are not due to changes in bulk soil $\delta^{15}N$, or internal fractionation.
453 Furthermore, the largest ^{15}N depletion was in one of the non-N_2-fixing trees suggesting that
454 the effect is not due to atmospheric N_2 fixation. This is good evidence to support our third
455 hypothesis, that the impacts of elevated CO_2 on N cycling will be evident in a decrease in leaf
456 $\delta^{15}N$ 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 ^{15}N depletion
458 is increased reliance on mycorrhizal derived N, which tends to be ^{15}N depleted (Hobbie et al.
459 2000; Mayor et al. 2008). Increased mycorrhizal infection under elevated CO_2 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 ^{15}N
462 enriched NH_4^+ or relatively ^{15}N depleted NO_3^- (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 $\delta^{15}N$ 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 CO_2 was elevated was supported by increased N_2 fixation. We found some evidence of
468 biologically significant CO_2 stimulation of N_2 fixation in mixed stands, despite there being no
469 statistically significant increase in growth. We found evidence of significant impacts of
470 elevated CO_2 on aspects of the N cycle, shown through differences in N_2 fixation and $\delta^{15}N$.
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 N_2 fixation in *A. glutionsa*, transfer of
474 fixed N_2 to non-N_2-fixing species, changes in leaf $\delta^{15}N$ 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 CO_2 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 CO_2 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 CO_2 on these N budgets.

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Table 1 Results of univariate GLM for characteristics of trees of three species (*Alnus glutinosa*, *Betula. pendula* and *Fagus sylvatica*) growing in monoculture or mixture (Mix/Mon) at ambient or elevated (ambient + 200 ppm) CO_2 growing in the BangorFACE experiment. Presented are *P*-values from the analyses of $\delta^{15}N$, soil-to-leaf nitrogen enrichment factor (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}). Significant (*P*<0.1) effects are in bold.

Effect	d.f.	$\delta^{15}N$	EF	N_{TOTAL}	N_{AREA}[a]	N_{MASS}[a]	Ndfs
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

[a]Data were Log_{10} transformed before analysis

669 **Table 2** Results of univariate GLM for impacts on N_2 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) CO_2 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.

		N_{dfa}		$\%N_{dfa}$	
Effect	d.f.	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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676

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Figure legends

Fig. 1 Difference in a) $\delta^{15}N$ and b) soil-leaf N enrichment factor (EF) of leaves of *Alnus glutinosa, Betula pendula* and *Fagus sylvatica* growing in the BangorFACE experiment. Presented are mean±SE of trees growing in monoculture (Mon) or in a mixture (Mix) of all three species at ambient (filled bars) or elevated (ambient + 200 ppm, open bars) atmospheric CO_2. Note that the x-axis minimum is -1.9. This is the expected $\delta^{15}N$ for *Alnus glutinosa* growing with no root N. Statistics results in Table 1

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

Fig. 3 $\delta^{15}N$ (mean±SE) of soil in the BangorFACE experiment at three depths at ambient (filled bars) or elevated (ambient + 200 ppm, open bars) atmospheric CO_2. Pooled data from three different stand types (*A. glutinosa*, *B. pendula* and *F. sylvatica* monoculture or in a mixture of all three species) are presented because there were no significant differences between stands. Bars with different letters are significantly different from each other (Fisher's protected LSD, *P*<0.05). Repeated Measures GLM results: Depth - *P*<0.001, CO2 - *P*=0.034; Stand - *P*=0.69, Depth×CO2 - *P*=0.32, Depth×Stand *P*<0.001, CO2×Stand - *P*=0.98, Depth×CO2×Stand - *P*=0.50

Fig. 4 Characteristics of three tree species growing in the BangorFACE experiment in monoculture (Mon) or in a mixture (Mix) of all three species at ambient (filled bars) or elevated (ambient + 200 ppm, open bars) atmospheric CO_2. a) total leaf N content per tree (upper parts of bars for *A. glutinosa* indicate N from atmospheric fixation (N_{dfa}), all other bars are N from soil (N_{dfs})); b) leaf N concentration on an area basis (N_{AREA}); c) leaf N concentration on a mass basis (N_{MASS}). Data for a are mean±SE, for b and c geometric mean±SE (note log y axis). Statistics results are in Table 1, results for N_{dfa} are in Table 2

Fig. 5 The percent of N derived from atmospheric fixation (N_{dfa}) in *A. glutinosa* grown in mixture (with *B. pendula* and *F. Sylvatica*) and in monoculture, under ambient CO_2 (filled bars) and elevated CO_2 (open bars). Presented are the mean±SE. Statistics results are in Table 2

Figure 1

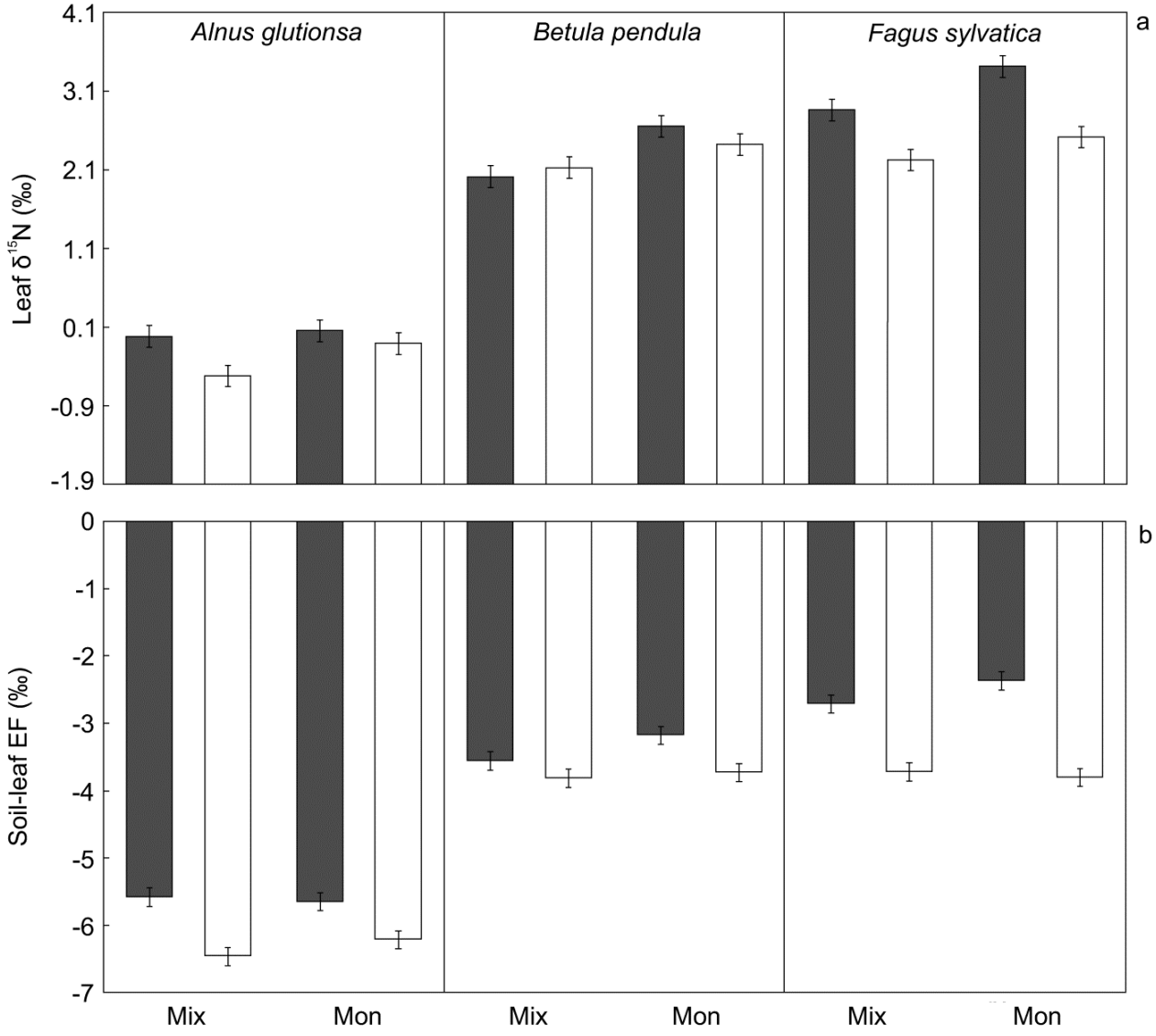
Alnus glutionsa
Betula pendula
Fagus sylvatica
a
b
Leaf δ15N (‰)
Soil-leaf EF (‰)
4.1
3.1
2.1
1.1
0.1
-0.9
-1.9
0
-1
-2
-3
-4
-5
-6
-7
Mix
Mon
Mix
Mon
Mix
Mon

Figure 2.

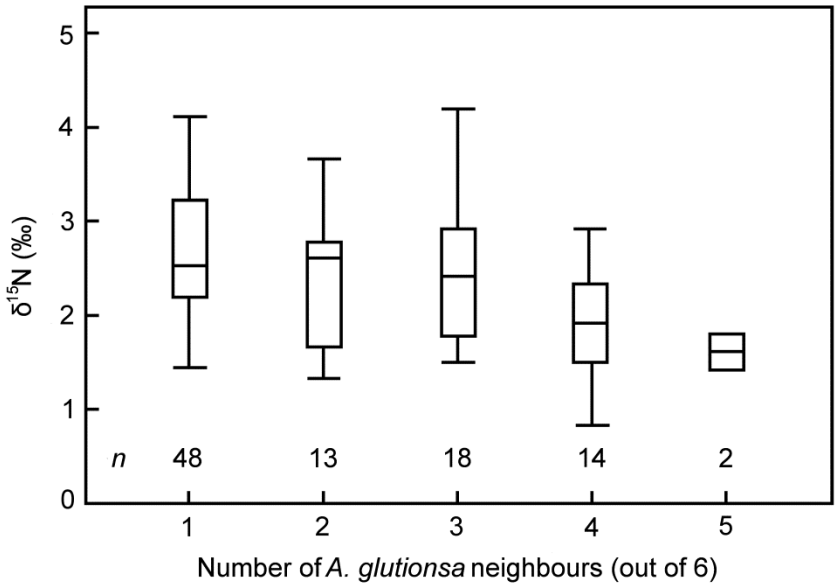
5
4
3
2
1
0
δ15N (‰)
n
48
13
18
14
2
1
2
3
4
5
Number of A. glutionsa neighbours (out of 6)

Figure 3.

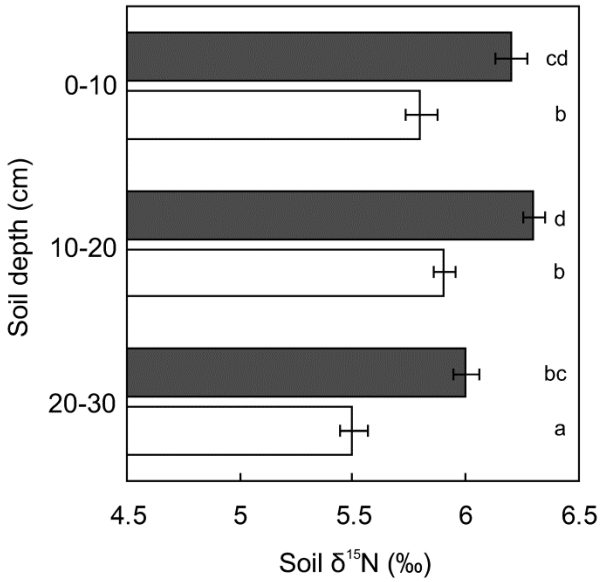

Figure 4.

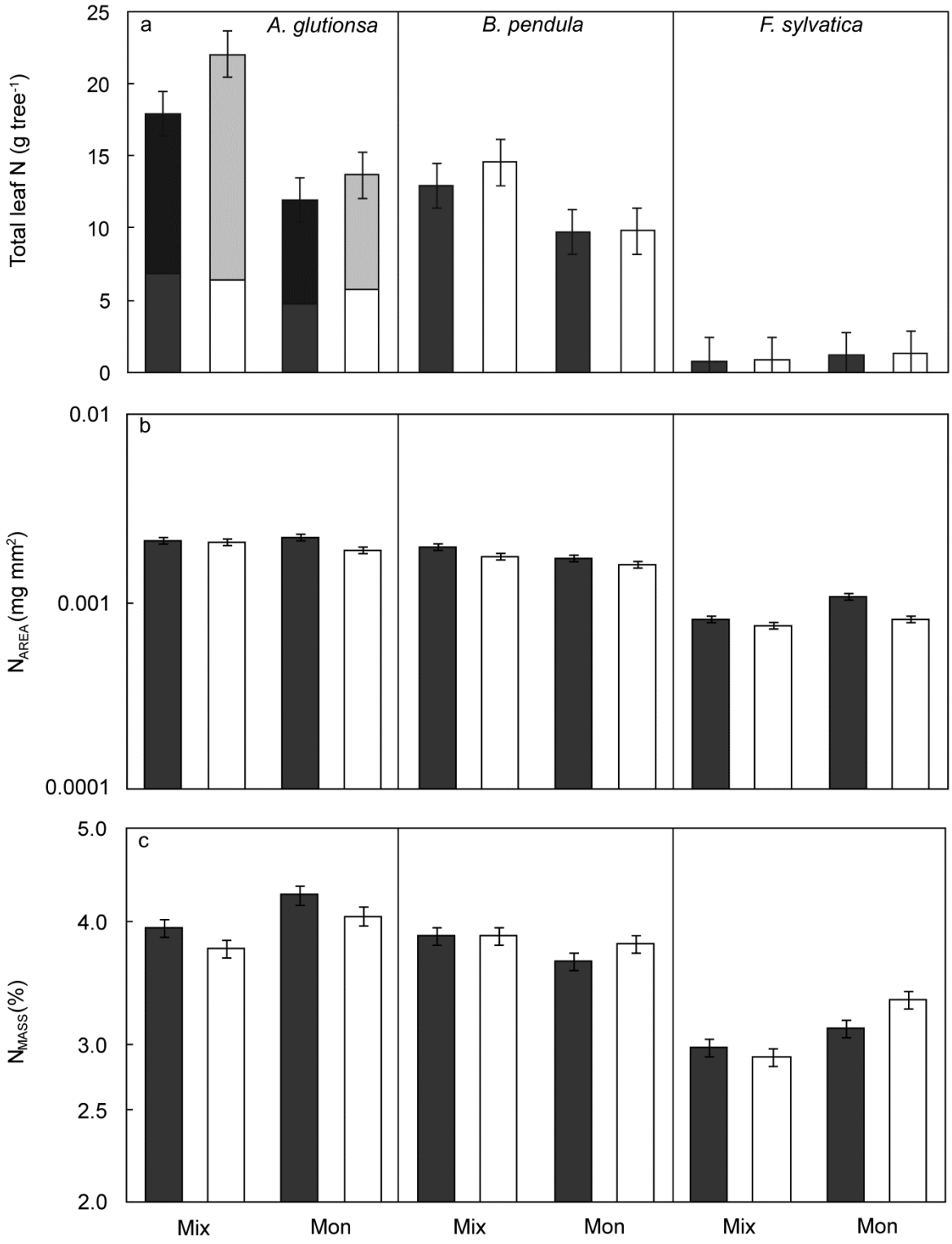
a
A. glutionsa
B. pendula
F. sylvatica
Total leaf N (g tree-1)
25
20
15
10
5
0
b
NAREA (mg mm2)
0.01
0.001
0.0001
c
NMASS (%)
5.0
4.0
3.0
2.5
2.0
Mix
Mon
Mix
Mon
Mix
Mon

Figure 5.