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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 (CO₂) 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 CO₂ with the atmosphere and are important sinks for anthropogenic CO₂ emissions (Pacala et al. 2001; Saugier et al. 2001; Janssens et al. 2003).

Tree growth is limited by present atmospheric CO₂ concentrations (Long et al. 2004) and so is predicted to be stimulated by elevated atmospheric CO₂ (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 CO₂ 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 CO₂ may be reduced (Oren et al. 2001; Ainsworth and Long 2005; Reich et al. 2006b). However, elevated CO₂ 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 CO₂ 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 N₂ fixation in plant and ecosystem responses to elevated CO₂ has been relatively little studied in forest ecosystems.

By directly accessing N fixed from the atmosphere by symbiotic bacteria, N₂-fixing plants are able to reduce their reliance on root-derived N to some extent (Postgate 1998; Vessey et al. 2005). Furthermore, N₂ 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
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 N$_2$-fixing tree species. Therefore, it is not clear how N$_2$-fixing and their non-N$_2$-fixing neighbours and will respond in mixed species stands.

When growing with N$_2$-fixing plants, non-N$_2$-fixing plants may be able to access some fixed N through direct transfer by release from nodulated roots, along common mychorrhizal 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-N$_2$-fixing plants. For example, significant amounts of the N in non-N$_2$-fixing species (*Pinus contorta* and *Dichanthium aristatum*) has been shown to originate from atmospheric fixation by their N$_2$-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 CO$_2$ on the transfer of fixed N between N$_2$-fixing and non-N$_2$-fixing trees.

The measurement of the relative abundance of the two most abundant stable isotopes of N ($^{14}$N, which constitutes approximately 99.6% of all N and $^{15}$N, 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 $^{15}$N enrichment (i.e. an increase in the proportion of $^{15}$N and therefore $\delta^{15}$N) or $^{15}$N depletion (i.e. a decrease in the amount of $^{15}$N and therefore $\delta^{15}$N). Thus, the $\delta^{15}$N of a tree reflects the $\delta^{15}$N 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 $\delta^{15}$N 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 δ15N of each is distinctly different, the δ15N 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
(%Ndff) for the N2-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 CO2 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: 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 δ15N 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 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\(_2\)
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
glutionsa* 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 δ¹⁵N 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 $\delta^{15}$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 ($N_{\text{AREA}}$) and mass ($N_{\text{MASS}}$) 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 80°C 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 ($\delta$) notation for stable isotopes. $\delta$ 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 (%00) ($\delta^{15}$N of air is therefore by definition 0‰). As such, $\delta = (R_{\text{sample}}/R_{\text{reference}}) - 1 \times 1000$, 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 ($N_{\text{dfa}}$) 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 $N_{\text{dfa}}$ 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 $%N_{\text{dfa}}$ and $N_{\text{dfa}}$ for $A$. glutinosa growing in ambient CO$_2$ concentrations. Reference plants growing in elevated CO$_2$ concentrations were used to calculate $%N_{\text{dfa}}$ and $N_{\text{dfa}}$ of $A$. glutinosa growing in elevated CO$_2$. Furthermore, the calculations of $N_{\text{dfa}}$ and $%N_{\text{dfa}}$ using each reference species are similar. There is good evidence that no fixed N is incorporated into the references 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 δ¹⁵N of the sources of all three species and in fractionation within the trees is assumed. The broad similarities of δ¹⁵N in *F. sylvatica* and *B. pendula* leaves suggests that this assumption holds (the difference in δ¹⁵N 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 δ¹⁵N did not differ from weighted whole tree δ¹⁵N for any of the three species (details in supplementary information). Thus, the use of leaf samples as representative of whole tree δ¹⁵N is supported. We therefore consider the quantification to remain robust.

**Data analysis**

The proportion of plant N derived from N₂ fixation (%Ndfa) was calculated from the δ¹⁵N of the leaves using a simple one-isotope, two-source, end-member mixing model as follows (after Shearer and Kohl 1986):

\[
\%N_{\text{dfa}} = \frac{(\delta^{15}N_{\text{REF}} - \delta^{15}N_{\text{TREE}})}{(\delta^{15}N_{\text{REF}} - B)} \times 100
\]

where %Ndfa is the percentage of leaf-N fixed from the atmosphere, δ¹⁵N_REF is the δ¹⁵N of trees for which the only source of N is through soil uptake (in this study the mean δ¹⁵N of leaves on *F. sylvatica* and *B. pendula* growing in monoculture in the same ring), δ¹⁵N_TREE is the δ¹⁵N of the tree of interest and B is the δ¹⁵N 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). %Ndfa and Ndfa were calculated separately using *F. sylvatica* or *B. pendula* as reference plants and using the mean value for the two species.
To isolate leaf $\delta^{15}N$ from differences in bulk soil $\delta^{15}N$, a soil-leaf N enrichment factor (EF) was calculated for the two non-N$_2$-fixing trees. The soil-leaf EF measures the relative $^{15}N$ depletion/enrichment from bulk soil to leaf. Thus it provides a sensitive qualitative measure of changes in N cycling in the plant-soil system where patterns in leaf $\delta^{15}N$ might be less sensitive due to changes in bulk soil $\delta^{15}N$ (Amundson et al. 2003; Kahmen et al. 2008). EF was calculated as follows for each tree (after Garten et al. 2007):

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

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

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

The measurements for the five leaf samples per tree were averaged over the whole tree to give one mean value per tree. These tree level data were analysed as a split-split-plot design ANOVA in SPSS (SPSS Inc., 2008) using the general linear model (GLM). Individual rings (Ring) were treated as ‘plots’ and were nested within CO$_2$ (CO2) treatments. Mixture/monoculture (MixMon) was treated as a sub-plot within ring and species was nested within mixture/monoculture. The model used was: CO$_2$ + Ring(CO2) + MixMon + Species + MixMon × Ring(CO2) + Species × Ring(CO2) + CO$_2$ × Species + CO$_2$ × MixMon + Species × MixMon + Species × MixMon × Ring(CO2) + CO$_2$ × Species × MixMon. Ndfa and %N$_{\text{dfa}}$ were only analysed for A. glutinosa, using the same model but with the terms containing
‘Species’ omitted. Soil $\delta^{15}$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 $\delta^{15}$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 $\alpha$ of 0.1 was used. While this increases the risk of a type I error this was considered an acceptable trade-off.

**Results**

Leaf $\delta^{15}$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 CO$_2 \times$Species interaction). Species composition had a significant impact on $\delta^{15}$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 $\delta^{15}$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
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\textit{AREA} and N\textit{MASS} were affected differently. There was no impact of elevated CO2 on N\textit{MASS}. However, elevated CO2 reduced N\textit{AREA} by an average of 5.3%. This reduction was consistent for all species.

When δ\textsuperscript{15}N was used to estimate the amount of fixed N in *A. glutinosa* the trees gained on average 10.5±0.9 g. tree\textsuperscript{-1} of N from fixation. For trees growing in mixture there was a trend towards increased N\textit{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\textit{dfa} but no overall effect of CO2 treatment (Table 2). This fixed N contributed on average 62.1±0.1 % of the total N in *A. glutinosa* leaves. As a result of the increased N\textsubscript{2} 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\textit{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 N\textsubscript{2} 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 N\textsubscript{2} fixation, either on an absolute (N\textit{dfa}) or relative (%N\textit{dfa}) basis. Thus
we cannot support our first hypotheses, that N$_2$ fixation in *A. glutinosa* will increase in response to increased atmospheric CO$_2$ concentrations, when growing in monoculture. Instead elevated CO$_2$ resulted in a slight (but not statistically significant) increase in root N uptake and decrease in leaf N concentration (thought this was only statistically significant on an area basis). Previous studies have shown that in some circumstances N$_2$ fixation increases to support higher growth rates under elevated CO$_2$. Norby (1987) and Vogel et al. (1997) found that *A. glutionsa* trees growing in elevated CO$_2$ were larger and fixed more N, but that this was due to their larger size rather than an increase in the rate of N$_2$ fixation per se.

However, Temperton et al. (2003) grew *A. glutionsa* trees in more realistic field conditions and found that elevated CO$_2$ had no statistically significant impact on N$_2$ fixation. Our study, with the findings of Temperton et al. (2003) suggests that when growing in single species stands, in ‘real world’ conditions *A. glutionsa* does not support CO$_2$ induced growth increase with N$_2$ fixation, but rather with an increase in root N uptake and nitrogen use efficiency. However, it is possible that over longer periods of time this might change.

Our study suggests fundamental differences in forest ecosystem function in mixed stands compared to single species stands. These differences have impacted on the response of N$_2$ fixation to elevated CO$_2$. Thus we cannot support our second hypothesis, that the impact of elevated CO$_2$ on N$_2$ fixation in *A. glutinosa* is the same in mixture and in monoculture. As such, our findings differ from patterns found in other systems. For example, Lee et al. (2003) found that N$_2$ fixation in *Lupinus* sp. was increased by elevated atmospheric CO$_2$ in both monoculture and in a mixed grassland system. We provide some evidence that N$_2$ fixation might have been stimulated by elevated CO$_2$ for *A. glutionsa* trees growing in mixture, despite there being no statistically significant impact of CO$_2$ on tree biomass. There were large differences in growth rate, N uptake and N$_2$ fixation for *A. glutinosa* trees growing in 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 $\delta^{15}N$ 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 $\delta^{15}N$ are explained by the incorporation of fixed N$_2$ into these tissues. This is consistent with other studies where $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 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
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. glutionsa* 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 N\textsubscript{2} fixation in *A. glutionsa*, transfer of fixed N\textsubscript{2} to non-N\textsubscript{2}-fixing species, changes in leaf $\delta^{15}$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 CO\textsubscript{2} 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 CO\textsubscript{2} 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 CO\textsubscript{2} on these N budgets.

**Acknowledgements**

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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 $\delta^{15}$N, soil-to-leaf nitrogen enrichment factor (EF), total leaf N per tree ($N_{\text{TOTAL}}$), leaf N per unit area ($N_{\text{AREA}}$), N per unit mass ($N_{\text{MASS}}$) and N derived from soil ($N_{\text{dfs}}$). Significant ($P<0.1$) effects are in bold.

<table>
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<th>Effect</th>
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<th>EF</th>
<th>$N_{\text{TOTAL}}$</th>
<th>$N_{\text{AREA}}$</th>
<th>$N_{\text{MASS}}$</th>
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aData were Log$_{10}$ 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 %Nₐdfa and Nₐdfa. 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.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Nₐdfa</th>
<th>%Nₐdfa</th>
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<tr>
<td></td>
<td>d.f.</td>
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<td>CO₂</td>
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**Figure legends**

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

**Fig. 2** δ\(^{15}\)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, \(\chi^2 = 12.94, P=0.01\); *B. pendula*: \(\chi^2 = 7.78, P=0.1\); *F. sylvatica*: \(\chi^2 = 5.57, P=0.135\)

**Fig. 3** δ\(^{15}\)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 - \(P<0.001\), CO\(_2\) - \(P=0.034\); Stand - \(P=0.69\), Depth×CO\(_2\) - \(P=0.32\), Depth×Stand \(P<0.001\), CO\(_2\)×Stand - \(P=0.98\), Depth×CO\(_2\)×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 (Ndfa), all other bars are N from soil (Ndfs)); b) leaf N concentration on an area basis (NAREA); c) leaf N concentration on a mass basis (NMASS). 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 Ndfa are in Table 2.

**Fig. 5** The percent of N derived from atmospheric fixation (Ndfa) 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

(A) Leaf $\delta^{15}N$ (

(B) Soil-Leaf EF (%)

Species: 
- Alnus glutinosa
- Betula pendula
- Fagus sylvatica

Mix: 
- Mon:
Figure 2.
Figure 3.
Figure 4.
Figure 5.