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

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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.
30

Abstract

We measured the effect of elevated atmospheric CO₂ on atmospheric nitrogen (N₂) fixation for the tree species *Alnus glutinosa* growing in monoculture or in mixture with the non-N₂-fixing tree species *Betula pendula* and *Fagus sylvatica*. We addressed the hypotheses that 1: N₂ fixation in *A. glutinosa* will increase in response to increased atmospheric CO₂ concentrations, when growing in monoculture, 2: the impact of elevated CO₂ on N₂ fixation in *A. glutinosa* is the same in mixture and in monoculture and 3: the impacts of elevated CO₂ on N cycling will be evident in a decrease in leaf $\delta^{15}\text{N}$ 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 CO₂ concentrations were maintained at either ambient or elevated (by 200 ppm) concentrations using a free-air CO₂ enrichment (FACE) system. Leaf $\delta^{15}\text{N}$ 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 CO₂, despite higher growth rates. However, N₂ fixation did increase for trees growing in mixture, despite the absence of significant growth stimulation. There was evidence that fixed N₂ was transferred from *A. glutinosa* to *F. sylvatica* and *B. pendula*, but no evidence that this affected their CO₂ response. This study shows that N₂ fixation in *A. glutinosa* may be higher in a future elevated CO₂ world, but that this effect will only occur where the trees are growing in mixed species stands.

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

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

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

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

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

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

The measurement of the relative abundance of the two most abundant stable isotopes of N (¹⁴N, which constitutes approximately 99.6% of all N and ¹⁵N, which constitutes approximately 0.4% of all N), provides a useful tool for investigating the N cycle. Some processes result in fractionation (i.e. the preferential movement or uptake of the heavier or lighter isotope) resulting in relative ¹⁵N enrichment (i.e. an increase in the proportion of ¹⁵N and therefore δ¹⁵N) or ¹⁵N depletion (i.e. a decrease in the amount of ¹⁵N and therefore δ¹⁵N). Thus, the δ¹⁵N of a tree reflects the δ¹⁵N of the N source(s) subject to any fractionation that occurs during movement from or to the tree, gains and losses of N and N pool mixing (Robinson 2001). As such changes in δ¹⁵N can indicate changes in these components of forest N cycling (Emmett et al. 1998; Robinson 2001; BassiriRad et al. 2003). While these changes 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 $\delta^{15}\text{N}$ of each is distinctly different, the $\delta^{15}\text{N}$ 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. glutinosa* 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 $\delta^{15}\text{N}$ 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 1-2° 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 CO₂ 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 (CO₂, 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 CO₂ from natural gas injected through laser-drilled holes in tubing mounted on eight masts (Miglietta et al. 2001). The elevated CO₂

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

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

Measurements and leaf samples were taken in 2008, when the trees were approximately 6.5 years old, after 4 growing seasons of the CO₂ treatment. Three individual trees were sampled from each species growing in monoculture and in mixture (i.e. $n=3$ trees per species per sub-plot, 18 trees per ring), in each of the 4 ambient and elevated FACE rings (total $n=144$ trees). The trees to be sampled were chosen from those in the centre of each sub-plot (i.e. monoculture or mixture), from where they were selected at random. For trees growing in monoculture all 6 nearest neighbours (accounting for the hexagonal planting design) were the same species. For trees growing in mixture the 6 nearest neighbours contained at least one individual from each of the three species. For each tree, diameter of the main stem (stem diameter at 22.5 cm height) and height were measured. Additionally, leaf samples ($n=5$ per tree) were taken. A stratified random sample of leaves was taken from the canopy of each tree to ensure that the leaf sample was representative. This is because $\delta^{15}\text{N}$ of tree leaves may 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 $\delta^{15}\text{N}$ 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 subsample of the soil from each depth was taken, dried at 80°C overnight, sieved <2 mm and ground to a fine powder. $\delta^{15}\text{N}$ 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 (‰) ($\delta^{15}\text{N}$ of air is therefore by definition 0‰). As such, $\delta = (R_{\text{sample}}/R_{\text{reference}}) - 1 \times 1000$, and $R = ^{15}\text{N}:^{14}\text{N}$. 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

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

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

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

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

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

Data analysis

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

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

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

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

Results

Leaf $\delta^{15}\text{N}$ differed significantly between species when growing in monoculture with *A. glutinosa* considerably lower than *B. pendula* which was slightly lower than *F. sylvatica* (Table 1, Fig. 1a). When compared with *A. glutinosa* across both CO_2 treatments, *B. pendula* and *F. sylvatica* were relatively ^{15}N enriched, by 2.5‰ and 2.9‰ respectively. The leaves of all species were ^{15}N depleted under elevated CO_2 , by on average 0.4‰ compared to those in ambient CO_2 , but this effect was only statistically significant for *F. sylvatica* (*A. glutinosa* = 0.3‰, *B. pendula* = 0.1‰, *F. sylvatica* = 0.8‰; Fig 1a, Table 1, CO_2 effect and $\text{CO}_2 \times \text{Species}$ interaction). Species composition had a significant impact on $\delta^{15}\text{N}$ values of trees grown in mixture, which were significantly ^{15}N depleted compared to those in monoculture (Fig. 1a, Table1). Furthermore, the leaves of the non- N_2 -fixing species became less ^{15}N enriched with increasing numbers of *A. glutinosa* neighbours (Fig. 2). Though when considering the two 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 $\delta^{15}\text{N}$ 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= $20.0\pm 1.6 \text{ g. tree}^{-1}$, monoculture= $12.8\pm 1.6 \text{ g. tree}^{-1}$, 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

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

When $\delta^{15}\text{N}$ was used to estimate the amount of fixed N in *A. glutinosa* the trees gained on average $10.5 \pm 0.9 \text{ g. tree}^{-1}$ of N from fixation. For trees growing in mixture there was a trend towards increased N_{dfa} under elevated CO₂, with *A. glutinosa* trees obtaining 46% more N from fixation than under ambient atmospheric CO₂ (Fig. 4a, Table 2, CO₂ × 'MixMon', $P=0.15$). While this effect is not statistically significant, the magnitude of the effect is likely to be biologically significant. As a result of this increase in mixture there was a significant effect of species composition on N_{dfa} but no overall effect of CO₂ treatment (Table 2). This fixed N contributed on average $62.1 \pm 0.1 \%$ of the total N in *A. glutinosa* leaves. As a result of the increased N₂ fixation under elevated CO₂ for trees in mixture, the percentage contribution of fixed N increased by 6.9% for these trees compared to those in ambient CO₂ (68.3% compared to 61.4%, Fig. 5). This effect resulted in a significant impact of species composition on %N_{dfa} and a trend towards an interaction (though not statistically significant) between species composition and the impact of elevated CO₂, but no significant effect of CO₂ overall (Table 2).

Discussion

Our study is the first to measure N₂ fixation in a tree species in FACE conditions. The observed increased growth of *A. glutinosa* in monoculture under elevated CO₂ was not 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

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

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

Furthermore, leaves of *F. sylvatica* and *B. pendula* with greater numbers of *A. glutinosa* trees as direct neighbours were significantly depleted in ^{15}N compared to those with fewer. It seems likely that these changes in $\delta^{15}\text{N}$ are explained by the incorporation of fixed N_2 into these tissues. This is consistent with other studies where $\delta^{15}\text{N}$ of N_2 -fixing trees has been compared with co-occurring non- N_2 -fixing species (e.g. Daudin and Sierra 2008) and where the transfer of fixed N_2 specifically has been measured. For example the contribution of transferred N to total N was on average 5-15% (Arnebrant et al. 1993) and 1.3-3% (Ekblad and Huss-Danell 1995) between *A. glutinosa* and *P. contorta* and *A. incana* and *P. sylvestris* respectively. These inputs of fixed N_2 do not translate into differences in $\delta^{15}\text{N}$ of the soil in stands containing *A. glutinosa*. This suggests that inputs of fixed N_2 are small relative to the ecosystem N pool, or that little fixed N_2 makes its way into the soil N pool, possibly due to a tightly coupled leaf-soil-plant N cycle. Additionally, the clear impact of *A. glutinosa* on $\delta^{15}\text{N}$ of these species in mixture highlights the importance of choosing reference plants that are not 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 N₂ contributed between 45% and 90% of total N. As a result of this uptake of fixed N₂, *A. glutinosa* in our study relied on root derived N to a far smaller extent than did the non-N₂-fixing species.

Ecosystem C and N pools are tightly linked (Chen and Coops 2009). Therefore, forest responses to elevated atmospheric CO₂ 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-N₂-fixing trees leaf $\delta^{15}\text{N}$ is determined by source (i.e. soil) $\delta^{15}\text{N}$ subject to any fractionation that occurs during uptake or within the tree. Thus, changes in leaf $\delta^{15}\text{N}$ might reflect changes in bulk soil $\delta^{15}\text{N}$, differential uptake of different forms of N (with different $\delta^{15}\text{N}$ signatures) or changes in fractionation during uptake. The impact of elevated CO₂ on N cycling can therefore be reflected in leaf $\delta^{15}\text{N}$, with a tendency towards a decrease in $\delta^{15}\text{N}$ when CO₂ 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 CO₂ in our study was matched by relative enrichment of soil by 0.4‰. Thus the $\delta^{15}\text{N}$ of the plant-soil system appears to have remained constant, but elevated CO₂ 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

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

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

species stands. This is suggested by higher rates of N₂ fixation in *A. glutinosa*, transfer of fixed N₂ to non-N₂-fixing species, changes in leaf $\delta^{15}\text{N}$ and large differences in tree N content. These different impacts have important consequences for how we consider the impacts of global environmental change and interactions with ecosystem function. Changes in atmospheric CO₂ will occur concurrently with changes in plant community species composition due to this and other drivers of global environmental change (Badeck et al. 2001). Thus forest species compositions that exist when the atmospheric CO₂ concentrations used in this and other studies are reached will be different to those at present. Our study shows that these changes can result in very real effects on forest N budgets and in the impact of elevated CO₂ 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) CO₂ growing in the BangorFACE experiment. Presented are *P*-values from the analyses of $\delta^{15}\text{N}$, 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.	$\delta^{15}\text{N}$	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

^aData were Log₁₀ transformed before analysis

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

Effect	d.f.	N _{dfa}		%N _{dfa}	
		F	<i>P</i>	F	<i>P</i>
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

Figure legends

Fig. 1 Difference in a) $\delta^{15}\text{N}$ 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 $\pm$ 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 $\delta^{15}\text{N}$ for *Alnus glutinosa* growing with no root N. Statistics results in Table 1

Fig. 2 $\delta^{15}\text{N}$ 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 25th and 75th 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, $\chi^2 = 12.94$, $P = 0.01$; *B. pendula*: $\chi^2 = 7.78$, $P = 0.1$; *F. sylvatica*: $\chi^2 = 5.57$, $P = 0.135$)

Fig. 3 $\delta^{15}\text{N}$ (mean $\pm$ 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 - $P < 0.001$, CO_2 - $P = 0.034$; Stand - $P = 0.69$, Depth $\times$ CO_2 - $P = 0.32$, Depth $\times$ Stand $P < 0.001$, $\text{CO}_2 \times$ Stand - $P = 0.98$, Depth $\times$ $\text{CO}_2 \times$ 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 CO₂. 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 CO₂ (filled bars) and elevated CO₂ (open bars). Presented are the mean±SE. Statistics results are in Table 2

Figure 1

Figure 2.

Figure 3.

Figure 4.

Figure 5.