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The effect of atmospheric CO_2 enrichment on biogeochemical cycling of temperate forest ecosystems

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Abstract

Anthropogenic activities are increasing atmospheric CO_2 concentrations and significantly altering the global carbon balance. Forest ecosystems occupy one third of the terrestrial surface of the earth, and store approximately 40% of terrestrial biosphere carbon. Despite the importance of forests in the global carbon cycle, the interactive effects of species diversity and elevated atmospheric CO_2 on temperate forest biogeochemistry and productivity remain poorly understood. This study utilised the Bangor Free Air CO_2 Enrichment (BangorFACE) facility to investigate how forest ecosystem biogeochemical cycling is altered by atmospheric CO_2 levels predicted for the year 2050. The experiment consisted of eight FACE rings in a 2 × 4 factorial design, four at ambient atmospheric CO_2 and four at elevated (580 μmol mol^{-1}) atmospheric CO_2. To elucidate the interactive effects of species diversity and elevated atmospheric CO_2, three broadleaved tree species, birch (*Betula pendula*), alder (*Alnus glutinosa*) and beech (*Fagus sylvatica*) were planted in monoculture and three species polyculture within each experimental plot.

Elevated CO_2 enrichment increased above-ground biomass of all species in monoculture, whilst in polyculture the positive effect was approximately four-fold smaller. Conversely, fine root biomass in the elevated CO_2 plots and averaged across all species was two-fold greater in polyculture than in species grown singularly. Detailed examination of fine root morphology revealed a greater to elevated CO_2 response with increasing depth. The effect of growing trees in mixture was accessed by comparing measured data with data predicted from trees grown in monoculture. Above-ground overyielding of trees grown in polyculture was strongly enhanced under ambient conditions, whereas the overyielding response of trees grown in an enriched CO_2 atmosphere was reduced by a third. In contrast to the aboveground results, below ground overyielding was greater in the elevated CO_2 plots suggesting that carbon sequestration may be greater in diverse tree species communities as atmospheric CO_2 concentration increases. Community level physiological profiles were determined using sixteen different low molecular weight substrates. Microbial utilisation kinetics were determined and related to carbon use efficiency and ecosystem function. When species were grown in monoculture elevated CO_2 mediated an increase in catabolic respiration that increased with depth, and was attributed to deeper prolific rooting, and greater mycorrhizal mycelium inputs. Whereas, when species were grown in polyculture a decrease in catabolic respiration and increase in carbon residence time was apparent. Environmental data correlations suggest that resource limitation may constrain microbial catabolic simulation in communities of higher diversity. Substrate utilisation profiles also indicated a shift in microbial structure and function to improve acquisition of nutrients such as P. P cycling was closely examined using the Hedley fractionation procedure. Elevated CO_2 induced a decrease in the labile P fractions, whilst soil organic P pools increased, and recalcitrant occluded P pools decreased throughout the experiment. A negative correlation with mineral P pool and fungal biomass suggested that enhanced mycorrhizal mediated mineral P dissolution may maintain ecosystem demand.

The results contained within this thesis provide some of the first clear evidence that tree biodiversity will not only mediate the aboveground response to high CO_2, but also make a major contribution to carbon input in to soils through the fine root biomass in a CO_2 enriched atmosphere. Reductions of tree biodiversity may weaken the ability of forests to sequester carbon, potentially creating a feedback loop with global implications.

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Abbreviations

ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CFIR-MS	Continuous Flow Isotope Ratio Mass Spectrometry
CLPP	Community Level Physiological Profiling
CO_2	Carbon Dioxide
DBH	Diameter at Breast Height
DOC	Dissolved Organic Carbon
DON	Dissolved Organic Nitrogen
ECM	Ectomycorrhiza
FACE	Free Air Carbon Enrichment
FAO	Food and Agriculture Organization
GLM	General Linear Model
HPLC	High Performance Liquid Chromatography
ICP	Inductively Coupled Plasma Spectrometry
IPCC	Intergovernmental Panel for Climate Change
IRGA	Infra Red Gas Analyser
LAI	Leaf Area Index
LMW	Low Molecular Weight
MRP	Molybdate Reactive Phosphate
MRT	Mean Residence Time
OTC	Open Top Chamber
PCA	Principle Component Analysis
PDB	Pee Dee Belemnite
PID	Proportional Integral Differential
PNL	Progressive Nitrogen Limitation
PPB	Parts Per Billion
PPM	Parts Per Million
RAI	Root Area Index
RLD	Root Length Density
SIR	Substrate Induced Respiration
SLA	Specific Leaf Area
SOC	Soil Organic Carbon
SOM	Soil Organic Matter
SRL	Specific Root Length
TCA	Tri-Carboxylic Acid

1 General Introduction

Anthropogenic activities, such as burning fossil fuels, land use change and functional perturbations of the biosphere's carbon (C) cycle are increasing the level of atmospheric CO_2. Since the industrial revolution beginning in 1750, atmospheric CO_2 concentrations have risen 39% from 280 μmol mol^{-1} to 390 μmol mol^{-1} CO_2 in 2011 (Solomon *et al.* 2007). Analysis of ocean sediment boron-isotope composition, supported by CO_2 trapped in glacial ice (Siegenthaler *et al.* 2005) has revealed that terrestrial flora is growing in atmospheric CO_2 levels not experienced for several million years (Pearson and Palmer 2000). Furthermore, atmospheric gas monitoring at Mauna Loa, Hawaii has provided empirical evidence that CO_2 concentrations are rising at a rate of 1-2 μmol mol^{-1} CO_2 yr^{-1} (Keeling and Whorf, 2005), and accelerating atmospheric CO_2 concentrations are expected to surpass 700 μmol mol^{-1} by the end of the century (Solomon *et al.* 2007).

The global C flux between atmosphere and terrestrial biosphere approximately equates to 60 Pg C yr^{-1}, and is balanced by two main processes: (i) C gain during photosynthetic CO_2 fixation, minus autotrophic respiration, and (ii) C loss during heterotrophic respiration of fungi, animals and bacteria (Chapin *et al.* 2009). However, terrestrial C efflux as volatile organic compounds, methane or dissolved organic C could also be significant (Heimann and Reichstein, 2008). Global C balance is maintained by tightly coupled proportional fluctuations in ecosystem net primary productivity and heterotrophic respiration. These feedback systems are governed by complex physiological processes that may be antagonistically altered by global environmental change. We currently have limited understanding of ecosystem function and indirect influences on atmosphere-biosphere feedback cycles that may alter global C balance.

Forest ecosystems cover an area of 3.9×10^9 ha are a major C store containing half of the all C of terrestrial ecosystems (Schlesing and Lichter, 2001). C stored in forest ecosystems is partitioned between vegetation (31%) 359 Pg C and soils (69%) 787 Pg C. These C pools serve as a sink, sequestering between 1.3 and 1.5 Pg C yr^{-1} (Dixon *et al.* 1994). The functioning of forest ecosystems has a significant role in the future global C cycle as the processes governing the flux of C stored in phytomass

and soil organic mater may be altered by global change. Environmental perturbations, at any magnitude, may fundamentally alter ecosystem processes maintaining the fastidious atmosphere-biosphere C balance. Thus evaluating the impact of climate change on forest ecosystems and terrestrial C dynamics is both scientifically and politically important.

1.1 Plant response to elevated CO_2

Many studies have shown that vegetation utilising the C_3 photosynthetic pathway are stimulated by increases in atmospheric CO_2 through enhanced carboxylation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and competitive inhibition of Ribulose-1,5-bisphosphate (RubP) oxygenation (Long *et al.* 2004). Elucidating the physiology of C assimilation response to elevated CO_2 has enabled modellers to precisely predict photosynthetic leaf responses and scale predictions to the canopy level (Farquhar *et al.* 1980). Modelling of ecosystem responses demonstrated increased productivity under high CO_2, with a greater potential for C storage in phytomass and soil. However, model linkage to belowground ecosystem processes and externalities was required to extrapolate to long-term prediction of global change requiring data from field studies (Amthor, 1994).

Physically studying the effects of elevated CO_2 on plants communities is technically difficult and often expensive (Pontailler *et al.* 1998). Studies involving elevated CO_2 started in earnest during the 1980s, and were instigated by concern over the affect that competition between species using the C_3 and C_4 photosynthetic pathway may have on food supplies (Patterson *et al.* 1984; Kimball, 1983). Initially studies focused on the physiological response of plants, while other researchers related data to broader issues, eventually leading to questions surrounding the impact on terrestrial ecosystem communities and the global C cycle (Eamus and Jarvis, 1989; Ceulemans and Mousseau, 1994). By the early 1990s short-term studies were not only being conducted on ecosystem level interactions of natural grasslands and agronomic crops plant species, but also tree seedlings (Bazzaz, 1990).

1.2 Scaling elevated CO_2 research to whole communities

Experimental methodology has developed considerably during the last few decades. Early studies were conducted with closed and open top growth chambers, branch bags, natural CO_2 vents, and more recently free air CO_2 enrichment (FACE) (Saxe *et al.* 1998). Chamber experiments provide a homogeneous environment for plant growth, at a relatively low fumigation cost compared to field conditions. Although excellent for studying plant level responses, physical size prevents realistic ecosystem studies. Additionally, microclimatic interference caused the chamber structure and control system often result in light attenuation, temperature, and humidity artefacts (Whitehead *et al.* 1995). FACE technology allows the exposure of entire freely rooting plant ecosystems in their natural environment, without significantly modifying the microclimate around the studied species (Nowak *et al.* 2004). FACE is now almost unanimously considered the best method to expose plant communities to future atmospheric CO_2 conditions (Calfapietra *et al.* 2009), despite the significant investment in establishing and maintaining a CO_2 enrichment infrastructure that generally prohibits large scale experimental replication.

1.3 Forest ecosystem responses to elevated CO_2

As current levels of atmospheric CO_2 are not yet sufficient to saturate photosynthesis, forests offer great potential for C sequestration through enhanced growth in future atmospheres (Millard *et al.* 2007). Thus far research has indicated that a 200 ppm increases of atmospheric CO_2 over ambient results in a highly conserved *ca.* 23% increase in net primary productivity (NPP) (Norby *et al.* 2005). However, ecosystem responses are governed by tightly coupled C and nitrogen (N) feedback cycles that are likely to be altered by climatic change. For example, NPP provides organic C detritus that sustains heterotrophic respiration, and the resulting mineralised N maintains NPP. In some ecosystems where P exhibits a limitation to productivity, mineralised P also influences heterotrophic respiration (Hobbie and Vitousek, 2000). NPP may also be influenced by a myriad of environmental factors, including pollutants, temperature, precipitation, and N deposition (Nemani *et al.* 2003). Therefore, stimulation of photosynthesis by increased CO_2 is often not sustained (Körner, 2006), does not always increase productivity (Oren *et al.* 2001), and does not always result in C sequestration (Carney *et al.* 2007).

As forests often occur on low nutrient soils, our ability to estimate forest C sequestration is confounded by our knowledge of nutrient cycling and indirect feedback mechanisms under elevated atmospheric CO_2 (Oren *et al.* 2001). Mature closed canopy forests rely on decomposition of plant detritus to provide 69-87% of the nutrients needed annually for forest growth (Sinsabaugh *et al.* 1993), the remainder is acquired as inorganic forms via primary mineral mineralisation (Leuschner and Rode, 1999). Enhanced C assimilation under elevated CO_2 increases plant tissue C:N ratio, lowering tissue quality (Reich *et al.* 2006). Consequently, negative feedback of enhanced growth response and N immobilisation in biomass may slowly deplete soil N reserves (Luo *et al.* 2004). The progressive N limitation hypothesis predicts that forest growth responses will become limited as N is immobilised in biomass (Hu *et al.* 2001). On the other hand, trees growing in elevated atmospheric CO_2 have been shown to increase C allocation belowground to roots, mycorrhizal symbionts and through exudation of low molecular weight substrates to improve nutrient supply (Körner, 2006; van Groenigen *et al.* 2006). Furthermore, although N is often considered the most limiting plant nutrient, its availability is believed to be one of the major drivers of fine root response to elevated CO_2 in forest ecosystems (Johnson *et al.* 2006).

Nutrient cycling processes within forest ecosystems are intrinsically linked to the soil microbial communities. Consequently, plant detritus of lower quality may impact upon soil organic matter quality and influence the composition and function of soil microbial communities (Finzi *et al.* 2006; Phillips *et al.* 2002). Microbial growth is generally constrained by the availability of C. Therefore, qualitative and quantitative changes in rhizodeposition are likely to alter the metabolism of heterotrophic microorganisms (Zak *et al.* 2000; Phillips *et al.* 2006). An increased flux of C compounds to the soil may retard or increase the rate of soil organic matter mineralisation. From one perspective faster SOM mineralisation may supply nutrients to enable C sequestration in biomass. Conversely, greater respiratory losses through microbial co-metabolism of old recalcitrant C may reduce the soil C pool (Kuzyakov *et al.* 2000; Fontaine *et al.* 2003).

1.4 Species diversity and elevated CO_2

Reductions in tree biological diversity may negatively affect forest ecosystem function and resilience (Millennium Ecosystem Assessment, 2005). Grassland studies have demonstrated that plant productivity generally increases with species richness, and may be positively correlated with ecosystem function (Tilman *et al.* 1996). However, most studies investigating the effect of elevated CO_2 on forests have focused on species specific responses. Therefore the role of tree biodiversity in ecosystem processes and functioning remains poorly understood, despite the overwhelming ecological significance of forests (Leuschner *et al.* 2009). In forests, species productivity is usually increased when growing in mixture. Pretzsch *et al.* (2010) used an ecological gradient and forests of *Picea abies* and *Fagus sylvatica* in mixture and monoculture to demonstrate a mixture response ranging between -46% to +138% across Northern Europe. Greater productivity in mixtures has been explained by ecological niche separation allowing exploitation of resources not available to co-existing species, or facilitation among functional groups (Coomes and Grubb, 2000). In support of the niche separation hypothesis Pretzsch (2005) showed that by combining light demanding pioneer, and shade tolerant late successional species resource utilisation can be improved by almost 30% in comparison to species grown in monoculture. Whilst conversely if species are selected with similar ecological niches competition may result in a negative growth effect (Bolte and Villanueva, 2006). Facilitative or competitive behaviour may have significant implications for processes operating at larger ecological scales (Novoplansky, 2009).

As forests are exceptionally important in the regulation of atmospheric CO_2 and the global C cycle it is necessary to improve our understanding of the physicochemical and biological processes that govern ecosystem responses. Critically, knowledge gaps exist in our understanding of indirect ecosystem feedback mechanisms between the biosphere and atmosphere. Improving our understanding of ecosystem processes and function in the global C cycle requires greater understanding of soil and microbial processes governing biogeochemical cycles that determine ecosystem C fluxes (De Deyn *et al.* 2008). Furthermore, biological diversity and species interactions may have a large impact on forest response to global environmental change warranting further research.

1.5 Aims and Outline

Forest productivity is determined by complex feedback mechanisms that regulate soil fertility. Nutrient cycling processes are regulated by soil microbial communities that rely on C inputs to sustain populations. Consequently trees have evolved elaborate mechanisms to recycle and store nutrients which include investing considerable amounts of C belowground to support microbial communities and nutrient cycling.

This thesis aims to elucidate how forest ecosystem biogeochemical cycling is altered by atmospheric CO_2 levels predicted for the year 2050. I utilised a broadleaved tree plantation fumigated with CO_2 using free air CO_2 enrichment technology to examine the poorly understood dynamics of mixed species forest ecosystem establishment in its natural environment. Central to this thesis are forest stand C dynamics and how species × CO_2 interactions affect C allocation, biomass accrual, soil C residence time and C fluxes. Intrinsically linked to C dynamics are the role of soil microbial communities in forest ecosystem function. Microbial communities regulate C and nutrient cycling processes through the mineralisation of organic matter and mineral weathering. These processes create feedback loops that ultimately determine forest ecosystem productivity and C fluxes. To elucidate the effect of elevated atmospheric CO_2 on these processes I first quantify above and belowground growth responses to determine if growth response and C allocation changes. I then relate this data to inter and intra species competitive and facilitative interactions both above and belowground. Secondly, I examine the impact of CO_2 treatment on soil microbial community labile C metabolism and turnover. Thirdly, I determined the size and availability of P pools to assess how P cycling is altered. Finally, I used C isotopes to identify the rate and source of new C accumulation. Understanding how intra and inter-species interactions affects forest ecosystem dynamics; and the complex interactions between microbially mediated C and nutrient dynamics are key to understanding how forest ecosystems will respond and function in a future climate.

1.6 Hypotheses

- *Elevated atmospheric CO_2 induces a differential growth response altering forest compositional dynamic to favour faster growing species.*

- *Elevated atmospheric CO_2 increases tree belowground C allocation resulting in larger and deeper root systems.*
- *Tree species diversity has a positive effect on above and below ground biomass accumulation when trees are grown under elevated atmospheric CO_2.*
- *Elevated atmospheric CO_2 enhances rhizodeposition altering the soil microbial community structure, function and soil C turnover.*
- *Elevated atmospheric CO_2 increases demand for P in forest ecosystems and depletes soil P pools.*
- *Elevated atmospheric CO_2 increases the accumulation of C through enhanced root and mycorrhizal hyphal production and turnover.*

1.7 Structure of the study

This thesis comprises twelve chapters; an introductory chapter, a methods chapter detailing the research facility, nine research chapters, and a concluding chapter drawing together the different threads of the thesis. All research chapters begin with a literature review on the chapter subject.

Chapter 2: **Bangor free air CO_2 enrichment facility** offers a detailed overview of the Bangor Free Air CO_2 enrichment site, together with a description of the basic characteristics of FACE technology setup and software modifications made that enabled remote monitoring and performance analysis using an expert system. An analytical assessment of the CO_2 enrichment system performance is provided.

Chapter 3: **Elevated atmospheric CO_2 enrichment induces a differential biomass response in a mixed species temperate forest plantation** discusses the effect of atmospheric CO_2 enrichment on species specific aboveground biomass accumulation. Inter and intra specific growth interactions are also examined when species are grown in monoculture and polyculture using allometric relationships developed at the site.

Chapter 4: **Elevated atmospheric CO_2 enrichment mediates a greater root response in temperature trees species grown in polyculture** describes differential belowground carbon allocation, root stratification and niche exploitation of species grown in monoculture and polyculture.

Chapter 5: **Species diversity enhances carbon sequestration of CO_2 enriched forests** examines how inter and intra-species competitive or facilitative interactions alters above and belowground carbon allocation when species in polyculture and monoculture are grown under elevated atmospheric CO_2

Chapter 6: **Elevated CO_2 alters the seasonal dynamic of low molecular weight root exudate mineralisation** concerns temporal changes labile carbon turnover and inter-specific differences in carbon mineralisation under elevated CO_2.

Chapter 7: **Species diversity decreases low molecular weight substrate turnover in temperate forest enriched by elevated atmospheric CO_2** examines how differences in mineralisation of labile carbon substrates relate to microbial function and how resource utilisation and niche differentiation impact turnover.

Chapter 8: **Temperate forest enriched by elevated atmospheric CO_2 increases phosphorus mineral weathering** determines how soil phosphorus pools change in response to differential demands of tree species in an establishing forest, and how mineral weathering meets these increasing demands.

Chapter 9: **The relative contribution of mycorrhizal extraradical mycelium and fine roots to soil carbon in CO_2 enriched temperate forest** quantifies new soil carbon from fine roots and mycorrhizal mycelium using a ^{13}C stable isotope mixing model and in-growth cores containing ^{13}C enriched C_4 soil produced under maize (*Zea mays*).

Chapter 10: **Elevated atmospheric CO_2 enrichment delays autumnal senescence of *Betula pendula* in an aggrading mixed species temperate forest** demonstrates an extension of growing season for *B. pendula* through a greater retention of leaves that is not observed in *A. glutinosa*.

Chapter 11: **N-fixation and N dynamics in *A. glutinosa*, *B. pendula* and *F. sylvatica* exposed to free air CO_2 enrichment (BangorFACE)** determines if nitrogen fixation is altered by elevated CO_2 and if symbiotically fixed N contributes to improved growth of *A. glutinosa*, and non N fixing tree species grown in polyculture with *A. glutinosa.*

Chapter 12: **General Conclusion** summarises the results of the thesis and relates the finding to the hypotheses defined within this thesis.

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2 Experimental Facility

The BangorFACE plantation and experimental facility was established in March 2004 at Bangor University research farm, Henfaes, Abergwyngregyn, Gwynedd, 12km east of the city of Bangor (53°14'N, 4°01'W) grid reference (SH 653 741 GB). The experiment covered an area of 2.36 ha and was located on two fields, a former agricultural field, and former agricultural pasture which had been used for small scale forestry experiments for the previous eight years. Soil parent material is described as postglacial alluvial deposits from the Aber river which comprised Snowdonian rhyolitic tuffs and lavas, microdiorites and dolerite in the stone fractions and Lower Palezoic shale in the finer fractions. The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Soils are fine loamy brown earth over gravel (Rheidol series) and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002), and as a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011) Soil texture is 63% sand, 28% silt and 9% clay determined by laser diffraction (Coulter LS particle size analyzer). Climate at the site is classified as Hyperoceanic. Mean annual temperature collected at hourly intervals throughout 2005-2008 was 11.5 °C with an annual rainfall of 1034 mm (Campbell Scientific Ltd, Shepshed, UK), (Figure 2.1).

In January and March 2004, before planting, soil reference samples were taken on a 10 metre grid throughout both fields. Soil pH was measured in water and calcium chloride according to Smith and Doran (1996). Plant available phosphorous (P) was extracted in 0.5 M acetic acid and determined colourimetrically as molybdate reactive P (Murphy and Riley, 1962) using a VERSA max micro plate reader (Molecular Devices). Potassium (K), Calcium (Ca) and Sodium (Na) were extracted in 0.5 M acetic acid and determined by Atomic Emission Spectroscopy (Sherwood Flame Photometer 410). Ammonium and nitrate were extracted in 1M KCl and measured colourmetrically using a Skalar continuous flow analyser. Total elemental C and N were determined by CHN-2000 analyser (LECO Corp, St Joesph, MI, USA.). Soil chemical and physical properties are shown in Table 2.1.

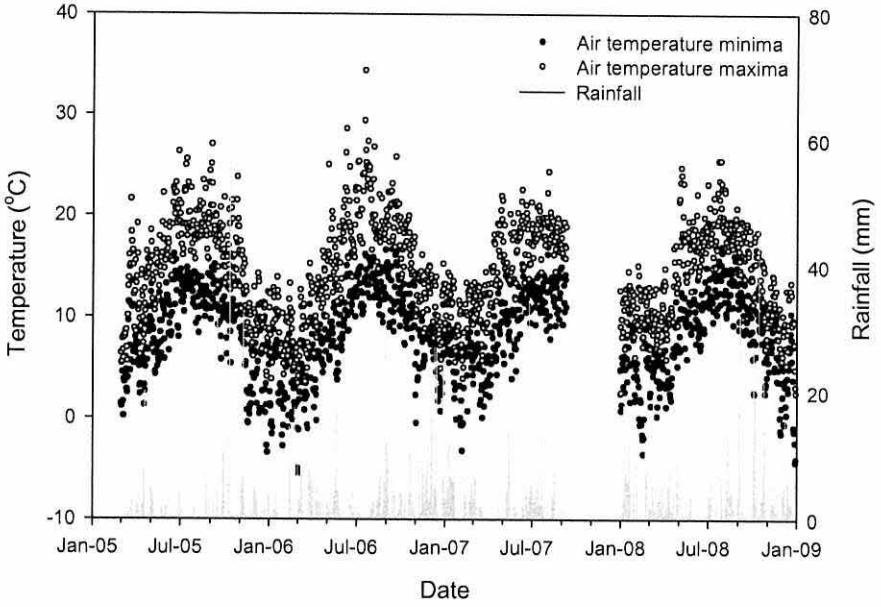


Figure 2.1 Monthly rainfall and air temperature minima and maxima collected throughout the CO_2 enrichment period at the BangorFACE facility, Henfaes. Due to a data logger failure no data is presented between September and December 2007.

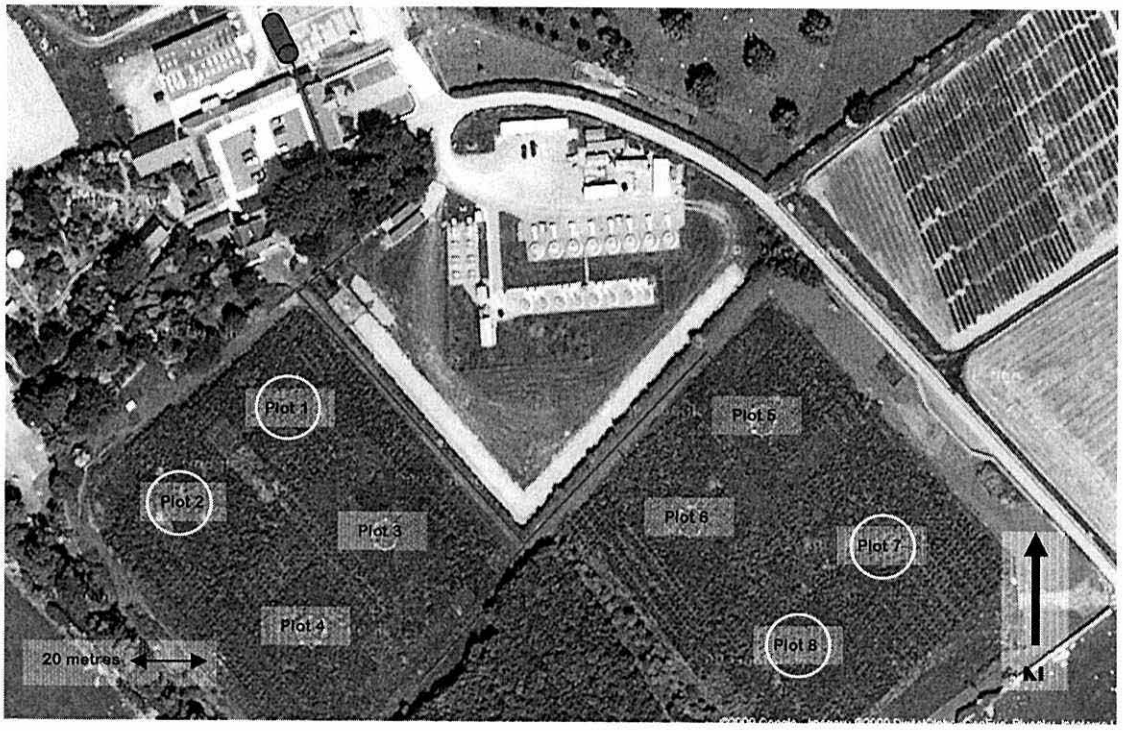


Figure 2.2 Layout of the BangorFACE plantation ambient plots are depicted using yellow circles and FACE plots using red circles. The CO_2 enrichment pipe line is highlighted as red lines leading to the 40 tonne CO_2 storage vessel. Plots are 8 m diameter and surrounded by a 10 m buffer of trees planted at a similar density to prevent experimental artefacts.

Table 2.1. Soil chemical and physical characteristics at BangorFACE prior to afforestation March 2004.

Field	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Na (mg kg^{-1})	pH	Moisture (%)	OM (%)	C (%)	N (%)
1	$25.6^{\pm 4.9}$	$108.2^{\pm 19.2}$	$658.6^{\pm 79.3}$	$87.7^{\pm 8.6}$	$5.6^{\pm 0.1}$	$18.4^{\pm 0.8}$	$6.0^{\pm 0.3}$	$2.8^{\pm 0.2}$	$0.3^{\pm 0.0}$
2	$08.4^{\pm 0.4}$	$075.3^{\pm 05.8}$	$381.6^{\pm 30.5}$	$33.8^{\pm 2.6}$	$5.2^{\pm 0.1}$	$22.8^{\pm 0.7}$	$6.1^{\pm 0.2}$	$2.6^{\pm 0.0}$	$0.2^{\pm 0.0}$

The plantation and FACE facility was established using three native broadleaved tree species in conjunction with the trend towards mixed planting continuous cover forestry in the UK. The three tree species alder (*Alnus glutinosa* [L.] Gaertner), birch (*Betula pendula* Roth.) and beech (*Fagus sylvatica* L.) were selected due to their contrasting shade tolerance, successional chronology and to represent a range of taxonomic, physiological and ecological types. The facility was comprised of eight octagonal plots, four ambient and four CO_2 enriched, creating a 2 × 4 factorial block design (Figure 2.2). Each plot encompassed an area approximately 50 m^2 and was divided into seven planting compartments and planted in a pattern creating areas of one, two and three species. However, for simplicity this experiment utilised three single species subplots of birch, alder and beech, and a fourth subplot containing a mixture of all three species (Figure 2.3). A triangular spacing design allowed each tree six equidistant neighbours. Plots were rotated by 90° to avoid potential artefacts introduced by prevailing wind, microclimate, soil and uneven growth rates of the different species. In addition, each plot was surrounded by a 10 m buffer of the same species planted at the same density. The remaining field has been planted at a 1 m spacing (10000 stems ha^{-1}) with a mixture of birch, alder, beech, ash (*Fraxinus excelsior* L.), sycamore (*Acer pseudoplatanus* L.), chestnut (*Castanea sativa* Mill.) and oak (*Quercus robur* L.). To protect the saplings the entire enclosure was fenced to prevent intrusion by rabbits.

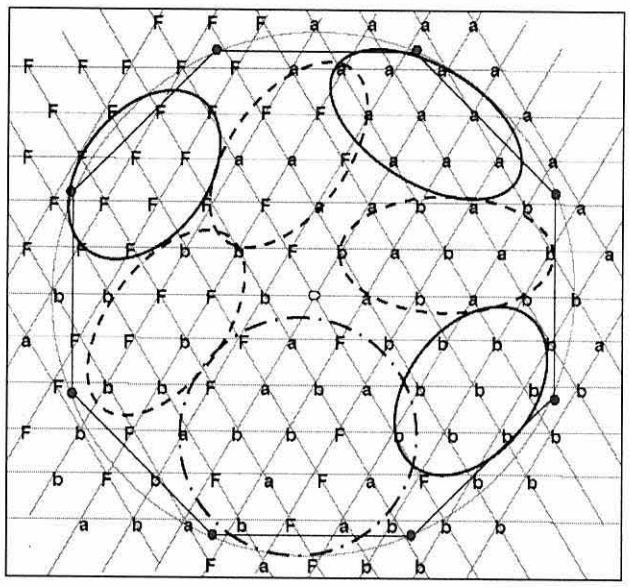


Figure 2.3 Layout of the ambient and FACE plots. a = alder, b = birch, F = beech. Each plot contains 27 trees per species. Single species area indicated by a solid lined oval, two species areas indicated using a broken line oval, and three species plots a dot-dash line oval.

2.1 CO_2 Enrichment Plots

Eight steel towers built on concrete foundations surround each plot and are extendable in 2 metre sections to a maximum height of 8 metres. CO_2 delivery was achieved through polyethylene horizontal pipes (diameter 25 mm) releasing pure CO_2. The enrichment pipes contained 0.4 mm laser drilled holes distributed equidistantly through which rapidly disperses CO_2 to the desired target concentration 580 parts per million (ppm), 200 ppm CO_2 above ambient atmospheric CO_2. Gas mixing was achieved by high velocity pure CO_2 injection (Okada *et al.* 2001). The pipes were suspended from the towers at mid canopy position. As the study species grew the CO_2 delivery pipe position was altered to ensuring accurate enrichment of the crown up to 8 metres height. Initially a single layer of gas delivery pipes was sufficient to obtain the desired atmospheric concentration. As the tree species grew and the canopy developed it was necessary to install a second layer of delivery pipes to ensure satisfactory enrichment of the entire crown of both the fast and slower growing species. The CO_2 was stored and delivered from a 30 ton storage tank with electrical vaporisers and Kurz regulatory valve. CO_2 used to elevate the atmospheric CO_2 was a byproduct of burning natural gas and was depleted in ^{13}C ($\partial^{13}C$ -39 ‰) relative to the international Pee Dee Belemnite standard.

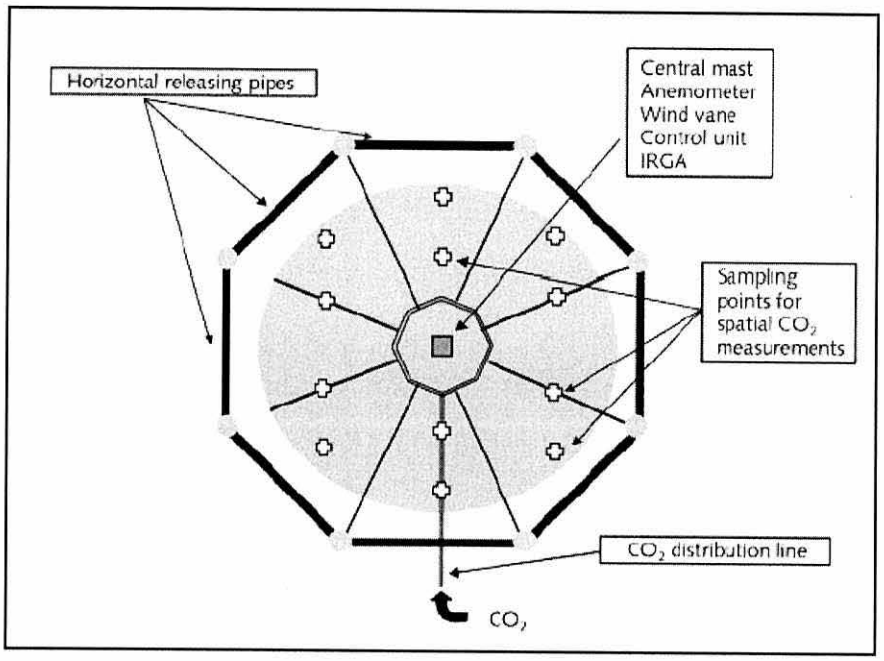


Figure 2.4 Schematic of the FACE array used at Euro-POPFACE (Miglietta *et al* 2001).

Control of CO_2 delivery was achieved using equipment and software modified from Euro-POPFACE (Miglietta *et al.* 2001; Figure 2.4). Located in the centre of each plot was an infra red gas analyser (IRGA) (WMA-4, PP Systems Ltd, Hitchin, Herts, UK) which draws air from the middle of the canopy. A sonic anemometer is integrated into the system supplying wind speed and direction information to a computer control system. The achieved CO_2 concentration determined at minute intervals by the IRGA is communicated with a computer control system which calculates the flow rate of CO_2 using a proportional integral differential (PID) algorithm incorporating wind speed and integrated difference from the target CO_2 concentration. Wind direction was used to control eight solenoid valves and maintain precisely supplied, high velocity upwind delivery. When wind speed was below 0.3 m s^{-1} each valve was opened sequentially to ensure the entire plots was enriched. The design is described in detail by Miglietta *et al.* (2001). Additionally FACE control system source code modifications to improve reliability are included in Appendix A.

Data from the instrumentation was logged and recorded at minute intervals on site providing data on atmospheric CO_2 concentrations, wind speed, direction and the solenoid valve that is open. Using a telemetry system data was downloaded from each plot control system at 15 minute intervals and transferred to Bangor University. At Bangor the telemetry data was automatically analysed using an expert system

(Appendix A). The project researchers were then automatically alerted to potential technical performance issues every 3 hours throughout the day by email.

2.2 Performance of Free Air CO_2 Enrichment System

The fumigation system was enabled in April 2005, immediately after planting and was maintained from bud burst until leaf senescence during the photosynthetically active daylight hours of the year during 2005 and 2007 (Table 2.2). Due to a lack of funding in 2008 CO_2 enrichment was delayed until all leaves were fully unfurled to obtain the largest effect of CO_2 fertilisation for the quantity of CO_2 expended. Subsequently enrichment started on the 12th May 2008, and it was necessary to stop enrichment on the 1st August 2008 when monies were no longer available to purchase CO_2 for enrichment. During the four year experiment the four FACE rings were kept operational for 85% of the time they were enabled. Interruptions to CO_2 delivery were mainly due to power cuts, CO_2 control valve hardware failures and CO_2 deliveries.

Table 2.2 Start and end date when the enrichment system was enabled and the total number of days that the CO_2 enrichment was active for each year.

Year	Start	End	Days
2005	07/04/05	28/10/05	204
2006	20/04/06	30/10/06	193
2007	27/04/07	19/10/07	175
2008	12/05/08	01/08/08	81

Average CO_2 concentration attained during each year the experiment was conducted and for each individual enrichment plot are shown in Table 2.3. In 2005 enrichment was achieved using a single horizontal CO_2 supply pipe. However, as the canopy developed it became necessary to add two more steel tower sections, raising the construction to 6 metres in 2006 and 8 metres in 2007. Additional horizontal supply pipes were also added to obtain satisfactory CO_2 enrichment across the entire canopy.

Figure 2.5 The first year of CO_2 enrichment The bangorFACE facility during the first year of CO_2 enrichment. Photo courtesy of Mike Bambrick, March 2005.

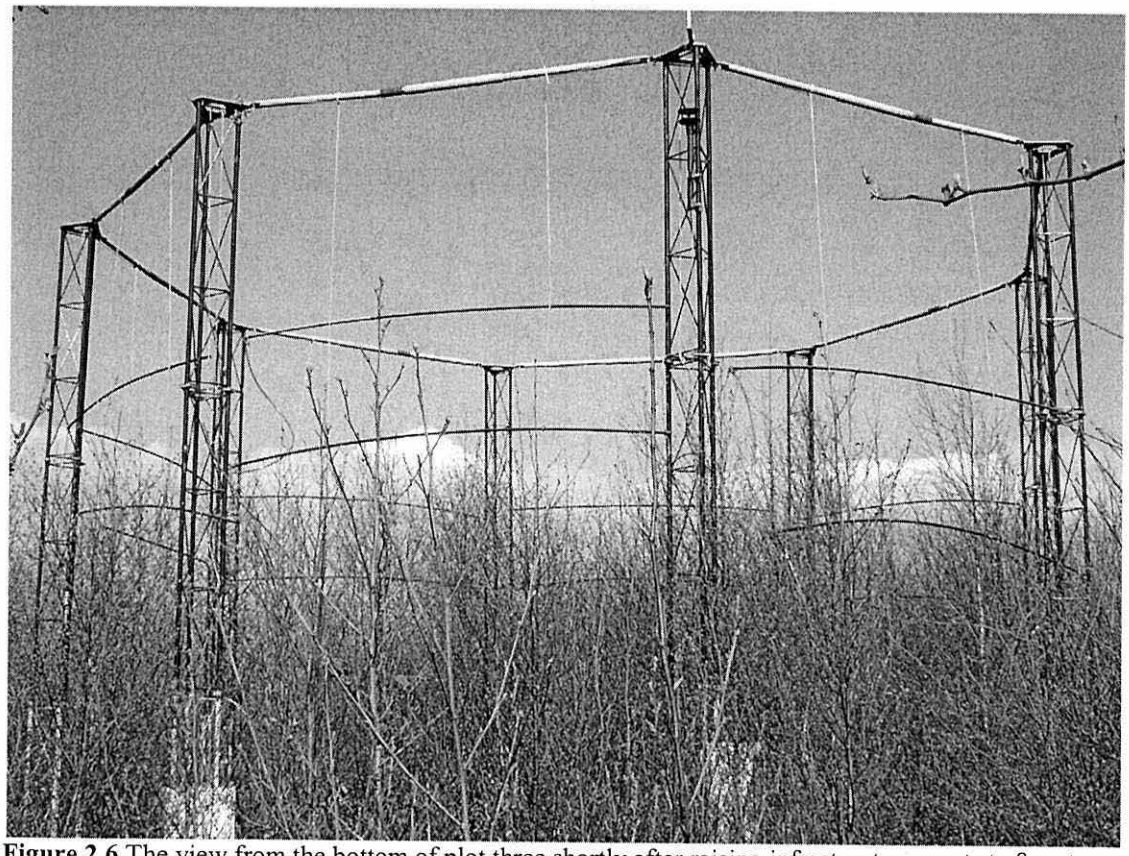


Figure 2.6 The view from the bottom of plot three shortly after raising infrastructure masts to 8 meters in February 2007.

Figure 2.7 The view from the top of plot three during the summer of 2007. Two layers of enrichment pipes can be seen suspended from the infrastructure supplying CO_2 to the middle of the canopy.

Table 2.3 Atmospheric CO_2 concentration attained for the four enrichment plots for the four years that the experiment was run. Values are mean ± SE (n=4) calculated from the daily average CO_2 concentration.

	CO_2 concentration (μmol mol^{-1})				
Year	Plot 3	Plot 4	Plot 5	Plot 6	Average
2005	587.5 ± 5.7	576.3 ± 3.4	556.2 ± 3.2	567 ± 3.1	571.7 ± 6.7
2006	543.4 ± 4.0	583.3 ± 6.2	549.9 ± 5.6	548 ± 3.6	556.2 ± 9.1
2007	599.0 ± 4.4	584.5 ± 3.4	566.8 ± 4.2	567 ± 3.0	579.5 ± 7.7
2008	578.1 ± 9.3	563.0 ± 4.3	560.5 ± 6.8	568 ± 7.5	567.7 ± 3.7

CO_2 concentration averages calculated at minute intervals were evaluated to determine fluctuations from the predetermined target concentration of 580 ppm. The system achieved CO_2 concentrations within 30% of our target concentration for between 75.0 to 84.6% of the time. The percentage of time spent with 5, 10, 20 and 30% of the target concentration can be seen in Table 2.4.

Table 2.4 Percent of time within 5, 10, 20 and 30% of the target concentration of 580 ppm calculated from daily average values. Values shown represent mean ± SE (n=4).

Year	5%	10%	20%	30%
2005	24.4 ± 2.7	45.1 ± 4.5	67.5 ± 4.1	79.2 ± 3.0
2006	17.6 ± 0.4	34.2 ± 0.7	58.6 ± 1.7	75.0 ± 2.3
2007	16.8 ± 1.2	32.6 ± 2.1	57.8 ± 3.0	75.3 ± 2.4
2008	17.6 ± 0.6	35.3 ± 0.9	64.5 ± 1.6	84.6 ± 2.5

The daily average CO_2 concentration measured from the centre of each enriched plot during the 2007 growing season is shown in Figure 2.8. Immediately apparent from Figure 2.8 plots 4 and 6 are the improvement in performance following July 2007 when modification to control software were applied that prevented a condition where

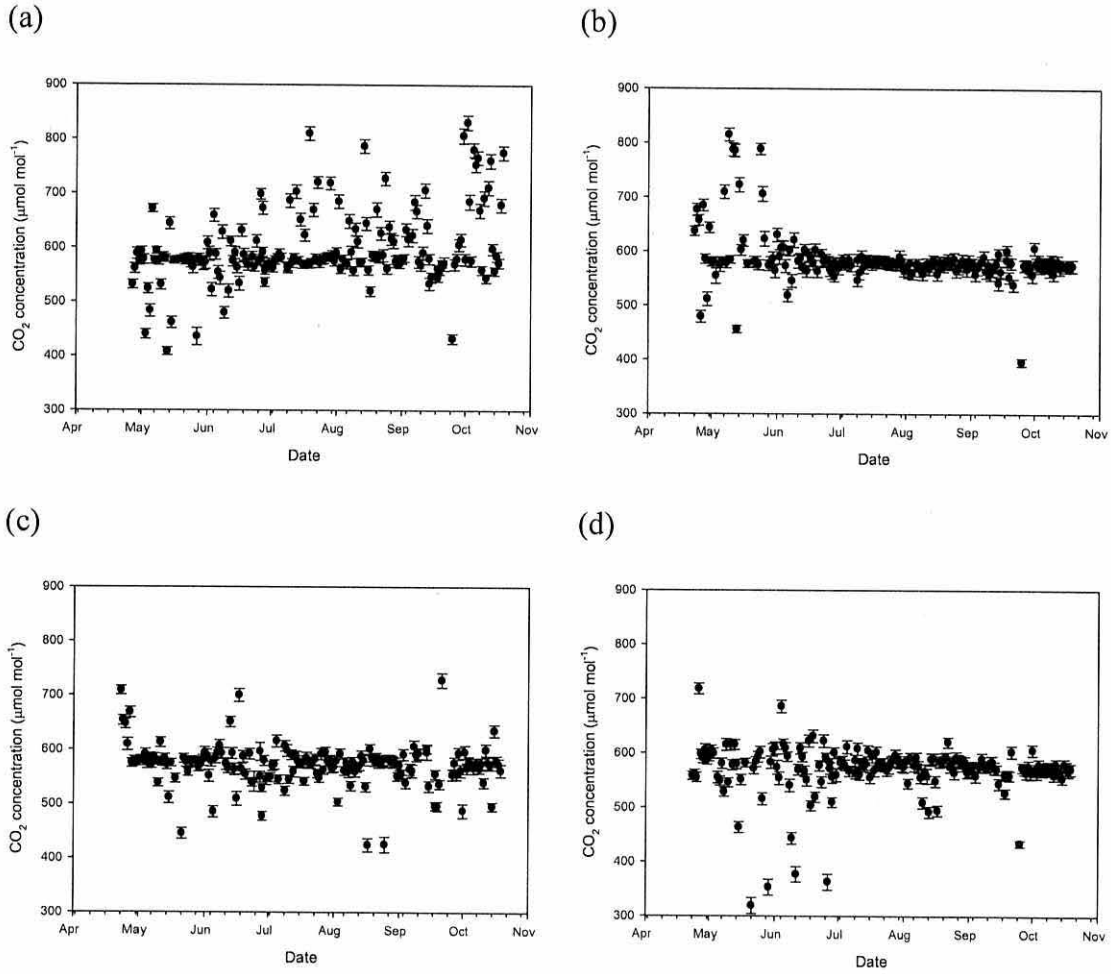


Figure 2.8. Daily average CO_2 concentration measured at the centre of each FACE plot during the 2007 growing season, a) plot 3, b) plot 4 , c) plot 5, d) plot 6

CO_2 solenoid control valves would become stuck open in low wind conditions leading to fluctuations in CO_2 concentration. Variation in CO_2 concentrations were greatest in plot 3 which correlates well with low wind conditions and a persistent problem with a control valve. The system design relies on wind to mix and disperse CO_2 gas over the

plots, thus performance is actually reduced in low wind conditions. The affect of wind speed on attainment of target CO_2 concentrations is shown in Figure 2.9 were it is apparent that high wind speed does not negatively affect the desired CO_2 concentration.

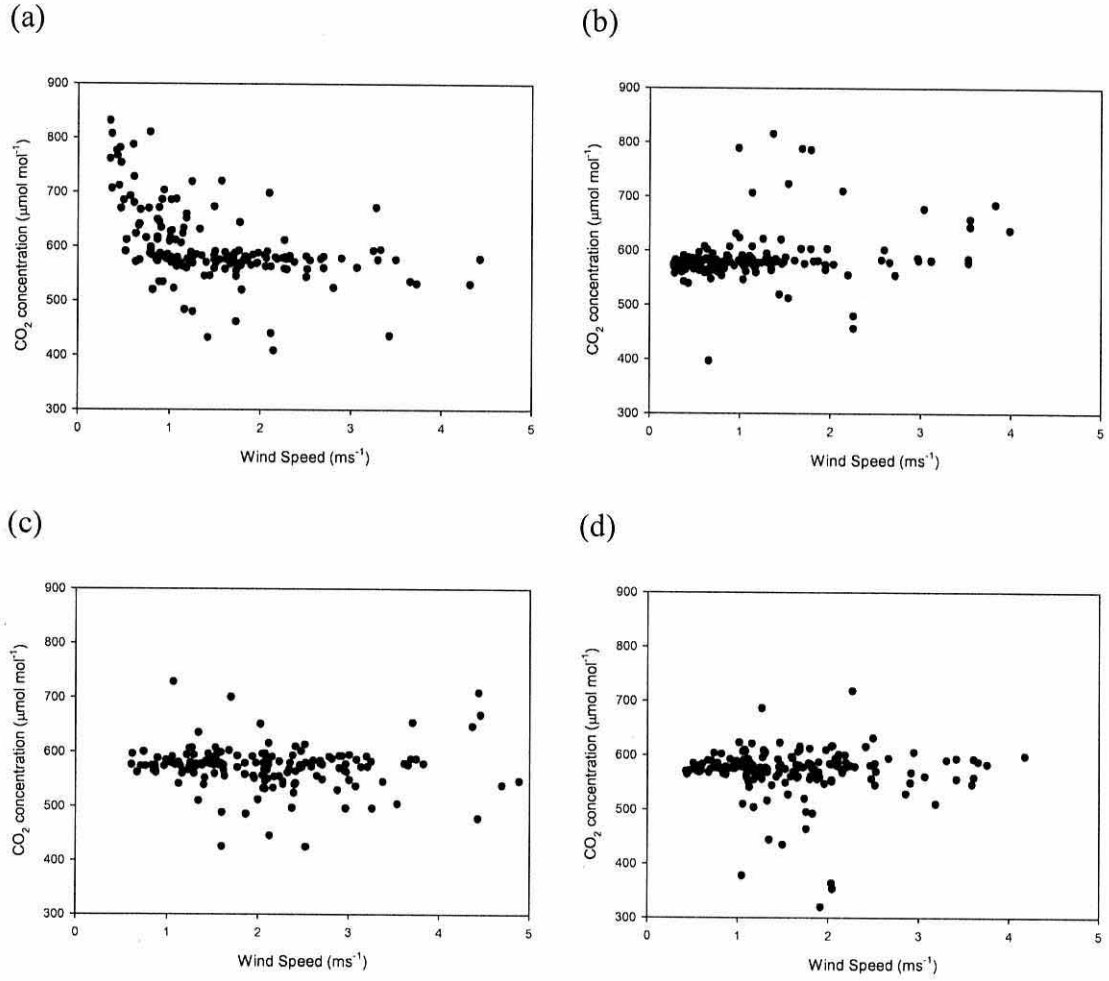


Figure 2.9. Daily average CO_2 concentration measured at the centre of each FACE plotted against wind speed during the 2007 growing season, a) plot 3, b) plot 4, c) plot 5, d) plot 6

The prevailing wind at BangorFACE was in the south westerly direction (Figure 2.10) with remarkably little variation in the direction across a growing season. The highest wind speeds seem consistent with a change in wind direction to the north east. Wind speed had a positive affect on the system performance as air-CO_2 mixing was enhanced. However, it should be noted that the wind direction is predominantly from a south westerly direction and thus plants growing on the south western side of the plots will have been exposed to marginally higher concentrations of CO_2 than those on the north eastern side. To remove this affect each replicate plot was rotated at by 90° relative to each other. Vertical CO_2 enrichment efficiency was confirmed by

measuring CO_2 concentrations at 50 cm intervals throughout the canopy as the canopy developed and whenever the horizontal enrichment pipes were relocated. Figure 2.11 shows the attained CO_2 concentration during a two hour period at three positions (100, 150 and 200 cm) throughout the canopy during July 2006. During the two hour period the mean CO_2 concentration achieved at 100, 150 and 200 cm was 564 ± 10, 621 ± 11, 623 ± 13 μmol mol^{-1} CO_2 respectively.

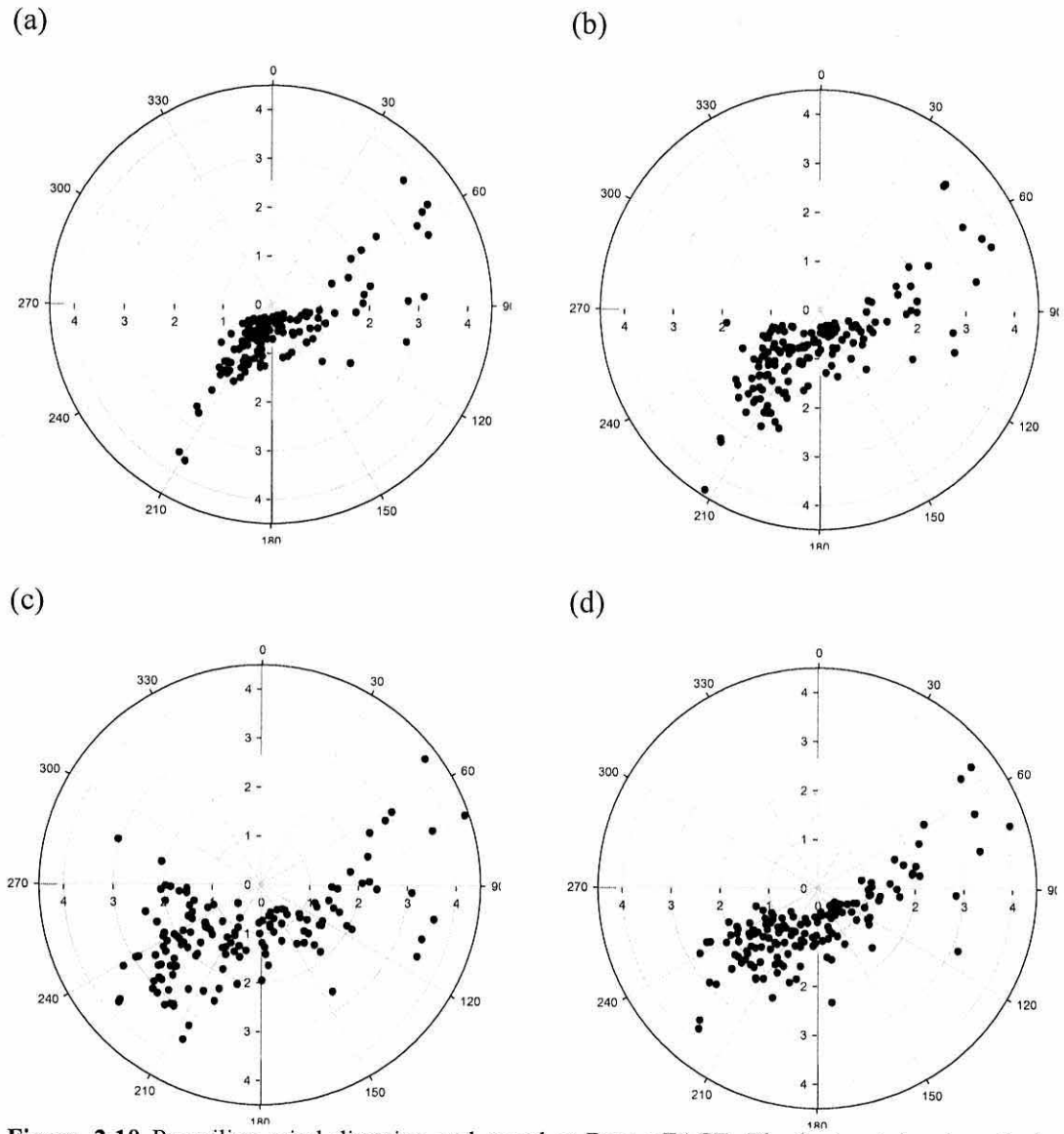
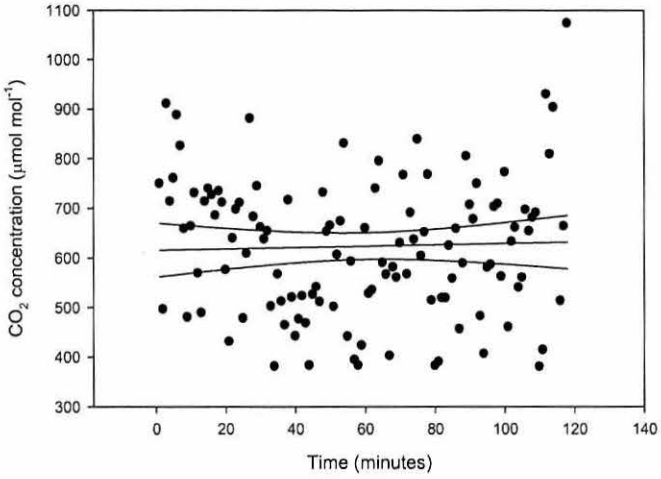
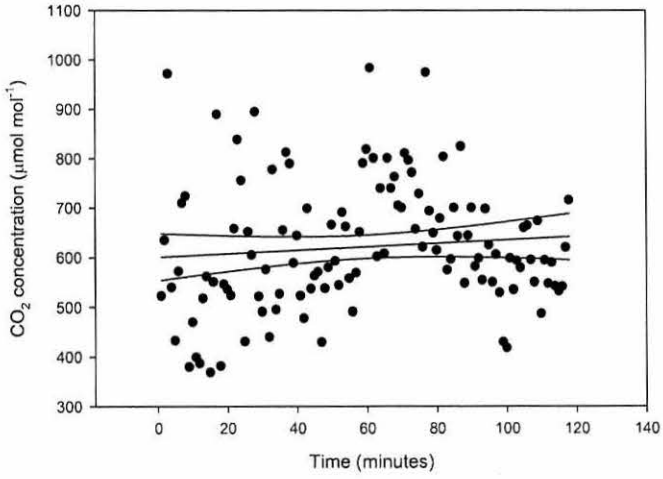


Figure 2.10 Prevailing wind direction and speed at BangorFACE. The horizontal and vertical axis represent wind speed (ms^{-2}) and the circular axis represent direction as degrees from north (a) plot 3, (b) plot 4 , (c) plot 5, (d) plot 6.

(a)



(b)



(c)

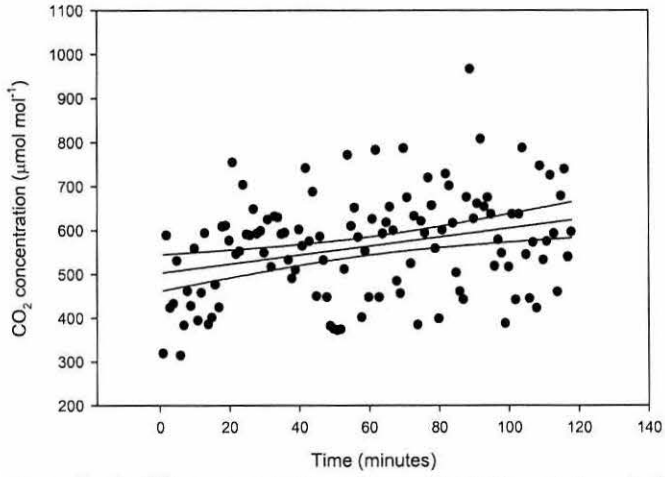


Figure 2.11 Vertical atmospheric CO_2 concentration measured at 50 cm intervals for 2 hours throughout the canopy during July 2006. A linear regression line and 95% confidence interval indicates the mean and variation around the mean panels show the atmospheric CO_2 concentration at a) 200 cm (623.0 ± 13.6 µmol mol^{-1}) b) 150 cm (621.2 ± 11.9 µmol mol$^{-1)}$ and c) 50 cm (564.0±10.9 µmol mol^{-1}).

2.3 References

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3 Elevated CO_2 Enrichment Induces a Differential Biomass Response in a Mixed Species Temperate Forest Plantation

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Contributions:

Andrew Smith wrote the manuscript, collected the data, performed analysis and maintained the FACE facility during 2006-2009. Dr. Martin Lukac designed, built and maintained the experiment during 2004-2006. Robin Hood collected data to determine allometric relationships during his MSc in 2006. Prof. Douglas Godbold is the project principle investigator; he also provided assistance with data interpretation, analysis and manuscript preparation.

3.1 Introduction

Forests occupy one third of the land surface of the Earth, and account for almost half of C stored in the terrestrial biosphere (Schlesinger and Lichter, 2001). Anthropogenic activities since the industrial revolution have accelerated atmospheric CO_2 concentrations to levels not experienced for 26 million years (IPCC, 2007; Pearson and Palmer, 2000). In elevated CO_2 forest net primary productivity has been shown to increase by *ca.* 23% due to greater efficiency of the carboxylation enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) (Long *et al.* 2004 ; Norby *et al.* 2002; Norby *et al.* 2005). Increasing atmospheric CO_2 concentrations may fundamentally alter forest ecosystem functioning by altering species growth, resource use and community interactions (Eamus and Jarvis, 1989). As forests are inextricably linked to the global C cycle, elevated CO_2 driven environmental change may impact upon global C storage in phytomass, complex biogeochemical feedback mechanisms and ultimately long term sequestration in soils.

Empirical studies on forests and elevated atmospheric CO_2 have almost unequivocally demonstrated that growth and aboveground biomass production in woody plants increases. In early work Eamus and Jarvis (1989) estimated a +40% phytomass response. While Ceulemans and Mousseau, (1994) reported a differential response between deciduous (+68%) and coniferous (+38%) tree species. However, many early studies were conducted using chambers with juvenile saplings that may not reflect conditions experienced in the field. More recent research and meta-analyses have reported a mean response of *ca.* +28% with a confidence interval ranging between 6-54% for trees grown in field conditions using free air CO_2 enrichment (FACE) (Curtis and Wang, 1998; Ainsworth and Long, 2005). However, this response is often dependent upon whether other factors are limiting, for example in nutrient deficient conditions a mean non-significant growth stimulation of only 15.5% was shown (Curtis and Wang, 1998). The large magnitude of variation in response to elevated CO_2 has been attributed to a large number of factors such as, the length of study, interactions with other environmental stresses, plant functional group, species morphological physiology (Poorter, 1993), symbiotic associations (Godbold *et al.* 1997) and community dynamics (Kozovitz *et al.* 2005).

Current research efforts have been focused on whole ecosystem responses in natural conditions without influencing environmental actors using FACE (Norby *et al.*

2001). Relatively few experiments have been conduced using field grown trees because of the huge costs of installing and maintaining field based FACE facilities (Karnosky *et al.* 2001). Indeed, only six forest ecosystem FACE experiments have been conducted to date. These FACE experiment have been suggested to consist of two developmental conditions termed by Körner, (2006) as type I, high abundance of major resources other than C – 'decoupled' systems and type II, near to steady-state nutrient cycle and full canopy development – 'coupled systems'.

There have been three, decoupled type I FACE experiments to date. These include the study presented here, and the Aspen FACE and POPFACE experiments. The Aspen FACE plantation was established with trembing aspen (*Populus tremuloides*) in monoculture and subplots containing trembling aspen in admixture with sugar maple (*Acer saccharum*) and paper birch (*Betula papyrifera*). At Aspen FACE, estimated tree volume, which was linearly correlated to biomass (Crow, 1988) was significantly (+28%) greater after 6 years. Growth enhancement continued throughout the study, with the exception of sugar maple trees that demonstrated no growth response to CO_2 enrichment (Isebrands *et al.* 2001). Whilst at the EuroFACE experiment three poplar species in a short rotation coppice agroforestry system responded by +27% (*Populus alba*), +15% (*Populus nigra*) and +26% (*P.* × *euramericana*) to 200 μmol mol^{-1} CO_2 enrichment over three years (Calfapietra *et al.* 2003).

The remaining three experiments (type II) have enriched forest stands using FACE initiating enrichment after canopy closure, Oak Ridge and Duke both started enrichment *ca.* 10-20 years after planting while Basel Web-FACE was conducted on mature deciduous forest comprised of four species greater than 100 years old. At Oak Ridge woody biomass estimates of sweetgum (*Liquidambar styraciflua* L.) were determined using allometric relationships (Norby *et al.* 2001). Aboveground woody biomass was initially 35% greater in CO_2 enriched plots. However, the response diminished to a non-significant difference by the third and subsequent years (Norby *et al.* 2001). Similarly at the Duke FACE facility loblolly pine (*Pinus taeda*) woody biomass stimulation was initially 24% and declined to a non-significant difference during 8 years of CO_2 enrichment (Schlesinger *et al.* 2006). Finally, in mature forest enrichment only resulted in initial basal area enhancement of one species which diminished to zero within 4 years (Körner, 2006). These results have led researchers to conclude that nutrient cycling and availability may lead to growth limitation. An

assertion supported by Oren *et al.* (2001) who reported only a 6% biomass increment over ambient CO_2 grown *Pinus taeda* after 4 years on poor fertility soils in whole tree chambers. However, following the addition of nutrients a 130% increment in biomass resulted.

The objectives of this manuscript is to (a) establish allometric relationships for three temperate deciduous species grown in monoculture and a three species mixture in ambient and elevated CO_2 (580 μmol mol^{-1}) and (b) determine the relative growth response of each species both in monoculture and in mixture. The planting design and species selected are of contrasting successional characteristics enabling analysis of CO_2 enhanced growth response and the interactions between three temperate species used in continuous cover forestry plantations.

3.2 Materials and Methods

3.2.1 Site Description

The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2 ° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Soils are fine loamy brown earth over gravel (Rheidol series) and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002), and as a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011). Soil texture is 63% sand, 28% silt and 9% clay determined by laser diffraction (Coulter LS particle size analyzer). Climate at the site is classified as Hyperoceanic with a mean annual temperature throughout 2005-2008 of 11.5 °C and an annual rainfall of 1034 mm (Figure 3.1).

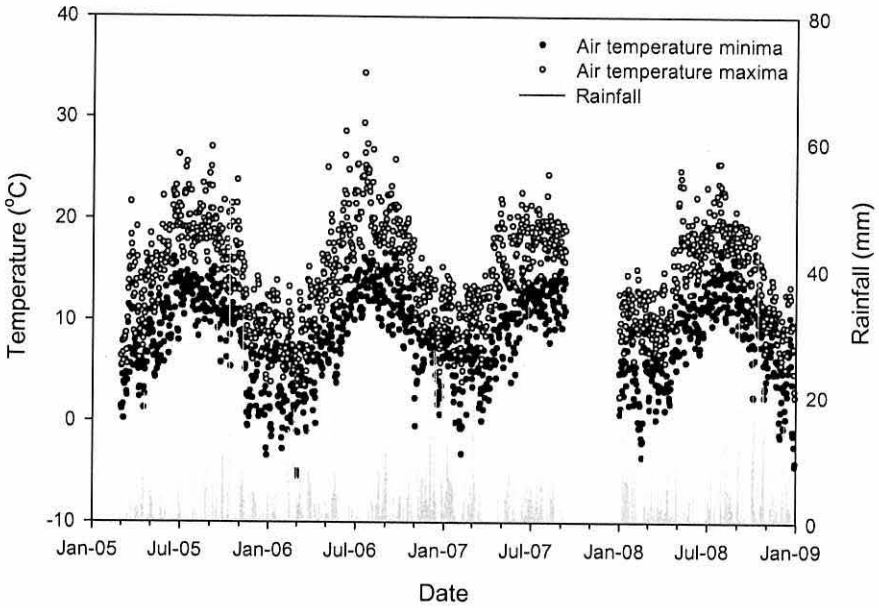

Figure 3.1 Monthly rainfall and air temperature minima and maxima collected throughout the CO_2 enrichment period at the BangorFACE facility, Henfaes. Due to a data logger failure no data is presented between September and December 2007.

Bangor University established a Free Air Carbon Enrichment (FACE) experiment in March 2004 at Henfaes Research Station, Abergwyngregyn (53°14'N, 4°01'W). The experiment covered an area of 2.36 ha and was located on two fields, a former agricultural field, and former agricultural pasture which had been used for small scale forestry experiments for the previous eight years. The experiments comprised of eight octagonal plots 8 m in diameter, four ambient and four CO_2 enriched, in a 2 × 4 factorial block design. We used three tree species (*Alnus glutinosa* [L.] Gaertner, *Betula pendula* Roth. and *Fagus sylvatica* L.), which were selected due to their contrasting shade tolerance, successional chronology and to represent a range of taxonomic, physiological and ecological types. Each plot was divided into seven planting compartments and planted in a pattern creating areas of one, two and three species. However, for simplicity this experiment utilised three single species subplots of *B. pendula, A. glutinosa* and *F. sylvatica*, in addition to a fourth subplot which contained a mixture of all three species. The experimental plots were surrounded by a 10 m buffer of the same species planted at the same density. The remaining field was planted with a mixture of species at 10000 stems ha^{-1}. The four FACE plots were enriched to 580 ppm using pure CO_2 depleted in ^{13}C (-39 ‰ PDB). CO_2 was supplied during the photosynthetically active part of the day through laser drilled holes in

horizontal pipes. Two layers of pipes were suspended from eight masts and positioned mid canopy (Miglietta *et al.* 2001). Elevated [CO_2] was measured at one minute intervals and was within 30% of the target concentration for 75-79% of the time during 2005-2008.

3.2.2 Biometric Measurements

Tree height and stem diameter at 22.5 cm were measured after tree establishment in March 2005 and then February of each following year during CO_2 enrichment (2006-2009). Tree measurements were taken during the winter dormant phase to prevent growth introduced variation. Tree height was determined using a telescopic pole, and two measurements of diameter were taken perpendicular to each other using digital vernier callipers. To account for elliptical stem shape a geometric mean was calculated. As the initial tree height was less than 137 cm it was only possible to measure diameter at breast height (DBH) in subsequent years as the stand developed. Basal area was determined for all species, because of the mixed planting pattern the contribution of individual species to polyculture basal area was determined assuming an 80 cm diameter circle around each tree and multiplying the number of trees contributing to the basal area of each species combination subplot and then expressed as m^{-2} ha^{-1}

3.2.3 Allometric Relationships, Stem Volume Index

Selection of a representative tree form for each species were based on average height and diameter data collected during the previous season. Two trees of each study species were selected for destructive harvest from the downwind buffer zone of each treatment plot. Tree height and stem diameter at 22.5 cm were measured and the trees were excavated to a root diameter of 3-4 mm then separated into leaves, branches stems and roots. Roots were washed free of adhering soil and stem cut into 15-20 cm sections and oven dried at 80 °C for 72 hrs prior to weighing for determination of biomass. Regression analysis revealed that height did not significantly contribute to an allometric model. A power regression of stem diameter and woody biomass was used to explain the allometric relationship for each species studied (Equation 1). Equation 2 shows the biomass allometric equation in its linear form. Where D is stem

diameter at 22.5 cm, with the power regression scaling coefficients a (amplitude) and b (exponent).

$$biomass = aD^{b} \quad \text{(Eqn. 1)}$$

$$\ln(biomass) = \ln(a) + b\ln(D) \quad \text{(Eqn. 2)}$$

Stem volume index (basal diameter^2 × height) was calculated and correlated against allometrically determined biomass to test the accuracy of predicted biomass values.

3.2.4 Statistical Analysis

Regressions and figure production was conducted using SigmaPlot v11.0 (Systat Software Inc, Chicago, IL.) and statistical procedures were undertaken with SPSS 17.0 (SPSS Inc., Chicago, IL) with $P<0.05$ used as the limit for statistical significance. Data were subjected to repeated measures ANOVA for time series analysis; equality of variance was tested using Mauchly's test of sphericity. A General Linear Model was used to calculate univariate analysis of variance for data determined at conclusion of the experiment. Data were tested for normality using Shapiro-Wilk's test and homogeneity of variance was determined using Levene's test.

3.3 Results

3.3.1 Allometric Equations

Initially tree biometric data gathered for elevated and ambient were subjected to stepwise regression predicting biomass from diameter, and height. During this analysis height was excluded as not significantly contributing to the regression model. Ultimately a simple power regression of diameter predicted biomass with the greatest accuracy. Comparison of treatment specific regressions revealed there were no changes in allometry at this stage of tree development and subsequently all species specific data were pooled to produce three allometric relationship equations shown in Figure 3.2 with coefficients of variation ranging from 0.78 to 0.85. Strong correlations between stem volume index with predicted biomass (Figure 3.3) confirm accuracy of predictions for *A. glutinosa* (r^2=0.98) and *B. pendula* (r^2=0.99) but highlight a small underestimate of predicted *F. sylvatica* biomass in elevated CO_2 plots (r^2=0.88).

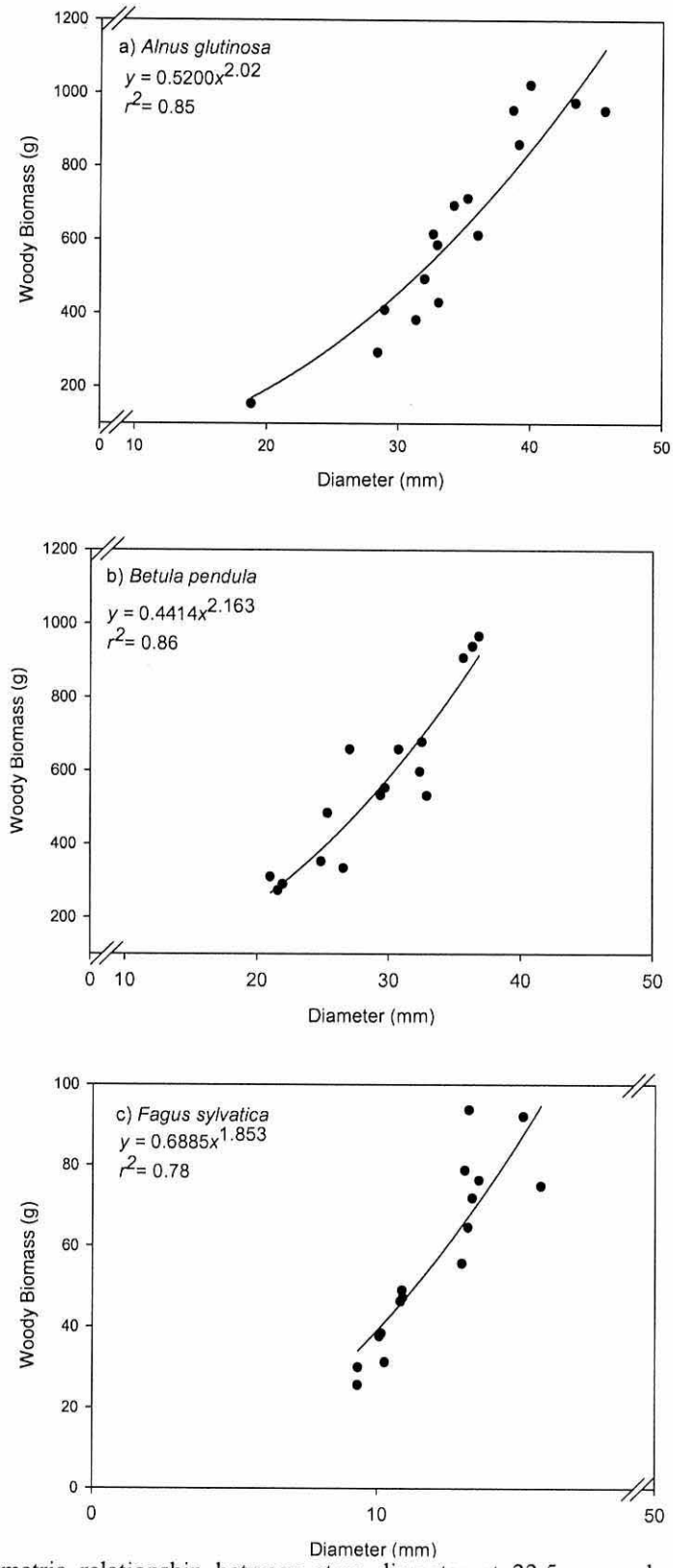

Figure 3.2 Allometric relationship between stem diameter at 22.5 cm and above ground woody biomass determined during the 2006 growing season. Data are pooled for both ambient and elevated CO_2 and fitted with power function regression. The allometric equation and coefficient of determination from each data set are inset in each panel.

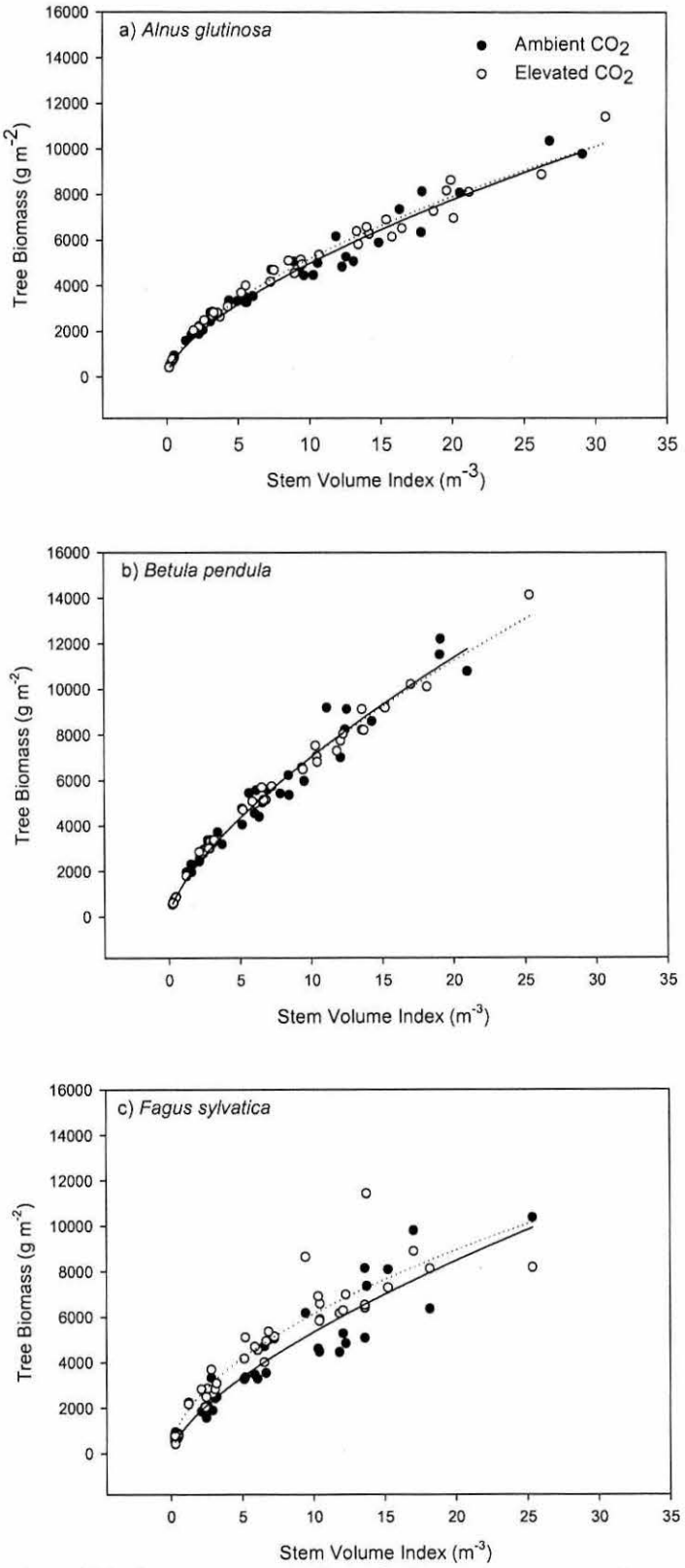

Figure 3.3 The relationship between stem volume index and allometrically predicted biomass of *A. glutinosa*, *B. pendula*, and *F. sylvatica* throughout four years growing in ambient and elevated atmospheric CO_2 Filled circles indicated ambient CO_2 conditions and hollow circles indicated elevated CO_2. Broken regression line are fitted to data from the elevated CO_2 plots and solid lines to ambient CO_2.

3.3.2 *Pooled Species Aboveground biomass*

At the conclusion of the experiment with all species pooled woody biomass was 5.5 ± 0.3 kg m^{-2} in ambient CO_2 plots and 6.5 ± 0.1 kg m^{-2} in elevated CO_2 plots, a significant increase of 17% (P=0.018). The contribution of total woody biomass within the FACE treatment plots followed the order *B. pendula* (10191 ± 22 g m^{-2}), *A. glutinosa* (8560 ± 635 g m^{-2}) and *F. sylvatica* (598 ± 28 g m^{-2}). Although not the contributing the most to biomass, the largest FACE effect was observed in *A. glutinosa* that produced 20% more biomass than in ambient conditions although this was not significant at the 5% level (P=0.055). A significant 16% (P=0.046) increase in woody biomass was observed in *B. pendula* in response to treatment whereas *F. sylvatica* biomass was not altered (-1%; P=0.817).

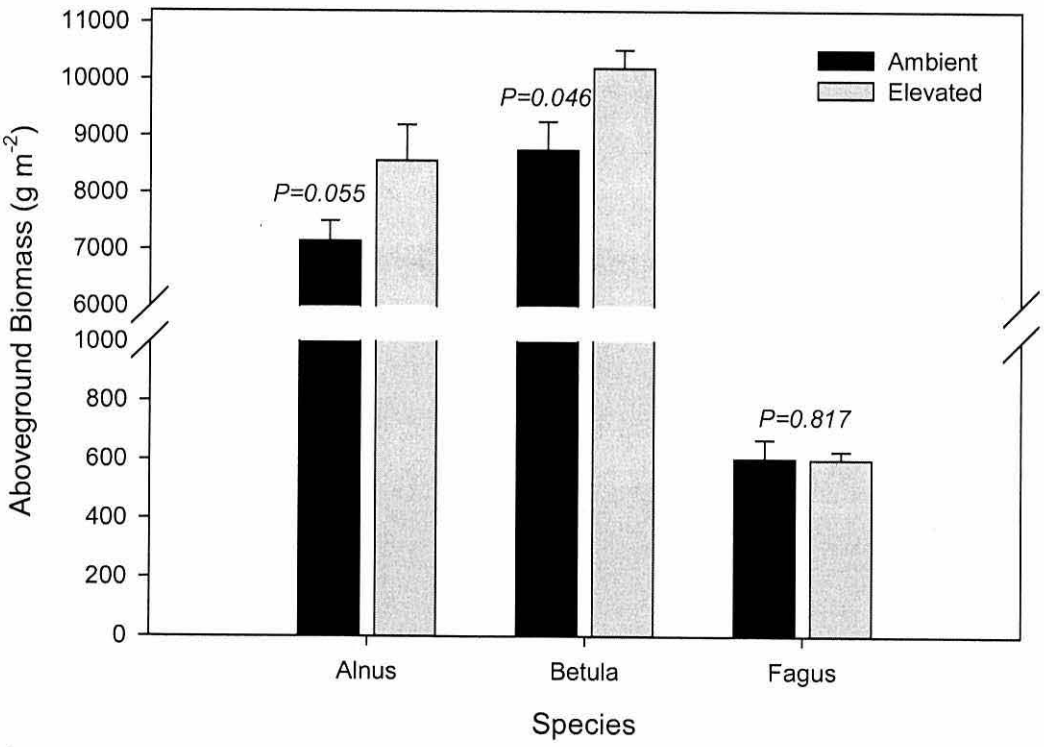

Figure 3.4 Above ground biomass of all trees within the ambient and treatment plots categorised by species at the conclusion of the experiment . Error bars are one standard error.

3.3.3 Aboveground biomass in monoculture and polyculture.

Species grown in single species subplots tended to exhibit a larger response to elevated CO_2 treatment than those grown in the three species mixture subplots. The mean response over four years of enrichment was 29, 22 and 16% for *A. glutinosa, F. sylvatica* and *B. pendula* respectively. Whereas in the three species mixture subplots the mean effect of FACE across all years was reduced to 10, 7, 0% whilst switching to the species order *A. glutinosa, B. pendula* and *F. sylvatica* respectively (Table 3.1). There were no significant differences in the elevated CO_2 treatment of the mixed species subplots throughout the experiment, however, the magnitude of treatment response was between -9-5% in *F. sylvatica*, 3-13% *A. glutinosa,* and 4-8% *B. pendula.* Species grown in monoculture exhibited a much stronger response ranging from between 9-33% *F. sylvatica,* 25-32% *A. glutinosa* and 9-27% *B. pendula.* Elevated CO_2 treatment produced a significant effect in *A. glutinosa* during 2005 (P=0.022), 2007 (P=0.025) and 2008 (P=0.002). The difference in CO_2 treatment effect between species grown in single and mixed species plots is shown in Figure 3.5. Sampling year explained the greatest amount of variation during repeated measures ANOVA being highly significant for all species in both single and mixed species subplots (Table 3.2). There were no significant year × treatment interactions for any species in the mixed species subplots or for *B. pendula* and *F. sylvatica* in the single species subplots. However, there was a significant year × treatment interaction for *A. glutinosa* (P=0.008). Figure 3.5 shows the relationship between time and biomass accruement for all species in ambient and elevated atmospheric CO_2. In both pioneer species biomass accumulated faster in the mixed species subplots (discussed in Chapter 5). Accumulation of biomass was altered by 9%, 8% and -8% by elevated CO_2 for *A. glutinosa*, *B. pendula* and *F. sylvatica* respectively. However, in the single species subplots the increase in biomass accumulation for *A. glutinosa* was four fold higher (32%) whereas there was *B. pendula* remained at a similar increment of 9% while *F. sylvatica* favoured growing in monoculture switching to 9% increment over ambient CO_2 conditions.

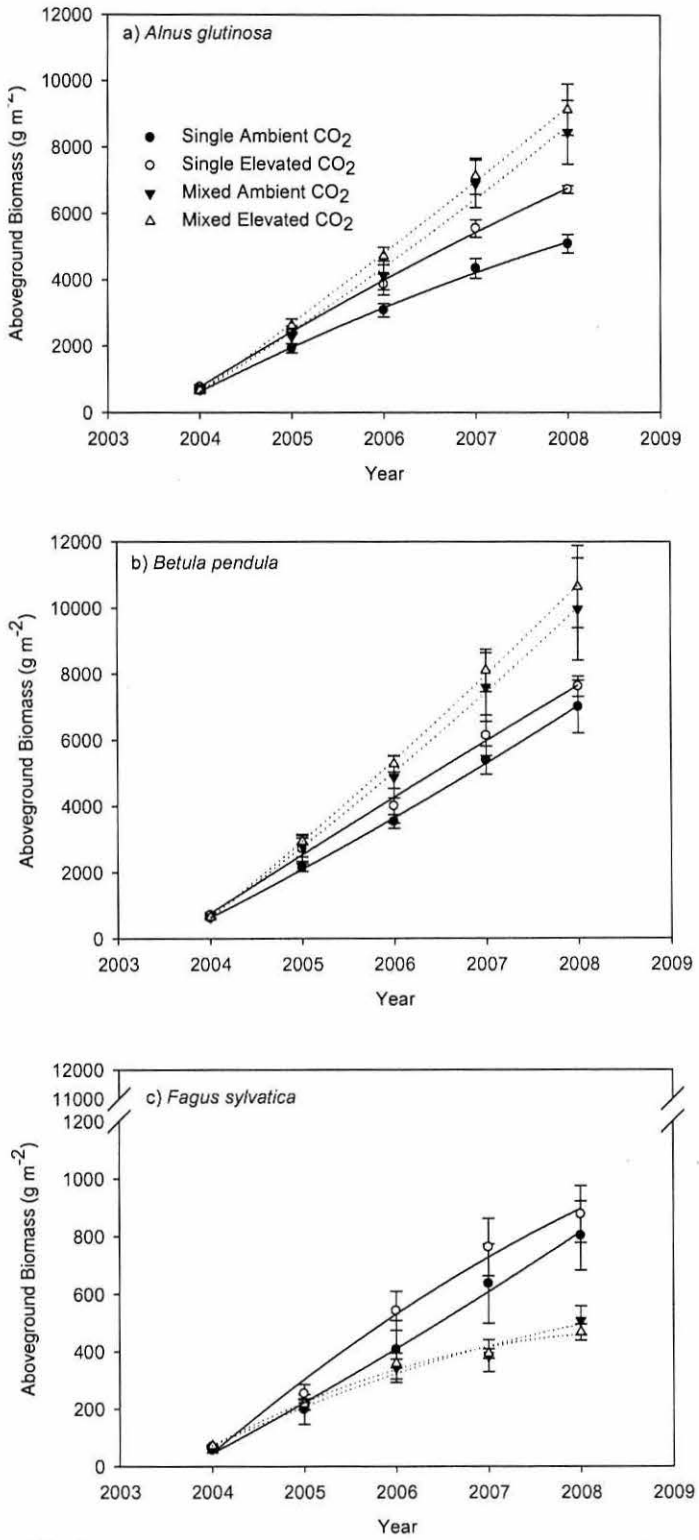

Figure 3.5 Mean ± SE aboveground woody biomass for the species grown in monoculture subplots under FACE and ambient CO_2 for four years. Woody biomass was calculated from allometric relationship determined from whole tree harvesting in 2006. Hollow circles indicated elevated atmospheric CO_2 and filled circles indicate ambient CO_2.

Table 3.1 The percentage FACE effect on woody biomass for each studied species studied grown singularly and in mixture with other species during the four years of CO_2 enrichment. Statistically significant results are denoted by $^{*}P<0.05$.

Planting	Species	2005	2006	2007	2008	Overall
Monoculture	*A. glutinosa*	***29%**	25%	***28%**	***32%**	29%
	B. pendula	27%	13%	14%	9%	16%
	F. sylvatica	28%	33%	20%	9%	22%
Polyculture	*A. glutinosa*	13%	12%	3%	8%	10%
	B. pendula	4%	8%	6%	6%	6%
	F. sylvatica	2%	5%	2%	-9%	0%

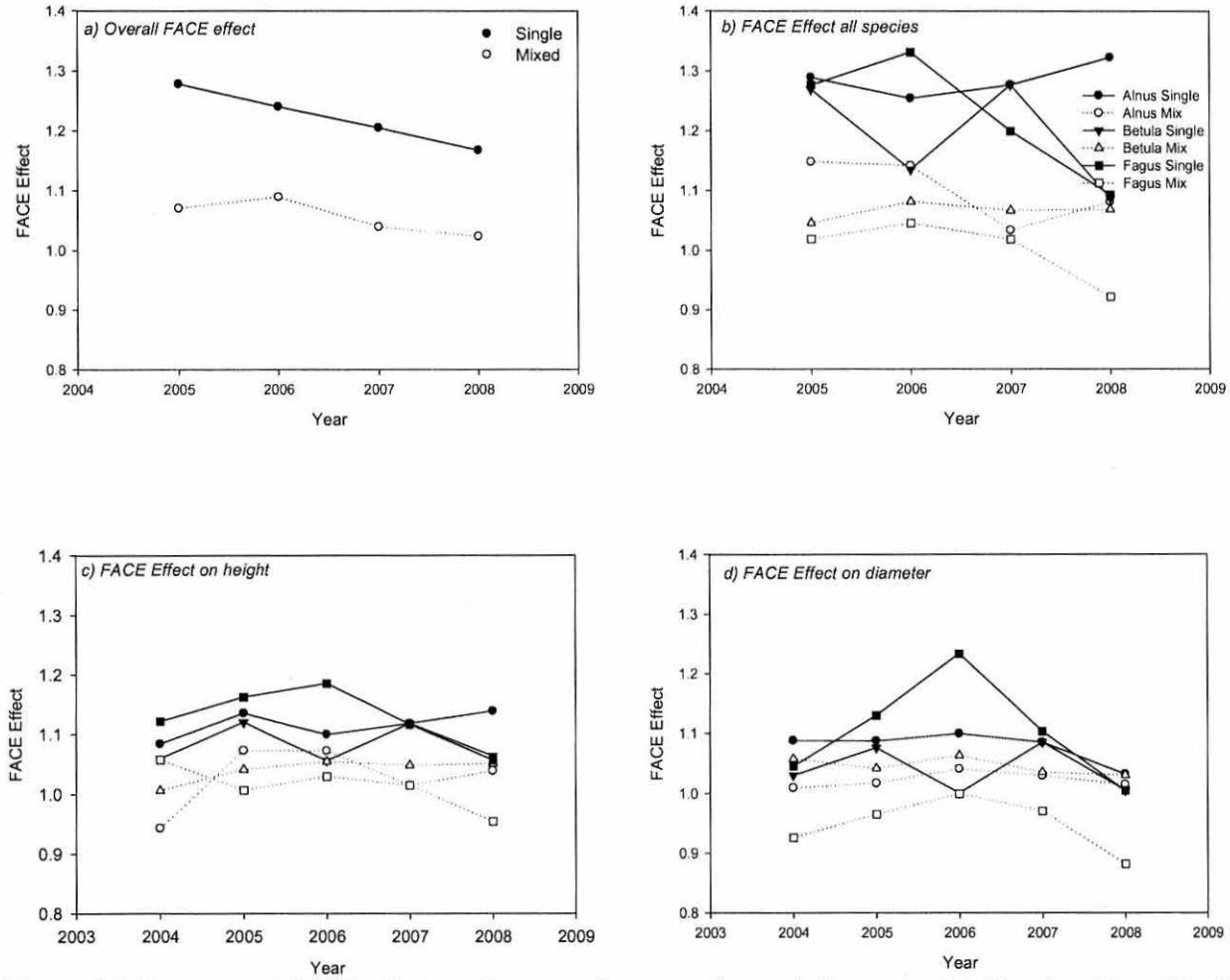

Figure 3.6 Aboveground FACE effect species grown in monoculture subplots under FACE and ambient CO_2 for four years. Aboveground woody biomass calculated using allometric relationships determined from whole tree harvesting in 2006. The continuous line indicates the FACE effect of species grown in single species plots and the broken line when species are grown in mixed species plots. Panel (a) shows the overall FACE effect, panel (b) shows the individual species contribution to the overall effect, panel (c) FACE effect on tree height and panel (d) FACE effect on tree diameter.

3.3.4 *Basal Area*

Basal area was significantly increased by 32% for *A. glutinosa* grown in monoculture and non-significantly increased by 5 and 9% in *B. pendula* and *F. sylvatica* respectively. In polyculture the effect of CO_2 enrichment was not significant in all species being reduced in *A. glutinosa* to 8% and negatively effecting *F. sylvatica* by 9% (Figure 3.7).

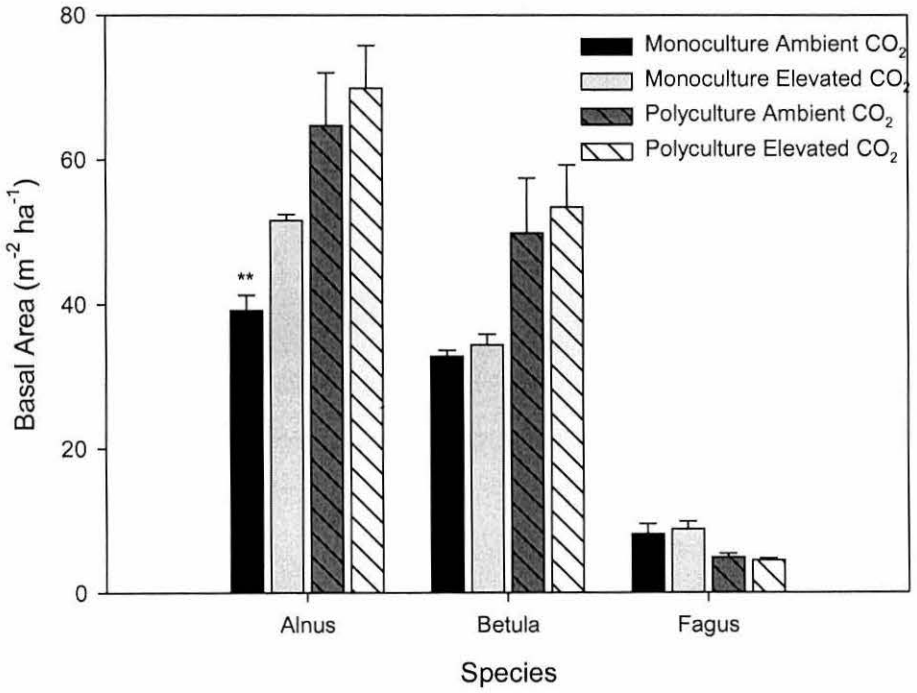

Figure 3.7 Basal area calculated for species in mono and polyculture under elevated CO_2 for four growing seasons at BangorFACE. Bars indicated mean ± SE ** denotes significance at the 5% level.

3.3.5 *Stem diameter in monoculture and polyculture*

The FACE effect on diameter was most pronounced in single species plots, with the largest effect observed within the *A. glutinosa* subplots. At the conclusion of the experiment the FACE effect for *A. glutinosa* was significantly +14% greater (P=0.007) whereas only a small non-significant increase of +6% was observed for *B. pendula* and *F. sylvatica.* Elevated CO_2 appeared to increase the magnitude of diameter response in *A. glutinosa* throughout the enrichment period from 8% to 14% at the end of the 2008 growing season with a slight dip during 2006 (Figure 3.8). The largest FACE effect on stem diameter was observed in *F. sylvatica* in monoculture which responded with a 12, 16 and 18% increase in 2004-06 a greater response than all other species in both mono and polyculture. However, following 2006 the elevated CO_2 rate of diameter enhancement decreased rapidly following canopy closure.

Table 3.2 F-values and probability of significance for sampling year and sampling year × treatment interactions from a repeated measures ANOVA of measured tree diameter, height and biomass determined using allometric equations for *A. glutinosa, B. pendula* and *F. sylvatica* grown in both monoculture and mixtures. Statistically significant differences are shown in bold and denoted by $^{*}P<0.05$.

Planting Pattern	Species	Source of Variation	Diameter		Height		Biomass	
			F	Probability	F	Probability	F	Probability
Single	*A. glutinosa*	year	506.525	**<0.001***	512.615	**<0.001***	253.786	**<0.001***
		year×treatment	2.689	0.055	0.603	0.664	5.546	**0.008***
	B. pendula	year	428.974	**<0.001***	394.712	**<0.001***	113.580	**<0.001***
		year×treatment	0.610	0.659	0.193	0.940	0.078	0.971
	F. sylvatica	year	123.828	**<0.001***	200.403	**<0.001***	47.454	**<0.001***
		year×treatment	0.454	0.769	1.124	0.368	0.250	0.860
Mix	*A. glutinosa*	year	377.886	**<0.001***	934.984	**<0.001***	125.788	**<0.001***
		year×treatment	0.818	0.526	0.223	0.923	0.179	0.909
	B. pendula	year	223.473	**<0.001***	351.368	**<0.001***	64.346	**<0.001***
		year×treatment	0.245	0.910	0.088	0.985	0.083	0.969
	F. sylvatica	year	205.838	**<0.001***	116.937	**<0.001***	101.798	**<0.001***
		year×treatment	0.651	0.632	0.950	0.453	1.240	0.325

Table 3.3 Overall FACE treatment effect and probability of significance following the 2008 growing season. FACE effect is expressed as a percentage relative to control plots measurements of tree diameter at 22.5 cm, height and biomass determined using allometic equations for *A. glutinosa*, *B. pendula*, *F. sylvatica*. Statistically significant differences are shown in bold and denoted by $^{*}P<0.05$.

Planting patterm	Species	Diameter		Height		Biomass	
		Effect	Probability	Effect	Probability	Effect	Probability
Single	*A. glutinosa*	+14%	**0.007***	+3%	0.706	+32%	**0.002***
	B. pendula	+6%	0.146	0%	0.935	+9%	0.502
	F. sylvatica	+6%	0.603	0%	0.965	+9%	0.649
Mix	*A. glutinosa*	4%	0.618	1%	0.837	8%	0.601
	B. pendula	5%	0.614	3%	0.728	7%	0.743
	F. sylvatica	-5%	0.483	-12%	0.333	-8%	0.518

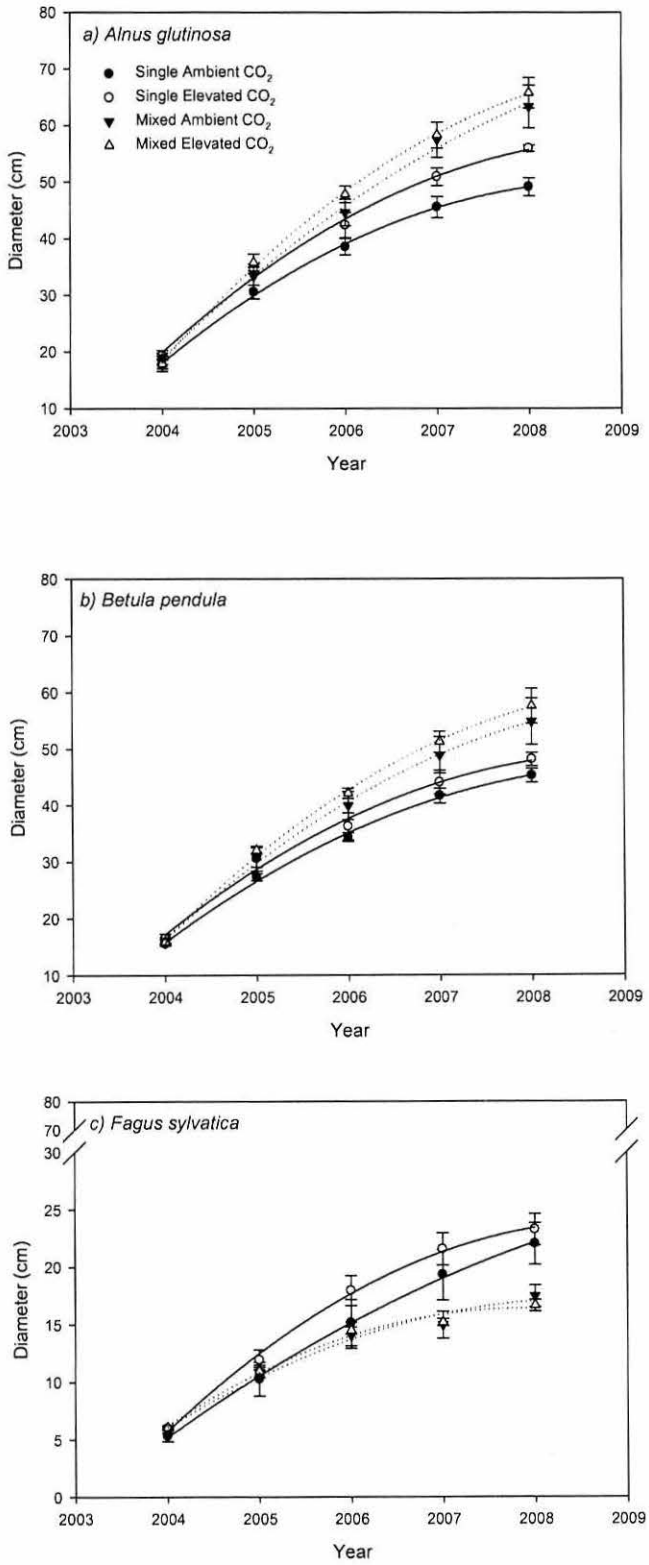

Figure 3.8 Mean±SE stem diameter at 22.5 cm of species measured in the single species and mixed species subplots during the dormant winter season (February of the proceeding season) . Filled circles indicated ambient CO_2 and hollow circles elevated CO_2 in panels (a)-(c). Fitted lines are 2nd order quadratic polynomial ($y = ax + bx^2$); r^2>0.99. Broken lines are regressed against mixed species subplots and continuous lines regressed against single species subplots.

3.3.6 Tree height in monoculture and polyculture

Tree height was unaffected by elevated CO_2 enrichment in both mono and polyculture (Figure 3.9). Despite the results being non-significant, a positive trend in height existed in monoculture plots enriched by elevated CO_2 of 8, 4, 10% respectively for *A. glutinosa*, *B. pendula*, *F. sylvatica.* Figure 3.9 shows that the greatest FACE effect was observed in the *F. sylvatica* plots where the response increased until canopy closure in 2006 after which a dramatic reduction in FACE effect was observed. In polyculture, non-significant increases of 2.5 and 4% were apparent in *A. glutinosa* and *B. pendula* respectively. Conversely there was a non-significant negative impact on the tree height of *F. sylvatica* which averaged over four growing seasons was -5%.

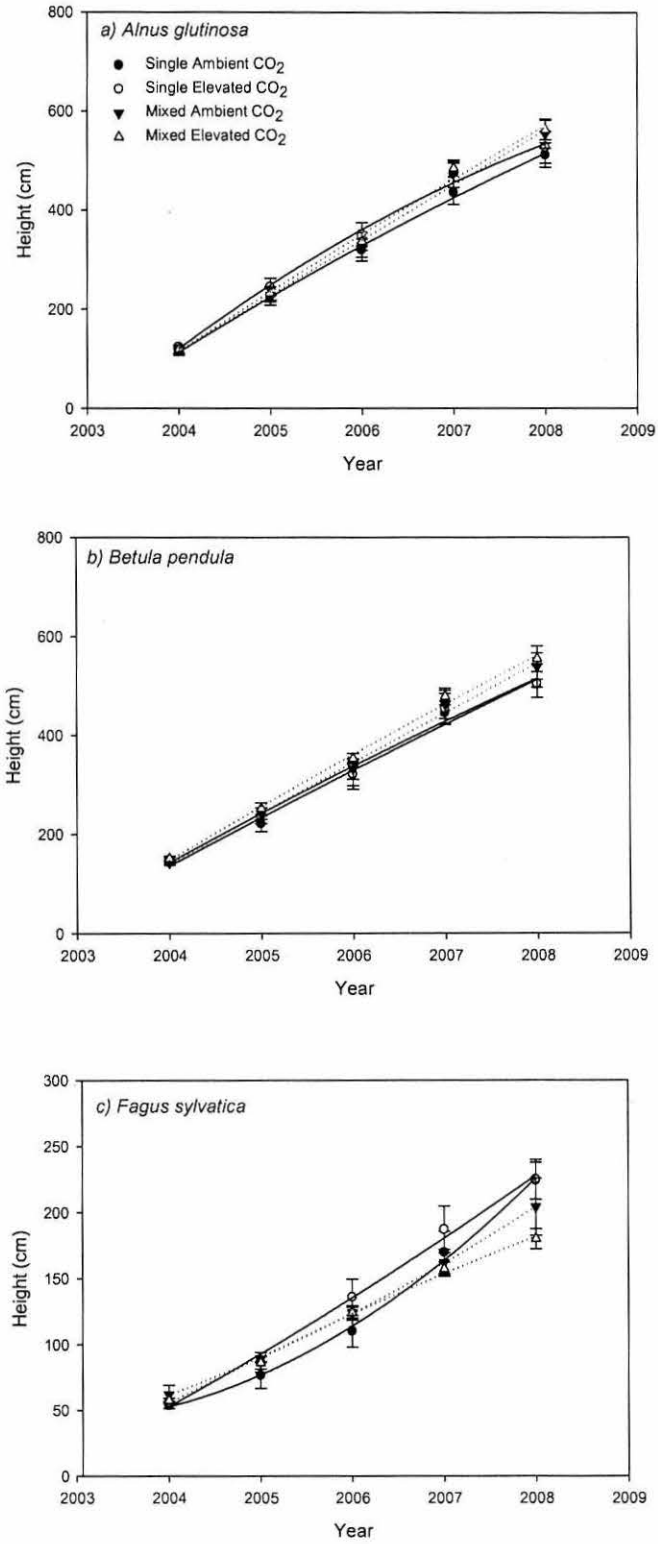

Figure 3.9 Mean±SE tree height of species measured in the single species and mixed species subplots during the dormant winter season (February of the proceeding season) . Filled circles indicated ambient CO_2 and hollow circles elevated CO_2 in panels (a)-(c). Fitted lines are 2nd order quadratic polynomial ($y = ax + bx^2$); r^2>0.99. Broken lines are regressed against mixed species subplots and continuous lines regressed against single species subplots.

3.3.7 Diameter class distributions

The highest frequency diameter class of species grown in monoculture was not altered by elevated CO_2 enrichment. The most often occurring diameter class was 50-60, 40-50 and 20-30 for *A. glutinosa*, *B. pendula* and *F. sylvatica* respectively. However there was a slight shift towards larger diameter boles in elevated CO_2, where 39% of trees had a diameter greater than 50-60 mm, which was in contrast to ambient plots, where only 11% of trees were in this diameter class. In polyculture *A. glutinosa* had a larger distribution of diameter classes under elevated CO_2, whereas diameter distribution for *F. sylvatica* was unchanged by treatment. Interestingly, a bimodal distribution was evident when *B. pendula* was grown in polyculture however this distribution was not affected by elevated CO_2 (Figure 3.10).

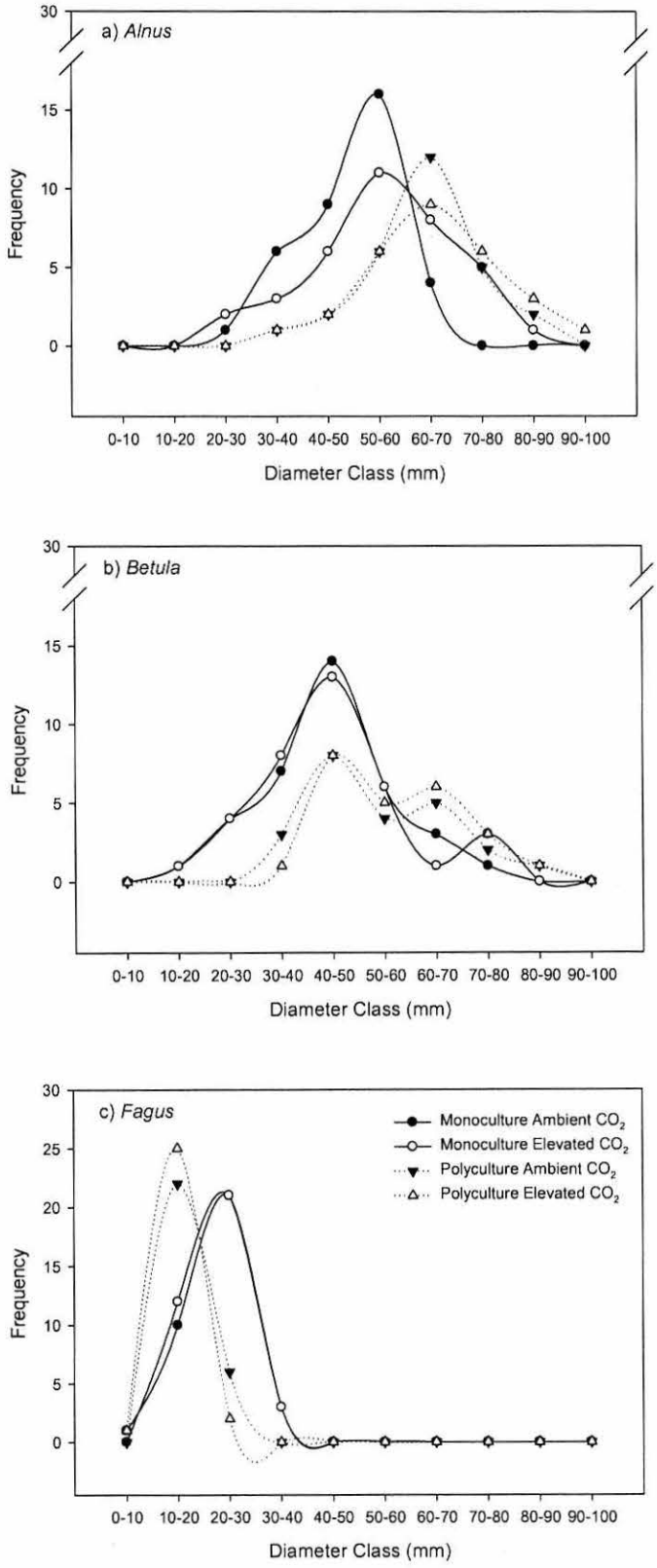

Figure 3.10 Diameter class distributions at the conclusion of the BangorFACE experiment of individual species grown in monoculture and polyculture (three species mixture) under ambient and elevated atmospheric CO_2 Broken lines indicate the frequency of diameter classes grown in polyculture and solid lines indicate the frequency of diameter classes grown in monoculture.

3.4 Discussion

During this four year study of CO_2 fumigation in field conditions all three species grown in monoculture were stimulated by an average of 22%. An elevated CO_2 induced response of this magnitude is consistent with previously reported woody plant response of 28% during meta-analyses of elevated CO_2 experiments (Curtis and Wang, 1998; Ainsworth and Long, 2005). Furthermore, at Oak Ridge an established closed canopy stand comparable results were also observed with *Liquidambar styraciflua.* Aboveground biomass was initially stimulated by 33% and was reduced to 15% in the subsequent year resulting in an overall mean of 24% (Norby *et al.* 2001). However, due to their developmental phase, FACE experiments conducted on establishing plantations prior to canopy closure are perhaps more comparable. During the first year of growth at EuroFACE all three poplar species were strongly stimulated *P. nigra* (121%), *P.* × *euramericana* (73%) and *P. alba* (30%) by elevated CO_2. Yet by the second year growth stimulation progressively decreased and converged at a response value of approximately 20% (Scarascia-Mugnozza *et al.* 2006). Surprisingly, no reduction in growth stimulation was observed at the AspenFACE experiment even after canopy closure was achieved (King *et al.* 2005). It is possible that nutrient limitation has a role in preventing the continuing growth response of some elevated atmospheric CO_2 forests experiments (Oren *et al.* 2001). However, in the present study the soil is of recent glacial origin providing a fertile base rich material with ideal chemical and physical properties for plant growth. We believe that our plantation soil would initially allow growth into unoccupied soil and enable exploitation of nutrients and water with little competition.

3.4.1 Allometry

Allometric relationships have commonly been used to estimate biomass of aboveground compartments. In the present study diameter explained the majority of variance in tree biomass without the necessary inclusion of height in accordance with the results of others; and supporting the observation that the mathematical model that best predicts aboveground biomass from diameter adheres to the form $biomass = aD^b$ (Zianis and Mencuccini, 2004). The power function allometric scaling coefficients generated were broadly similar to previously published coefficients (Table 3.4), with

the exception of *F. sylvatica*. Dimorphic growth characteristics of juvenile *F. sylvatica* under different light regimes during canopy development may explain the difference observed.

Alternative non destructive methods to estimate stand biomass include stem volume index (SVI) (Crow, 1988). An incredibly robust relationship between biomass and stem volume index (SVI) has led to its use in estimation of stand biomass in several studies (Isebrands *et al.* 2001; Karnosky *et al.* 2001). Strong correlations between SVI and allometrically predicted aboveground biomass in this study suggest that predicted values are accurate. The application of species and site specific allometric relationship seem to be valid for *A. glutinosa* and *B. pendula*. However, the relationship for *F. sylvatica* appears a little weaker and may benefit from closer examination of the differences in morphology when trees are shade suppressed and growing in full light.

Table 3.4. Previously published allometric relationship power function scaling coefficients for the three species utilised in this study.

Species	*a*	*b*	Reference
A. glutinosa	0.3251	2.0220	Johansson (1999)
B. pendula	0.2511	2.7142	Hughes (1971)
F. sylvatica	0.0798	2.6010	Bartelink (1997)

3.4.2 FACE effect in monoculture

In the first growing season before canopy closure, all species responded to elevated CO_2 enrichment by increasing biomass by 27-29%. Stimulation of *B. pendula* began to decline during the second growing season, whereas the response of *F. sylvatica* declined during the last two growing seasons. Although the decline in growth response of *B. pendula* and *F. sylvatica* may suggest a loss of CO_2 stimulation or acclimatisation to elevated CO_2 in these species, forest plantation developmental phase and species ontogeny must also be considered. During ontogeny, species physiological traits are differentially altered to maximise photosynthetic efficiency (Lintunen and Kaitaniemi, 2010). Initially, saplings of each species were not influenced by intra-specific competition for light and space allowing a greater response to elevated CO_2. Each species used in this study possesses differing shade tolerating characteristics. Ellenberg (1991) characterised *F. sylvatica, A. glutinosa* and *B. pendula* as shade tolerant (3), intermediate (5) and light demanding (7) respectively. Low leaf mass per leaf unit area and high rate of C assimilation per unit leaf area of

light demanding species allow rapid occupancy of available space and some canopy light penetration (Niinemets, 2006). However, the photosynthetic physiology of shade intolerant species does not allow optimum C assimilation at low light potentially leading to a reduced elevated CO_2 response. Whereas the loss of treatment response in *F. sylvatica* could be explained by leaf morphology and crown architecture that minimises canopy light penetration. The resulting deep shade cast likely induces strong intra-specific competition at high planting densities when grown in monoculture. C assimilation of *F. sylvatica* is slower relative to the other species studied resulting in a slower space occupancy that may explain the rate at which growth stimulation was lost. In contrast, *A. glutinosa* sustained a stimulation ranging between 25-32% throughout the four year experiment. Claessens *et al.* (2010) described *A. glutinosa* as fast growing when juvenile, but as a poor competitor that does not produce shade leaves. Consequently, respirational losses of crown shaded leaves are greater than C assimilation rates leading to rapid death. In this ecosystem, fast juvenile growth coupled with rapid self pruning and increased LAI enable *A. glutinosa* to fully utilise elevated levels of atmospheric CO_2.

3.4.3 FACE effect in polyculture

The growth response to elevated CO_2 was dramatically reduced when species were grown in polyculture. Initial increases in biomass of *F. sylvatica* were marginal, eventually becoming suppressed by -8% in the last growing season. An impact of belowground competition for resources between tree species is unlikely during the first growing season, as establishment of roots into unoccupied soil would initially allow unconstrained access to soil resources. Lack of belowground competition suggests that light and neighbour induced modification of crown profile is the most likely cause of differences in growth response. Negative growth response to elevated CO_2 of *F. sylvatica* is most likely simply due to faster canopy occupation by *A. glutinosa* and *B. pendula* under FACE. Furthermore, changes in leaf area index (LAI) may influence canopy light penetration and inter-specific competition under FACE. Okasanen *et al.* (2001) found that elevated CO_2 consistently increased leaf area index throughout the growing season in aspen, birch and maple stands which was attributed to larger leaves. In contrast, Gielen *et al.* (2001) found that leaf area index of *P. nigra* increased by 225% during the first growing. However, subsequent analysis after

canopy closure using a fish-eye canopy analyser revealed no increase in leaf area index, which is in agreement with data obtained at the Oak Ridge deciduous closed canopy FACE experiment (Norby *et al.* 2003; Gielen *et al.* 2001).

Inter-specific differences in the absorption and transmission spectra of leaves may also impact the response of neighbouring trees to elevated CO_2. Leaf characteristics such as leaf size, morphology and pigmentation differentially alter the quantity and quality of light that enters the canopy (Oliver and Larsen, 1996; Kimmins, 1997). Changes in the amount of light or red:far red photon ratio as a result of neighbouring species are detected by plant photoreceptors. These signals induce a variety of responses through mechanisms such as increased specific leaf area and epicormic sprouting (Aphalo and Rikala, 2006). The stronger growth response in *A. glutinosa* relative to other species under FACE may be due to differences in ability to alter crown architecture. Crown form plasticity depends on neighbour growth rates, resource use, crown architecture, light inception and ability to cause physical disturbance (Oliver and Larson, 1996; Aphalo and Rikala, 2006). Typically *A. glutinosa* grown in monoculture produces a straight bole and round crown, whereas when grown in admixture with species of differing growth rates light penetration significantly alters crown architecture to form a more structured canopy (Claessens *et al.* 2010). Indeed, in *Populus* spp. a 5-16% deeper crown with fewer proleptic branches and greater inter-nodal length was reported in response to elevated CO_2 at EuroFACE (Gielen *et al.* 2002).

Although *A. glutinosa* positively affects soil due to its N fixing capacity (Prescott, 1996), during the four years of this experiment *B. pendula* or *F. sylvatica* did not appear to benefit from *A. glutinosa* as a neighbour species. However, as the trees were planted into a young soil, previously used for agricultural experiments, we did not expect N limitation to plant growth within the first four years. Furthermore, *Alnus* spp. has been shown to have no positive influence over other broadleaved tree species at fertile sites (Prescott *et al.* 2002).

3.4.4 C Allocation

Aboveground growth response of all species was dramatically reduced when grown in polyculture. Physiologically given the same light, nutrient and water status as species grown in monoculture, the rate of C assimilation through photosynthesis is unlikely to be reduced by growth in polyculture. This strongly suggests that C is either being lost through higher respiration rates or that C is being allocated elsewhere.

3.5 Conclusion

Species specific allometric relationships were calculated for trees grown in ambient and elevated. Atmospheric CO_2 enrichment did not alter species specific allometric relationships. Estimation of aboveground biomass using the determined allometric relationships revealed a differential response to elevated atmospheric CO_2. Aboveground biomass responses to CO_2 enrichment were species specific and strongly reduced when species were grown in polyculture. In monoculture *A. glutinosa* produced the largest and most consistent response maintaining growth response until the experiment conclusion. Contrastingly the growth response of *B. pendula* and *F. sylvatica* diminished throughout time. Whilst in polyculture the aboveground growth response to elevated CO_2 was strongly reduced in comparison to species grown in monoculture. A negative CO_2 effect growth response was observed in the final growing season for *F. sylvatica* as the species became rapidly overtopped and suppressed. Determining how the biomass response of deciduous species grown in polyculture differs over single species plantations is imperative to improving out understanding of future CO_2 will impact natural forest ecosystems.

3.6 Acknowledgements

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4 Elevated Atmospheric CO_2 Enrichment Mediates a Greater Root Response in Temperate Tree Species Grown in Polyculture

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Contributions:

Andrew Smith wrote the manuscript, collected data, performed analysis and maintained the FACE facility during 2006-2009. Dr. Martin Lukac designed, built and maintained the experiment during 2004-2006, and collected root data during 2005. Dr. Franco Miglietta designed, built and installed the CO_2 enrichment control environment. Prof. Douglas Godbold is the project principle investigator; he also provided assistance with data interpretation, analysis and manuscript preparation.

4.1 Introduction

Atmospheric concentrations of CO_2 are rising at an unprecedented rate as a consequence of land use change and the burning of fossil fuels (IPCC, 2007). Through photosynthesis forest ecosystems assimilate CO_2 in biomass and transfer C to soils. A process which is thought to partially offset atmospheric CO_2 increases (Houghton *et al.* 1999). Fine root production of forest ecosystems comprise one third of global annual net primary productivity (NPP) in terrestrial ecosystems, highlighting the importance of roots in the global C cycle (Jackson *et al.* 1997). In comparison to other tree biomass components, fine roots have a relatively short lifespan, and fast turnover rate which is sensitive to environmental change (Hendrick and Pregitzer, 1992; Gill and Jackson, 2000; Pregitzer, 2003). Consequently, experiments manipulating atmospheric CO_2 in forests are important in determining the impact of environmental perturbations upon the global C balance.

Early elevated CO_2 experiments using closed and open top chambers demonstrated an increase in plant productivity (Ceulemans and Mousseau, 1994). However, these experiments were often limited to individual juvenile plants physiologically constrained by their environment and root volume (Saxe *et al.* 1998). In the last decade, large scale field experiments using the free air CO_2 enrichment (FACE) technique have enabled the study of entire ecosystems growing unconstrained in their natural environment (Zak *et al.* 2000; King *et al.* 2005; Iversen *et al.* 2008; Norby *et al.* 2004; Pritchard *et al.* 1999). An often reported response to elevated atmospheric CO_2 is enhanced allocation of assimilate to fine root production and biomass (Rogers *et al.* 1994). Norby *et al.* (2005) analyzed the response of NPP to elevated CO_2 in four FACE experiments and found that its response is well conserved across a broad range of productivity with a stimulation of 23 ± 2%. Indeed, a meta-analysis of woody vegetation conducted by Curtis and Wang (1998) found that elevated atmospheric CO_2 increased root biomass by *circa.* 40%. More recently and using three genotypes of fast growing *Populus* trees in field conditions, fine root biomass was stimulated by 35-84% and fine root turnover increased by between 27-55% following three years of FACE (Lukac *et al.* 2003). Whereas in a review of coniferous trees species enriched with CO_2, a median root response of 23% was reported for trees grown in field conditions (Tingey *et al.* 2000)

The inherent location of roots within the soil profile increases the probability that decomposition and bioturbation will result in translocation of root derived C to the soil organic carbon (SOC) pool. Therefore any increase in belowground biomass may increase the sequestration potential of forest ecosystems (Iverson *et al.* 2008; Gale *et al.* 2000). Enhanced root production, could however, result in an increased flux of labile C that stimulates microbial mineralisation of old C resulting in a positive feedback on atmospheric CO_2 through increased microbial respiration (Heath *et al.* 2005; Zak *et al.* 2000). On the other hand, deeper and larger root systems under elevated atmospheric CO_2 may offset soil C losses of increased respiration (Norby *et al.* 2004; Iverson *et al.* 2010). In a review, Eissenstat *et al.* (2000) correctly predicted that elevated atmospheric CO_2 may increase root longevity by increasing fine root diameter (Pritchard *et al.* 2008a; Eissenstat *et al.* 1997), increasing mycorrhizal infection (Godbold *et al.* 1997), increasing rooting depth (Iverson *et al.* 2010) and increasing the nutrient gain per unit C cost with the effect of decreasing root turnover. The aforementioned root physiological and morphological changes have the huge potential to alter soil C cycle processes.

Field based research elucidating the response of tree roots to elevated CO_2 has hitherto been conducted on species grown individually or in monoculture. Understanding how species respond to elevated CO_2 when grown in polyculture is essential to further our knowledge of forest growth dynamics and improve parameterisation of global C cycle models. During our research we attempted to address this current knowledge gap by characterising temporal and spatial root dynamics of three temperate tree species, chosen with contrasting successional characteristics, grown in mono and polyculture for four years under atmospheric CO_2 enrichment in field conditions. Testing the hypotheses that (i) deciduous trees growing in elevated atmospheric CO_2 increase their assimilate allocation belowground to maintain a C sink and meet the increased demand for nutrients and water, and (ii) that species grown in polyculture would allocate more C belowground as a result of competitive or facilitative interactions.

4.2 Materials and Methods

4.2.1 Site Description

The BangorFACE experimental site was established in March 2004 two former agricultural fields with a total area of 2.36 ha at the Bangor University research farm (53°14'N, 4°01'W), 12 km east of the city of Bangor. Both fields were originally pastures, for the last 20 years one field was used for small scales forestry experiments, the other field was ploughed and planted with oil seed rape in 2003. The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Soils are fine loamy brown earth over gravel (Rheidol series) and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002), and as a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011). Soil texture is 63% sand, 28% silt and 9% clay determined by laser diffraction (Coulter LS particle size analyzer). Climate at the site is classified as Hyperoceanic. Mean annual temperature collected at hourly intervals throughout 2005-2008 was 11.5 °C with an annual rainfall of 1034 mm (Campbell Scientific Ltd, Shepshed, UK),.

In January and March 2004, before planting, soil reference samples were taken on a 10 metre grid throughout both fields. Soil pH was measured in water and calcium chloride according to Smith and Doran, (1996). Plant available phosphorous (P) was extracted in 0.5 M acetic acid and determined colourimetrically as molybdate-reactive P (Murphy and Riley, 1962) using a VERSAmax micro plate reader (Molecular Devices). Potassium (K), Calcium (Ca) and Sodium (Na) were extracted in 0.5 M acetic acid and determined by Atomic Emission Spectroscopy (Sherwood Flame Photometer 410). Ammonium and nitrate were extracted in 1M KCl and measured colourmetrically using a Skalar continuous flow analyser. Total elemental C and N were determined by CHN-2000 analyser (LECO Corp, St Joseph, MI, USA.). Soil chemical and physical properties are shown in Table 4.1.

Table 4.1. Soil chemical and physical characteristics at BangorFACE prior to afforestation March 2004.

Field	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Na (mg kg^{-1})	pH	Moisture (%)	OM (%)	C (%)	N (%)
1	25.6 $^{\pm 4.9}$	108.2 $^{\pm 19.2}$	658.6 $^{\pm 79.3}$	87.7 $^{\pm 8.6}$	5.6 $^{\pm 0.1}$	18.4 $^{\pm 0.8}$	6.0 $^{\pm 0.3}$	2.8 $^{\pm 0.2}$	0.3 $^{\pm 0.0}$
2	08.4 $^{\pm 0.4}$	075.3 $^{\pm 05.8}$	381.6 $^{\pm 30.5}$	33.8 $^{\pm 2.6}$	5.2 $^{\pm 0.1}$	22.8 $^{\pm 0.7}$	6.1 $^{\pm 0.2}$	2.6 $^{\pm 0.0}$	0.2 $^{\pm 0.0}$

4.2.2 Free Air CO_2 Enrichment

At the BangorFACE site eight octagonal plots, four ambient and four CO_2 enriched were established, creating a 2 × 4 factorial block design across the two fields. Each plot encompassed an area approximately 50 m^2 and is divided into four planting arrangements, comprising three singles species subplots of (*Betula pendula* Roth.), alder (*Alnus glutinosa* (L.) Gaertner) and beech (*Fagus sylvatica* L.), and a fourth subplot contained a mixture of all three species. The site was planted with 60 cm saplings of each species. A hexagonal planting design means each tree has six equidistant neighbours. Within a treatment, the planting pattern was plots were rotated by 90° between the plots to avoid potential artefacts introduced by microclimate, soil and uneven growth rates of the different species. Each plot is surrounded by a 10 m border of *B. pendula*, *A. glutinosa* and *F. sylvatica* at the same density. The remaining field was planted at a 1 m hexagonal spacing with a mixture of birch, alder, beech, ash (*Fraxinus excelsior* L.), sycamore (*Acer pseudoplatanus* L.), chestnut (*Castanea sativa* Mill.) and oak (*Quercus robur* L.). To protect the saplings the entire enclosure was fenced to prevent intrusion by rabbits

Eight steel towers built on concrete foundations surround each plot and were extendable to 8 metres. CO_2 enrichment was carried out using high velocity pure CO_2 injection (Okada *et al.* 2001), delivered from horizontal pipes perforated with 0.4 mm laser drilled holes distributed equidistantly along the pipe. In the first two growing seasons CO_2 was delivered from 1 pipe held at 50 cm below the top of the canopy. In the third and fourth growing seasons an additional pipe was used held at 2 m from the ground to evenly distribute CO_2 across the entire canopy. Control of CO_2 delivery is achieved using equipment and software modified from EuroFACE (Miglietta *et al.* 2001). At the centre of each plot an infra red gas analyser (WMA-4, PP Systems Ltd, Hitchin, Herts, UK) drew air from the middle of the canopy. Wind direction measure by a sonic

anemometer was used to maintain a precisely supplied, high velocity upwind delivery, when wind speed is below 0.3 m s^{-1} each valve was opened sequentially. The design is described in detail by Miglietta *et al.* (2001). The target concentration in the FACE plots was ambient plus 200 ppm. The elevated CO_2 concentrations, measured at 1 minute intervals, were within 30% deviation from the pre-set target concentration of 580 ppm CO_2 for 75-79% of the time during the photosynthetically active part of 2005–2008. Vertical profiles of CO_2 concentration measure at 50 cm intervals through the canopy showed a maximum difference of 7%. The CO_2 used for enrichment originated from natural gas and had a $\delta^{13}C$ of -39 ‰.

4.2.3 Root Biomass

Sampling was conducted from January 2005 through 2009 using randomly located soil cores. Eight 8 cm diameter cores were taken from each plot, two cores from each of the three single species sub plots and two from the three species sub plot at three depths, 0-10 cm, 10-20 cm and 20-30 cm (192 cores during each sampling). Roots cores were transported back to the laboratory on the day of field collection and stored at 4 °C before being washed free of soil, and separated into two size classes, fine (≤2 mm), coarse (>2 mm) and necromass. Necromass determination was based on black or dark brown colour and a decaying fragmented appearance. Live fine roots were scanned using an Epson 4990 scanner at a resolution of 300 dpi. Images were analysed with WinRhizo (version 2005c Regent Instruments Inc, Quebec, Canada) to determine specific root length, size class distribution and root length density. Finally, roots mass was determined after drying at 80 °C for 72 hours.

To determine the effect of root heterogeneity and accuracy of coarse root biomass estimation by auger coring, during November 2007 coarse root biomass was also assessed by excavating 30×30×30 cm pits on the perimeter of each single species subplot and three species mixture subplot (32 pits in total). Each pit was excavated in 10 cm horizons, roots and stones where removed by sieving to 8 mm in the field, finer roots were carefully hand picked from the sieved soil. Roots were transported back to the laboratory on the day of field collection and stored at 4 °C before being washed free of soil, and separated into two size classes, fine (≤2 mm), coarse (>2 mm) and necromass. Root mass was again determined after drying at 80 °C for 72 hours.

4.2.4 Fine Root Production

Fine root production (< 2 mm) was determined during the 2008 growing season using a root mesh technique (Godbold *et al.* 2003; Lukac and Godbold, 2010). In February 2008, 192 individual nylon meshes (1 mm opening, 10 cm width and 25 cm length) per sub-plot were inserted vertically into the soil with the help of a steel plate and a hammer. The root meshes were then left in the soil for 10 months and extracted in December 2008. To extract the meshes, the soil was first cut 5 cm from each side of the mesh with a knife. The 10 cm thick soil block thus created, with the mesh inside it, was then extracted from the soil and carefully shaken free of soil. Using a binocular microscope, root fragments were classified into species, shortened to 1 cm on either side of the mesh to create a 'virtual' core 2 cm thick, 10 cm wide and 15 cm deep, then removed from the mesh, dried at 80 °C and weighed. Using this technique minimises disturbance to the soil and problems associated with the exploitation of new unoccupied soil associated with in-growth cores. Annual fine root turnover rate was calculated as the ratio of annual fine root production and maximum fine root biomass (Dahlman and Kucera, 1965).

4.2.5 Statistical Analysis

The BangorFACE experiment was designed as a replicated split-plot design with eight whole plots (4 FACE and 4 ambient plots) in four blocks. Each plot is split into seven sub-plots which contain one, two and three species. For this research however, the three single species plots and the three species mixture where studied. The data file was then split with groups based on species and depth. Repeated Measures ANOVA conducted with SPSS 17.0 (SPSS Inc., Chicago, IL) were used for the analysis and equality of variance was tested using Mauchly's test of sphericity. The first model was built with factors as CO_2 treatment (fixed) and block (random). The second model included CO_2 treatment (fixed), block (random) and year (fixed) as factors. Main and interactive effects were considered to be significant at $P<0.05$. During the work a glacial moraine was identified running across one field, for this reason plots that had high stone content were blocked together as (1+4, 2+3, 5+8, 6+7). Coarse root data was log transformed and fine roots square root transformed to satisfy normality.

4.3 Results

4.3.1 Fine Root Standing Biomass

At the final sampling conducted during January 2009 fine root biomass at 0-30 cm was 115.6 ± 28.4 g m^{-2} and 140.8 ± 23.4 g m^{-2} in the elevated CO_2 plots for *B. pendula* in monoculture and in the three species mixture, corresponding to significant increases of 59 and 35% ($P<0.05$) respectively. Whereas, at the same depth, fine root biomass of *A. glutinosa* and *F. sylvatica* was 90.3 ± 8.4 g m^{-2} and 107.8 ± 17.7 g m^{-2} and showed a non-significant increase of 11 and 59% ($P>0.20$) respectively. Fine root biomass for the duration of the experiment at 0-30 cm, and for each for individual species and the three species mixture is shown in Figure 4.1, a detailed breakdown of fine root biomass is shown in Table 4.2.

Repeated measures analysis of variance over the four sampling years at 0-10 cm depth revealed a positive and significant increase in biomass of *F. sylvatica* ($P=0.015$) and marginally non-significant increase for *B. pendula* ($P=0.056$), and the three species mixture ($P=0.067$). Whereas *A. glutinosa* fine roots were most stimulated by elevated CO_2 in 10-20 cm horizon ($P=0.047$). Interactions with year for all species and depths are shown in Table 4.3. The FACE effect on fine root biomass is shown in Figure 4.2 and clearly demonstrates that elevated CO_2 has a larger effect with increasing depth. It is apparent from this figure that *A. glutinosa* fine roots were not stimulated in the top horizon but allocation was strongly stimulated (68%) in the bottom horizon. Additionally both *B. pendula* and the three species mixture were stimulated in all horizons whilst increasing with depth. FACE appeared to have no stimulatory affect on the fine roots of *F. sylvatica*.

Table 4.2 Mean ± SE (g m^{-2}) fine root biomass determined during January 2009 at three 10 cm increments throughout the soil profile.

		0-10 cm		10-20 cm		20-30 cm	
Treatment	Species	Mean	SE	Mean	SE	Mean	SE
Ambient	*A. glutinosa*	36.6	5.3	19.3	12.7	8.3	4.6
	B. pendula	47.7	11.7	14.0	4.6	11.4	3.7
	F. sylvatica	41.3	3.7	43.6	1.6	16.4	11.5
	Mixture	53.3	15.0	15.9	1.7	10.7	7.3
Elevated	*A. glutinosa*	49.6	2.4	23.2	2.3	18.8	9.2
	B. pendula	48.2	15.4	27.6	6.1	14.3	2.3
	F. sylvatica	36.3	0.3	31.0	1.7	21.6	2.9
	Mixture	57.3	19.0	35.9	25.9	32.9	5.7

Table 4.3 Repeated measures ANOVA of fine root biomass across four sampling years at BangorFACE. Data was square root transformed. Significance is denoted by $^{*}P<0.05$, $^{**}P<0.01$.

		0-10 cm		10-20 cm		20-30 cm	
Species	Effect	F	Sig.	F	Sig.	F	Sig.
A. glutinosa	year	3.951	0.144	30.792	**0.009****	5.518	0.097
	year × treatment	0.851	0.551	9.671	**0.047***	1.357	0.404
B. pendula	year	5.439	0.099	0.828	0.560	10.824	**0.041***
	year × treatment	8.514	0.056	0.487	0.715	2.445	0.241
F. sylvatica	year	7.753	0.063	3.033	0.193	4.599	0.121
	year × treatment	22.093	**0.015***	2.384	0.247	0.351	0.794
Mixture	year	0.848	0.552	0.645	0.636	1.377	0.399
	year × treatment	7.440	0.067	1.150	0.456	0.240	0.864

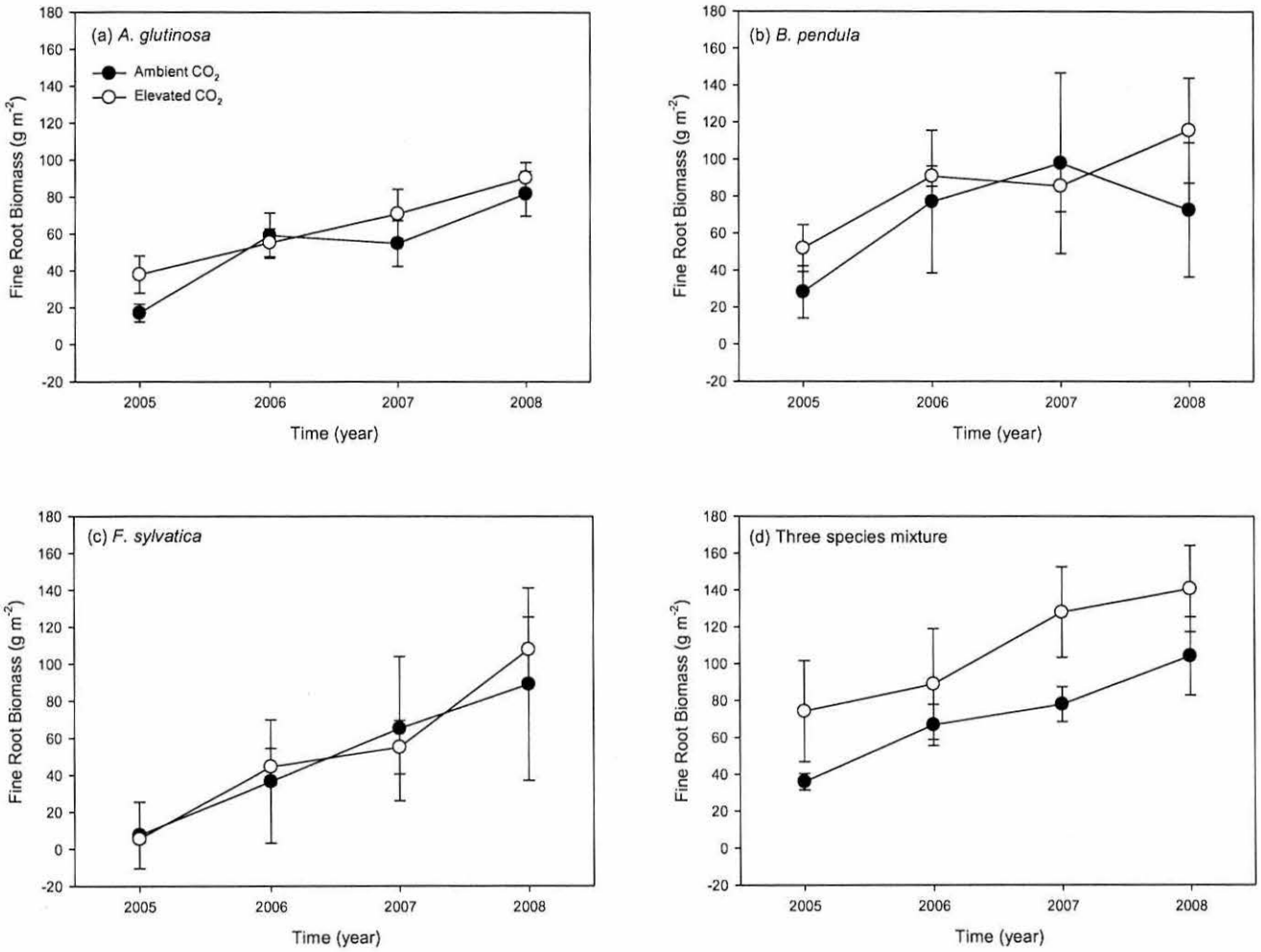

Figure 4.1 Mean standing fine root biomass 0-30 cm for single and mixed species plots measured by auger corer at the end of each growing season between 2005 and 2008. Panel (a) *A. glutinosa* single species plots (b) *B. pendula* single species plots (c) *F. sylvatica* single species plots and (d) Three species mixture plots. Filled circles denote control plots and hollow circles denote elevated CO_2 plots. Error bars indicated are ± SE.

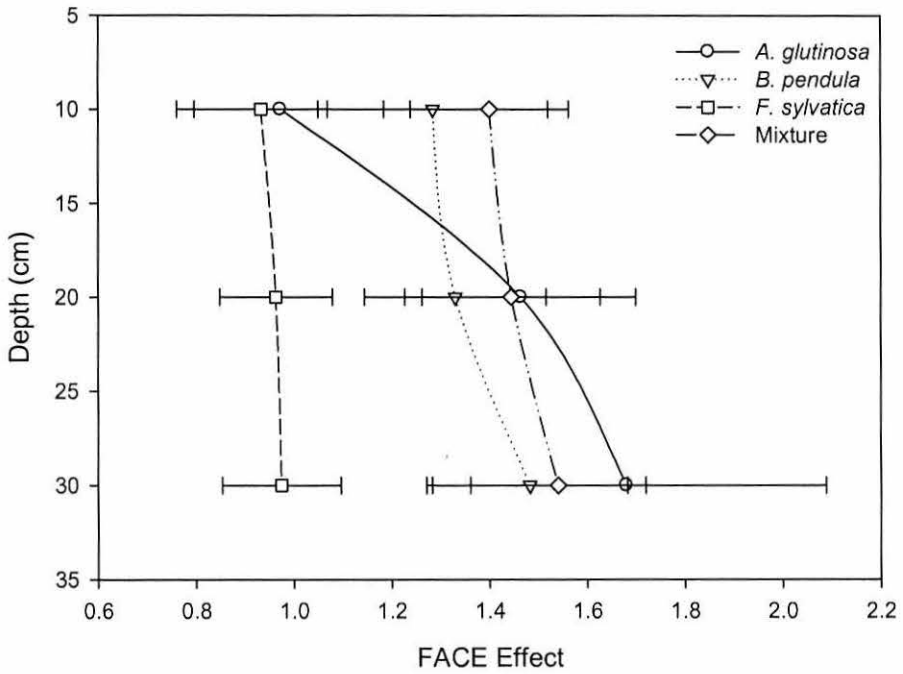

Figure 4.2 Mean ± SE FACE effect on fine root biomass at three depths for *A. glutinosa, B. pendula* and *F. sylvatica* grown in monoculture, and polyculture following four years CO_2 enrichment. Values greater than one indicate a positive affect of CO_2 enrichment.

4.3.2 Coarse Root Standing Biomass

At the end of the 2007 growing season coarse root biomass averaged across all species and treatments was 46.7 ± 14.1 g m^{-2} determined by excavating 30×30×30 cm soil pits, and 57.7 ± 18.0 g m^{-2} when determined by 8 cm diameter auger coring to a depth of 30 cm. There was a large degree of variance between measuring techniques with auger coring over estimating biomass by 24% compared to 30×30×30 cm pits. However, there was a good correlation (r^2=0.40; P<0.001) between the data obtained by both techniques (Figure 4.3).

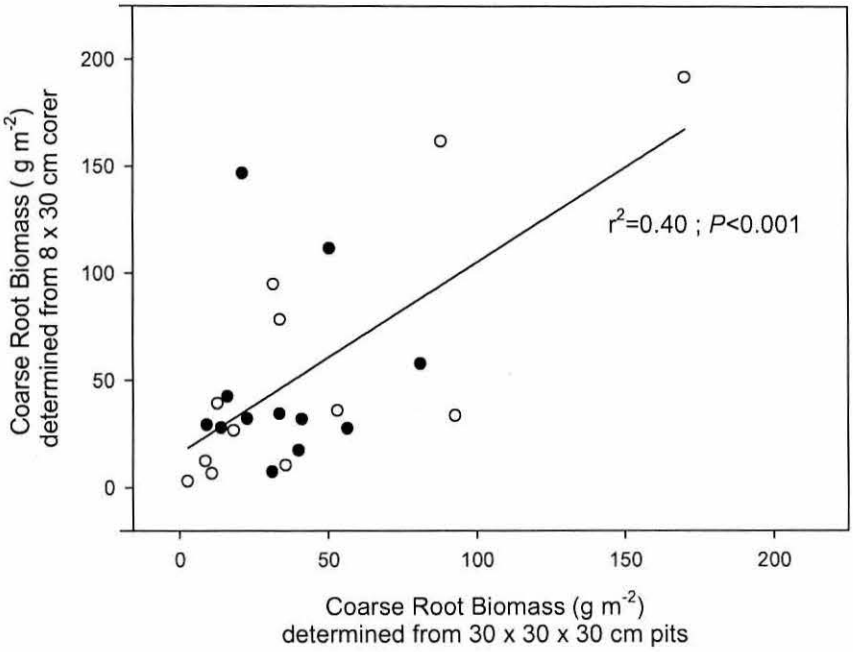

Figure 4.3 Correlation of coarse root biomass (g m^{-2}) determined in 2007 using two different techniques, 30×30×30 cm pits, and 8 cm diameter auger coring to a depth of 30 cm. Filled circular symbols ● denote ambient CO_2 plots and hollow circular symbols O denote elevated CO_2 plots.

4.3.3 Coarse Root Biomass Determined by 30×30×30 cm Pits

In response to elevated CO_2 coarse root biomass in the three species mixture was stimulated by 110% (P=0.054) in the 0-10 cm horizon, and 161% (P=0.032) in the 10-20 cm horizon. In the *B. pendula* subplots a 236% increase in biomass was observed in the 10-20 cm horizon (P=0.082) and a significant depth × treatment interaction (P=0.043) was also observed. In the *A. glutinosa* subplots, a 20-30 cm a decrease of 72% (P=0.097) was observed, however these observations were not statistically significant. Finally, the coarse root biomass of *F. sylvatica* in the 0-10 cm horizon showed a 67% (P=0.036) reduction, and in the 20-30 cm horizon a 69% (P=0.024) decrease (Table 4.4). Additionally, there were no interactions between depth × treatment for *F. sylvatica, A. glutinosa* or the three species mixture.

Table 4.4 Mean ± SE standing coarse root biomass for *A. glutinosa, B. pendula, F. sylvatica* and the three species mixture grown under ambient and elevated CO_2 at three depths throughout the soil profile. Coarse root biomass was determined from 30×30×30 cm pits at the end of the 2008 growing season.

		0-10 cm		10-20 cm		20-30 cm	
CO_2 treatment	Species	g m^{-2}	SE	g m^{-2}	SE	g m^{-2}	SE
Ambient	*A. glutinosa*	16.2	6.0	40.2	11.9	31.3	17.9
	B. pendula	50.6	9.1	21.3	10.8	14.1	4.0
	F. sylvatica	56.7	40.4	22.8	14.1	9.2	7.8
	Mixture	81.3	23.8	33.8	12.7	41.3	29.5
Elevated	*A. glutinosa*	33.3	26.6	35.8	16.8	8.7	3.3
	B. pendula	53.3	16.6	92.9	49.6	12.9	6.3
	F. sylvatica	18.3	10.7	10.9	4.5	2.9	1.4
	Mixture	170.4	38.8	88.2	15.0	31.7	15.4

4.3.4 Coarse Root Biomass Determined by 8 cm Diameter Auger Corer

Averaged across all four enrichment years there was a positive enhancement of 111% (*A. glutinosa*), 34% (*B. pendula*), 16% (*F. sylvatica*) and 96% in the three species mixture to elevated atmospheric CO_2. Total standing coarse root biomass determined at the end of the 2008 growing season was 307.4 ± 103.0 g m^{-2}, 312.2 ± 91.1 g m^{-2}, 261.0 ± 57.9 g m^{-2} and 280.0 ± 92.3 g m^{-2} for *A. glutinosa, B. pendula, F. sylvatica* and the three species mixture respectively. Repeated measures ANOVA revealed a statistically significant response in the three species mixture (P=0.044). Furthermore, in the *A. glutinosa* subplots, elevated CO_2 appeared to produce a positive trend on coarse root biomass (Figure 4.4). However, an interaction between year × treatment was apparent within 0-10 cm of the soil profile when coarse root biomass was stratified by depth and species, which were significant at 10% level. (*B. pendula*, P=0.098; *F. sylvatica*, P=0.096; *Mixture*, P=0.060). Deeper in the soil profile at 10-20 cm there was a significant interaction between year × treatment for coarse root biomass of *F. sylvatica* (P=0.044).

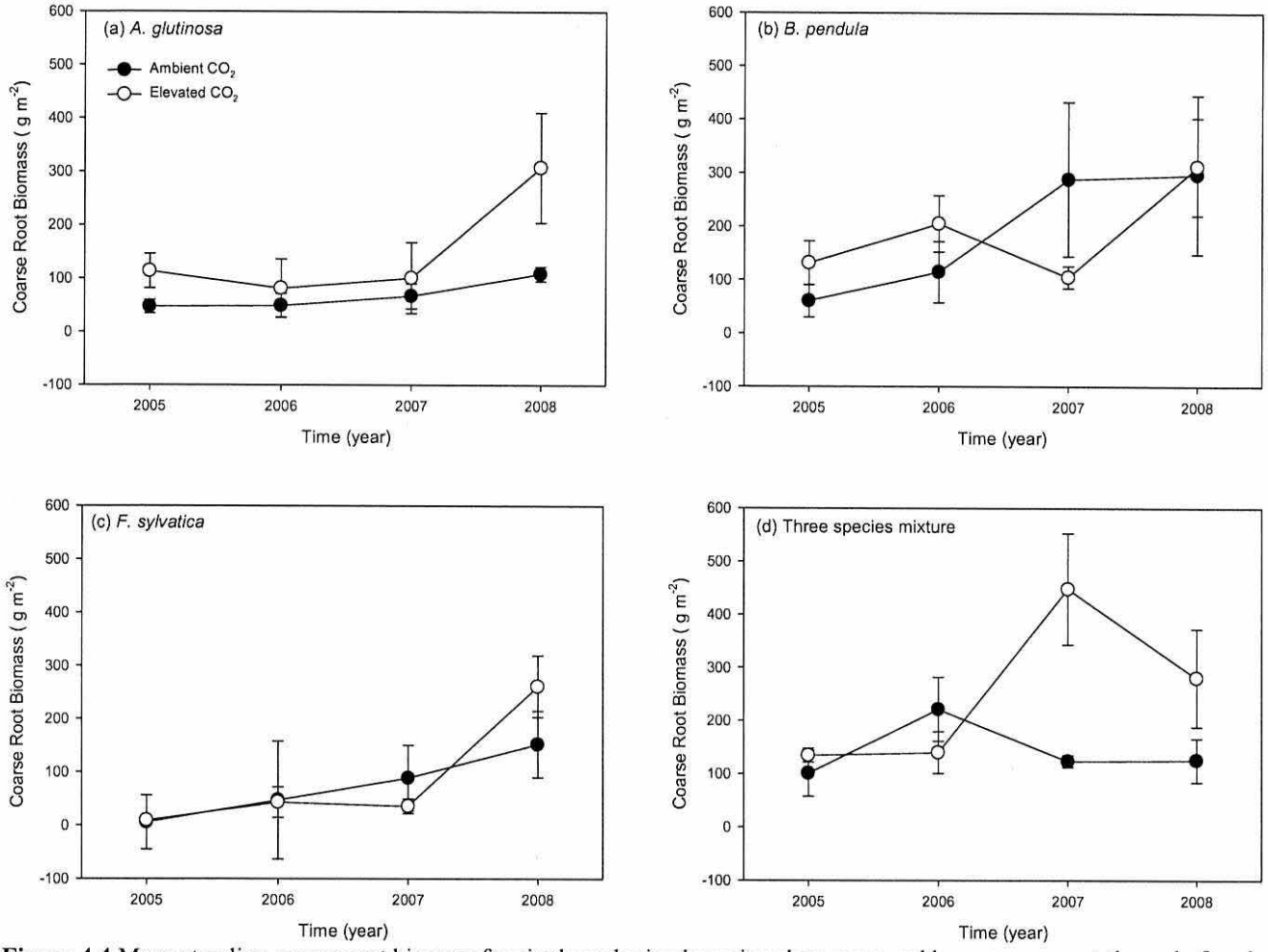

Figure 4.4 Mean standing coarse root biomass for single and mixed species plots measured by auger corer at the end of each growing season between 2005 and 2008. Panel (a) *A. glutinosa* single species plots (b) *B. pendula* single species plots(c) *F. sylvatica* single species plots and (d) Three species mixture plots. Filled circles ● denote control plots and hollow circles ○ denote elevated CO_2 plots. Error bars indicated are ± SE.

4.3.5 Root Characteristics

Mean root area index (RAI), specific root length (SRL), specific root area (SRA), and root length density (RLD) at 10 cm increments to a depth of 30 cm are shown in Table 4.5. In response to elevated atmospheric CO_2, and by the end of the 2008 growing season the RAI of *B. pendula* showed an non-significant (P=0.164) increase of 39%, and in the three species mixture there was a significant (P=0.037) increase of 40%. Elevated atmospheric CO_2 did not significantly increase the RAI of either *A. glutinosa* (12%) or *F. sylvatica* (5%). Specific root length has remained relatively unchanged between the 2006 and 2008 growing season. However, there was a statistically significant interaction between treatment × year for *A. glutinosa* within the 10-20 cm horizon (P=0.009). There were no significant changes in fine root length density. Additionally, *B. pendula* increased RLD by 43% (P=0.295), 35% (P=0.136) and 58% (P=0.554). Whereas in *F. sylvatica* there was an non-significant stimulation of 10% within the 0-10 cm horizon and an non-significant reduction of 19% and 27% for the 10-20 and 20-30 cm horizons respectively (Figure 4.5).

Table 4.5 Root characteristics, specific root length (SRL; Ostonen *et al.* 2007), root area index (RAI), specific root area (SRA; Lõhmus *et al.* 1989) and root length density (RLD) of species grown in monoculture plots and as a mixture of all three species.

Fine Root		2006		2007		2008	
Characteristic	Species	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated
SRL ($m\ g^{-1}$)	*A. glutinsoa*	1.67 ± 0.36	1.67 ± 0.23	3.84 ± 1.04	2.45 ± 0.24	3.45 ± 0.64	2.49 ± 0.22
	B. pendula	1.41 ± 0.32	1.86 ± 0.21	3.33 ± 0.72	3.47 ± 0.56	3.10 ± 0.54	3.49 ± 0.67
	F. sylvatica	2.54 ± 0.29	3.35 ± 0.23	4.94 ± 0.72	4.03 ± 0.64	4.49 ± 0.64	4.02 ± 0.48
	Mixture	1.59 ± 0.07	1.66 ± 0.23	2.94 ± 0.54	3.23 ± 0.56	3.43 ± 0.29	3.25 ± 0.36
RAI ($m^2\ m^{-2}$)	*A. glutinsoa*	0.27 ± 0.05	0.39 ± 0.05	0.69 ± 0.12	0.50 ± 0.08	1.03 ± 0.19	1.15 ± 0.13
	B. pendula	0.37 ± 0.09	0.45 ± 0.06	0.85 ± 0.14	0.82 ± 0.08	0.84 ± 0.12	1.17 ± 0.12
	F. sylvatica	0.38 ± 0.07	0.43 ± 0.09	1.06 ± 0.44	0.57 ± 0.16	1.38 ± 0.10	1.45 ± 0.28
	Mixture	0.36 ± 0.05	0.58 ± 0.03	0.75 ± 0.05	0.90 ± 0.41	1.21 ± 0.28	1.70 ± 0.17
SRA ($m^2\ kg^{-1}$)	*A. glutinsoa*	41.98 ± 3.93	30.27 ± 1.91	70.58 ± 12.27	55.08 ± 6.22	114.05 ± 30.18	124.39 ± 7.98
	B. pendula	38.67 ± 4.81	34.74 ± 11.34	39.16 ± 4.63	55.97 ± 1.26	114.43 ± 18.41	130.28 ± 18.03
	F. sylvatica	86.36 ± 14.19	47.13 ± 4.87	70.06 ± 4.43	72.72 ± 5.33	168.33 ± 25.33	132.86 ± 9.46
	Mixture	51.00 ± 15.22	33.04 ± 5.39	46.75 ± 1.40	50.68 ± 4.22	120.16 ± 8.79	128.01 ± 15.53
RLD ($cm\ cm^{-3}$)	*A. glutinsoa*	0.55 ± 0.21	0.74 ± 0.18	1.07 ± 0.34	0.87 ± 0.14	1.44 ± 0.45	1.51 ± 0.34
	B. pendula	0.85 ± 0.38	1.10 ± 0.31	1.28 ± 0.47	1.84 ± 0.18	1.48 ± 0.46	2.22 ± 0.33
	F. sylvatica	0.93 ± 0.18	1.13 ± 0.39	2.08 ± 1.09	1.20 ± 0.61	2.59 ± 0.52	2.70 ± 1.14
	Mixture	0.79 ± 0.20	1.31 ± 0.15	1.18 ± 0.15	1.68 ± 1.27	2.12 ± 1.02	2.85 ± 0.63

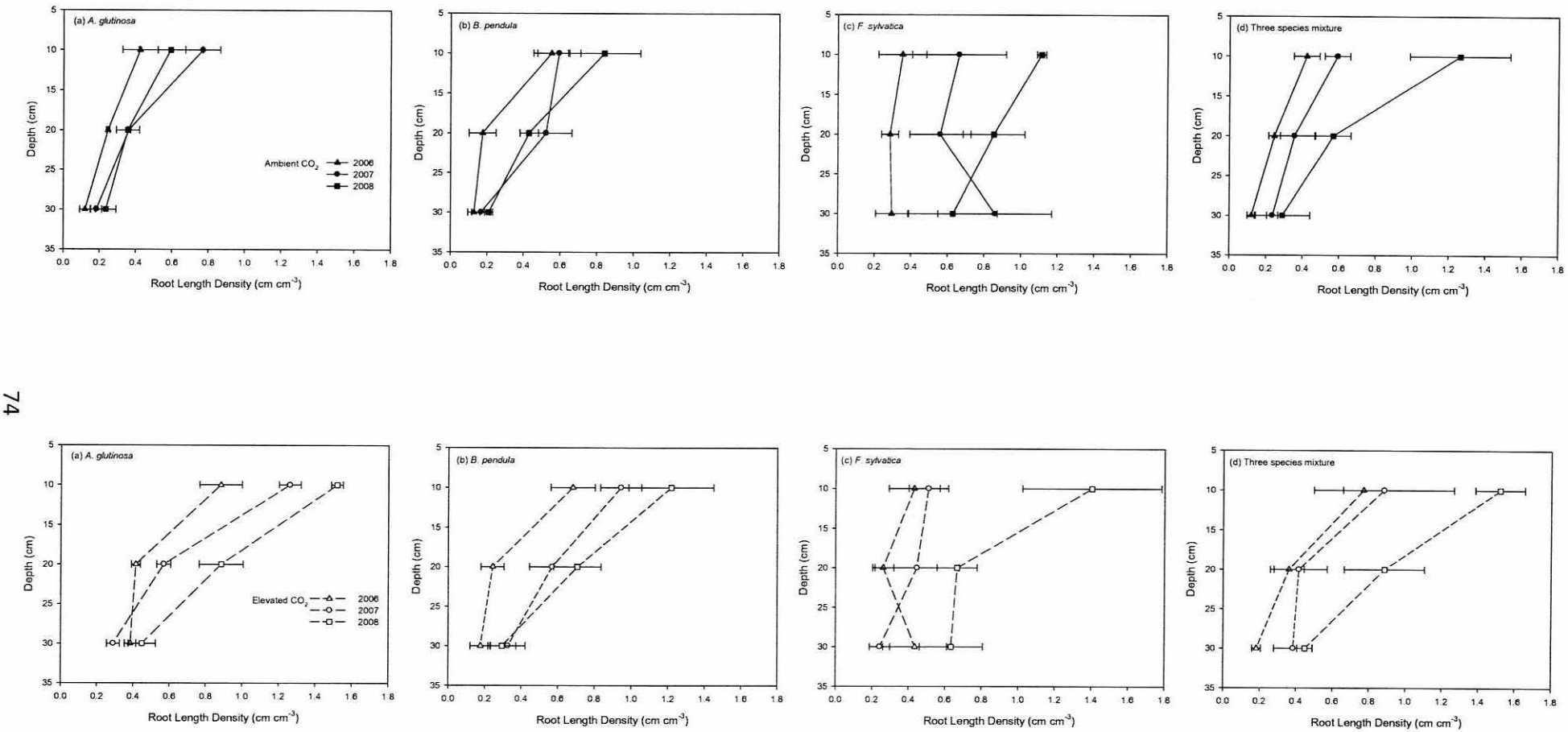

Figure 4.5 Mean ± SE root length density for (a) *A. glutinosa,* (b) *B. pendula,* (c) *F. sylvatica,* and (d) three species mixture at three depths in 10 cm increments from the soil surface. The bottom four panels with broken lines are elevated CO_2 plots and the top four panels with solid lines show ambient CO_2 plots, triangular symbols (2006), circular symbols (2007) and square symbols (2008).

Between 2006 and 2008 specific root area increased dramatically in both the ambient and elevated CO_2 plots, in *A. glutinosa* by 172% and 311%, in *B. pendula* by 196% and 275%, in *F sylvatica* by 95% and 182% and in the three species mixture by 136% and 287% respectively. The rate at which specific root area increased relative to the ambient CO_2 plots was 81%, 40%, 92% and 112% for *A. glutinosa, B. pendula, F. sylvatica* and the three species mixture, but was not statistically significant.

4.3.6 Root Production and Turnover

During the 2008 growing season annual root production in the elevated CO_2 plots for *A. glutinosa* was 52.6 ± 8.9 g m^{-2}, *B. pendula* 38.0 ± 7.3 g m^{-2}, *F. sylvatica* 15.0 ± 2.7 g m^{-2}, and the three species mixture 32.2 ± 9.2 g m^{-2} a stimulation of 132% (P=0.044) for *A. B. pendula* (Table 4.6). In the three species mixture, *A. glutinosa* and the *F. sylvatica* subplots there were no significant changes. Calculated root was 0.7 ± 0.2 yr^{-1} for *A. glutinosa*, 0.4 ± 0.1 yr^{-1} for *B. pendula*, 0.2 ± 0.2 yr^{-1} for *F.* sylvatica, and 0.3 ± 0.2 yr^{-1} in the three species mixture. However, elevated CO_2 enrichment did not alter the root turnover in any of the species studies.

Table 4.6 Mean annual root production during the 2008 growing season determined by root mesh ingrowth for each species grown in monoculture and a three species polyculture.

Species	Ambient [CO_2]		Elevated [CO_2]			
	Root biomass (g m^{-2})	SE	Root biomass (g m^{-2})	SE	FACE Effect	Sig.
A. glutinosa	41.1	15.2	52.6	8.9	28%	NS
B. pendula	16.4	4.5	38.0	7.3	132%	**0.041**
F. sylvatica	17.9	6.5	14.9	2.7	-17%	NS
Mixture	35.5	11.2	32.2	9.2	-9%	NS

4.4 Discussion

The primary goal of this research was to establish how deciduous temperature forest species of contrasting ecological characteristics responded to elevated CO_2 when planted in monoculture and polyculture. After four years of CO_2 enrichment our data clearly indicated differential species stimulation of fine root biomass. The two pioneer species, *B. pendula* and *A. glutinosa,* grown singularly increased belowground C allocation to fine roots with contrasting root growth patterns throughout the soil profile. Whereas, *F. sylvatica,* a late successional species did not positively respond to elevated CO_2 enrichment. However, it should be noted that species ontogeny has a major role in determining responses to elevated CO_2 particularly when examining juvenile species with contrasting successional characteristics (Norby *et al.* 1999). Surprisingly, root biomass of species grown in polyculture exhibited a greater response to elevated CO_2 than species grown in monoculture. Furthermore, not only was elevated CO_2 enhanced root production maintained throughout the experiment, a greater root response was evident with increasing soil depth (Figure 4.2).

4.4.1 Standing root biomass

After four growing seasons standing fine root biomass averaged across all species grown in monoculture plots was 81 g m^{-2} in ambient, compared to 105 g m^{-2} in elevated CO_2, whereas fine roots of species grown in polyculture averaged 104 g m^{-2} in ambient and 141 g m^{-2} in elevated CO_2 (0-30 cm depth). The corresponding FACE effect on fine root biomass averaged over four years for all species was 29% in monoculture and 60% in polyculture. Our results are consistent with that of Tingey *et al.* (2000) who found a median 54% response to 700 μmol mol^{-1} CO_2 enrichment. However, 75% of the studies considered in the analysis by Tingey *et al.* (2000) were conducted in conditions constricting root growth and may have biased results (Ceulemans and Mousseau, 1994). Exclusion of container-grown plant data from the analysis conducted by Tingey *et al.* (2000) reduced fine root response to 48%. In contrast to these large magnitude results, at the Duke FACE experiment, field grown loblolly pine (*Pinus taeda*) fine root biomass response was 24%, averaged over the 10 year experiment duration (Jackson *et al.* 2009). Deciduous species have been shown to exhibit similar responses to coniferous species, for example in a chamber experiment *Betula pendula* increased total root biomass by

50% (Ineson *et al.* 1996). Furthermore, in field conditions fine root biomass of three species of *Populus* determined from destructive coring increased by 33-145% across three growing seasons (Lukac *et al.* 2003). In stark contrast to aggrading forests, the fine root biomass of mature deciduous woodland dominated by *Fagus sylvatica* declined by 30% at the Swiss Canopy Web-FACE site (Bader *et al.* 2009). Variation in the magnitude of fine root responses to elevated CO_2 may often be simply explained by differential species response, further confounded by environmental conditions such as weather, soil conditions and length of the growing season (Norby *et al.* 1999). Forest developmental stage has also been highlighted as a major influential factor. Indeed, in a review, Körner (2006) stressed that biogeochemical processes differ between tightly coupled, fully occupied closed-canopy forests exhibiting limited resources, and decoupled aggrading forests where space and nutrients allow relatively unconstrained resource utilisation.

4.4.2 Fine root response and resource acquisition

Stimulatory effects of elevated CO_2 can be broadly explained by functional equilibrium models stating that plants will allocate more C to the plant organ responsible for acquiring the most limiting resource (Thornley, 1972). However, a plethora of inextricably linked biotic and abiotic factors also influence this equilibrium. Inter and intra-species competitive interactions have been shown to be the most influential biotic factor affecting C allocation in plant species (Wilson and Tilman, 1995), while abiotic factors include resource availability and environmental stresses (Wang and Taub, 2010). Ecophysiological theory dictates that CO_2 enriched forests should increase root biomass if water and nutrients remain the limiting resources. Therefore, enhancements in root biomass can be explained by three inextricably linked mechanisms (i) increased nutrient demand, particularly N, (ii) increased water demand and (iii) increased C available influencing source-sink relationships.

Lack of root response to elevated CO_2 in mature forest has been ascribed to greater tree water use efficiency (WUE) following reduced stomatal conductance, and thus reduced canopy transpiration (Medlyn *et al.* 2001). In this tightly coupled mature forest ecosystem, Bader *et al.* (2009) conjected that fine roots have a seminal role in water acquisition, and that improved WUE may have caused a reduction of fine root biomass. In this case nutrient foraging could be maintained by changes in root

mycorrhization under elevated CO_2 (Tresder *et al.* 2004). Root symbionts may also help to maintain plant source-sink relationships by providing a large carbohydrate sink in exchange for nutrients (Arp, 1991). However, the majority of studies on coupled and de-coupled systems have demonstrated enhanced fine root biomass (Curtis and Wang, 1998). Furthermore, in a recent meta-analysis Wang and Taub, (2010) showed that leaf production and leaf area index increases under elevated CO_2 may maintain water demand through alterations in the balance between WUE and greater transpiration losses resulting from enhanced productivity.

Nitrogen is often the most limiting plant nutrient and its availability is believed to be one of the major drivers of persistent fine root response to elevated CO_2 in forest ecosystems (Johnson, 2006). Ubiquitous carbohydrate accumulation in plant tissues under elevated CO_2 increases tissue C:N, lowering quality. Subsequent negative feedback of enhanced growth response slowly depletes soil N reserves (Luo *et al.* 2004). Progressive N limitation mediated through immobilisation of N in soil and plant biomass pools are projected to decrease soil N availability and forest productivity (Iverson *et al.* 2008). Thus far, forests productivity has not been constrained by soil N availability (Norby and Iverson, 2008), as root and mycorrhizal proliferation has facilitated greater N acquisition in forests ecosystem (Iverson *et al.* 2008; Pritchard *et al.* 2008).

4.4.3 Greater root response deeper in the soil profile

In a variety of experimental conditions and ecosystems a greater elevated CO_2 response has been observed in deeper fine roots (Iversen, 2010). Using minirhizotrons to a depth of 30 cm, Pritchard *et al.* (2008) found a greater elevated CO_2 stimulatory effect on root production between 15 and 30 cm than compared to 0 to 15 cm depth and have subsequently installed deeper rhizotron tubes to further investigation. Iverson *et al.* (2008) found a similar response in a CO_2 enriched loblolly pine plantation, where 60 cm mini-rhizotrons tubes permitted the observation of a two-fold stimulation of root response at 30-60 cm compared to 0-30 cm in the final year of sampling, following 9 years of CO_2 enrichment. Although using a different, destructive coring technique, to a depth of 30 cm our data is in strong agreement with the mini-rhizotron data of Iverson *et al.* (2008). We observed a greater root response to CO_2 enrichment with dept, in addition to a strong differential species effect (Figure 4.2). For example, in *A. glutinosa* exhibited no response

in the top 10 cm but was strongly stimulated by 68% at 30 cm in contrast *B. pendula* exhibited a response of 29% at the soil surface increasing to 48% at 30 cm. Facilitative interactions in the species mixture resulted in a strong (>40%) stimulatory effect increasing with depth throughout the soil profile. Lukac *et al.* (2003) also found species specific effects, with only *P. alba* increasing root response at depth in three *Populus* species.

4.4.4 Root turnover

Our root turnover estimates were not altered by elevated CO_2 treatment and ranged between 0.2 and 0.7 yr^{-1}, these values are consistent with temperate forest in Germany of 0.3 and 1.0 yr^{-1} (Godbold *et al.* 2003) but smaller than those found in *Populus* species (1.4-2.3 yr^{-1}) grown in Italy (Lukac *et al.* 2003). Estimates of root turnover in forests vary considerably, a fact reflecting inconsistencies in the definition of fine roots diameter, which are further confounded by environmental, methodological and intra-specific variation (Majdi *et al.* 2005; Pritchard and Strand, 2008b). An analysis of global root turnover based mainly on destructive coring estimated turnover at 1.4 yr^{-1} (Gill and Jackson, 2000), whereas, stable isotope methods deployed at Duke and ORNL FACE sites revealed turnover time between 1.3-3.0 yr^{-1} and 4.2-5.7 yr^{-1} for deciduous and coniferous species respectively (Matamala *et al.* 2003). Furthermore, the use of the atmospheric ^{14}C pulse produced during bomb experiments yielded estimates between 4.2 and 32 yr^{-1} (Trumbore *et al.* 2006). Elevated CO_2 had been expected to decrease root turnover (Eissenstat *et al.* 2000). Thus far, results have been inconclusive, Lukac *et al.* (2003) found a significant increases between 27-55% in *Populus* genotypes, supported by a 46% increase in loblolly pine at Duke FACE (Matamala and Schleisinger, 2000; Pritchard *et al.* 2008). On the other hand, and in agreement with our data, no response was initially observed in the FACE sweetgum stand at ORNL. However, subsequent analysis four years after minirhizotron installation revealed a significant increase and depth interaction in root turnover of between 1.1 and 1.3 yr^{-1} in elevated and ambient respectively at 0-15 cm, decreasing to 0.8 yr^{-1} in elevated and 1.1 yr^{-1} in ambient at 15-30 cm (Iverson *et al.* 2008). A myriad of biotic and abiotic factors such as reduced tissue quality (N), temperature, water, nutrient availability, microbial interactions eg. mycorrhization and respiration costs have been shown to influence root longevity

(Eissenstat *et al.* 2000). Surprisingly, tissue N has not been shown to decline in fine roots, and several authors have suggested the most likely mechanism thought to have the greatest influence is increased mycorrhizal colonisation (Iverson *et al.* 2008, Prichard *et al.* 2008b).

4.4.5 Root characteristics

Root area index at our site was between 0.84 and 1.70 m^2 m^{-2} which is considerably lower than the mean RAI of 9.8 m^2 m^{-2} for temperate deciduous forest (Jackson *et al.* 1997) enabling us to deduce that soil space and resources by fine roots were not fully exploited by the conclusion of the experiment despite canopy closure being achieved during 2006. Root area index was consistently greater in the three species mixture, supporting our assertion that differential niche exploitation was facilitating greater root proliferation within a volume of soil. Within the *A. glutinosa* plots specific root length was consistently increased suggesting that *A. glutinosa* had altered its root morphology in response to elevated CO_2. Pregitzer *et al.* (2008) had previously described how plant root morphology can be altered to forage for sufficient nutrients to sustain plant growth in elevated CO_2

4.4.6 Species mixture effect on root production.

The only other FACE experiment to investigate field afforested broadleaved forest of mixed species communities was conducted at the Aspen FACE facility, Rhinelander, Wisconsin. Fine root biomass determined by root coring in aspen monoculture plots increased by 45%, and in aspen-birch, and aspen-maple mixed species communities by 64% and 29% respectively, when averaged across 1997 and 2003 (King *et al.* 2005). Our fine root responses to elevated CO_2 are of a similar magnitude, but yielded a greater response when species were in polyculture, which we attribute to competitive interaction altering soil exploitation and spatial distribution to better complete for resources. In our experiment, we utilised two pioneer species and a third late successional species with contrasting root growth patterns in polyculture. When combining light demanding with shade tolerant late successional species, community dynamics are likely to have a large impact on facilitative or competitive interactions. By using species with contrasting characteristics, species resource utilisation can be improved by 30% in comparison to

species grown in monoculture (Pretzsch, 2005). Moreover, the survivorship strategy of late successional species such as beech is to produce more roots throughout the soil strata enabling rapid exploitation of canopy gaps when they occur. However, conversely, if species are selected with similar ecological niches competition may result in a negative effect (Bolte and Villanueva, 2006). Our data shows that mixed species facilitate a greater CO_2 response, through mechanisms such as ecological niche separation allowing exploitation of resources not available to co-existing species, and facilitation among functional groups (Coomes and Grubb, 2000).

4.5 Conclusion

Forest ephemeral fine roots have a fundamental role in determining the response function and feedbacks of the global C cycle to environmental change. Here we showed that the magnitude of fine root response increased with soil depth and was strongly differentiated by species. When species were grown in polyculture a greater response to elevated CO_2 was observed at all depths throughout the soil profile, indicating that species grown in mixtures were able to better exploit soil resources. Our data challenges our current understanding of biogeochemical cycling in response to elevated CO_2 as root proliferation in species diverse forets may deplete soil resources faster than previously thought and alter C cycles feedbacks. Existing biogeochemical cycling models parameterised with data from species grown in monoculture may be dramatically underestimating the belowground response to global change.

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5 Species Diversity Enhances Carbon Sequestration of CO_2 Enriched Temperate Forest

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Contributions:

Andrew Smith wrote the manuscript, collected the data, performed the analysis and maintained the FACE facility during 2006-2009. Dr. Martin Lukac designed, built and maintained the experiment during 2004-2006. Iftekhar Ahmed provided specific leaf area measurements collected during his MSc in 2006. Prof. Douglas Godbold is the project principle investigator who designed and constructed the experiment. He also provided assistance with data interpretation, analysis and manuscript preparation.

5.1 Introduction

Forest ecosystems have recently attracted considerable interest in an effort to understand the global C budget. Globally, forests cover one third of the terrestrial surface of the Earth and their soils store almost half of the C of terrestrial ecosystems *ca.* 2100 Pg C (FAO, 2001; Schulze, 2006). Through photosynthesis, trees assimilate CO_2 in biomass and transfer C to soils predominantly by means of fine roots, mycorrhizae and rhizodeposition. Indeed, fine root production of forest ecosystems comprises one third of global annual net primary productivity (NPP) in terrestrial ecosystems (Jackson *et al.* 1997) and mycorrhizal hyphal input has been suggested to be a dominant pathway of C to soil (Godbold *et al.* 2006). The ubiquitous and ephemeral nature of fine roots and mycorrhizal extramatrical hyphae emphasises their importance in the translocation of C to soil C pools (Gill and Jackson, 2000; Pregitzer, 2003).

Until recently research investigating species diversity, ecosystem functioning and productivity have mainly been limited to grasslands (Hooper *et al.* 2005). Early experiments showed a positive relationship between productivity and increased biodiversity (Tilman *et al.* 1996; Tilman *et al.* 1997); conversely, other studies reported a negative relationship (Hooper and Vitousek, 1997). In forests, controversy surrounding the benefits of mixed species stand productivity dates back to the 18th century (Hartig, 1791), with silvicultural practice of mixed species forests has being subject to much conjecture. Only recently have rigorous scientific studies been initiated to elucidate the precise mechanisms mediating the productivity differences of trees grown in polyculture (Pretzsch, 2005). For example, in Southern Germany, mixed stands of *Fagus sylvatica* and *Picea abies* produced up to 59% more above ground biomass than adjacent pure stands (Pretzsch and Schütze, 2009). In contrast, Jacob *et al.* (2010) found decreases in aboveground biomass of *F. sylvatica* with increasing species richness in comparison to *F. sylvatica* in monoculture. Authors have explained these conflicting results through contrasting ecological philosophies, demonstrating that complex environmental interactions and species ontogeny strongly influence observed results. Mixed species aggrading forest may be measurably more productive for three reasons: (i) species specific ecological niche complementarities that enable species rich plantations to more efficiently access and utilise limiting resources, (ii) facilitation by species improving the growing conditions, such as N fixation, that result in greater overall productivity (Richards *et al.* 2010; Coomes and

Grubb, 2000), and finally (iii) the sampling effect, where an inherently overyielding cohort of species within the community are responsible for increases in production due to their greater resource use efficiency compared to other species (Loreau *et al.* 2001). In contrast to the positive effect of mixed species, direct competition for a limiting resource by species with similar ecological niches may reduce overall productivity (Bolte and Villanueva, 2006). The majority of studies investigating forest overyielding have focused on above-ground responses. Subsequently, little is known about the belowground effects of tree species diversity on the structure and function of roots (Meinen *et al.* 2009). Available data from forest stands grown in ambient conditions reveals that generalised mechanisms explaining these observations are still lacking (Pretzsch *et al.* 2010). However, ecophysiological theory dictates that when species are grown in mixture, roots may respond by allocating more biomass and altering spatial distribution as plants compete for resources.

Atmospheric concentrations of CO_2 are increasing as a consequence of land use change and the burning of fossil fuels (IPCC, 2007). Experiments manipulating atmospheric CO_2 in forests are important in determining the impact of these environmental perturbations upon the global C balance. Thus far, elevated CO_2 research has tended to focus on tree growth response in monoculture or aboveground mixture effects (Kozovits *et al.* 2005). Data from these studies has extensively demonstrated that biomass accruement is stimulated by elevated atmospheric CO_2 (see reviews by Ceulemans and Mousseau, 1994; Curtis and Wang 1998; Saxe *et al.* 1998; Long *et al.* 2004; Ainsworth and Long, 2005). In a comparison of field CO_2 fumigated forests, net primary productivity was shown to increase by 23 $\pm$ 2% in response to a 200 $\mu mol\ mol^{-1}$ increase in atmospheric CO_2 (Norby *et al.* 2005). Belowground response to elevated CO_2 has also been shown to allocate more C to roots, producing larger systems with a greater response deeper in the soil profile (Lukac *et al.* 2003; Iverson *et al.* 2010). Fine roots are not only responsible for water and nutrient uptake, but also represent an important component of the ecosystem C cycle (Fogel and Hunt, 1983, Jackson *et al.* 1997). Therefore, interactions between elevated CO_2 and species diversity are likely to have a major impact upon nutrient and C cycling. However, the interaction between elevated atmospheric CO_2, species diversity and belowground productivity is poorly understood.

We examined how growth responses of three species (*Alnus glutinosa, Betula pendula* and *Fagus sylvatica*) varied in monoculture and a three species polyculture comprised of the aforementioned species, under both ambient and elevated atmospheric CO_2 using a free air CO_2 enrichment (FACE) system. Specifically we examined how species interactions affected above and belowground C allocation, fine root biomass and root morphological characteristics. To ascertain how these interactions affected productivity in polyculture we tested the hypotheses that (i) elevated CO_2 and species diversity increases aboveground biomass, (ii) elevated CO_2 and species diversity increases belowground C allocation and positively alters root morphology relative to species grown in monoculture.

5.2 Materials and Methods

5.2.1 Site Description

BangorFACE experimental facility is located at Henfaes Research Station, Abergwyngregyn, North Wales, UK (53°14'N, 4°01'W). The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Soils are fine loamy brown earth over gravel (Rheidol series) and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002), and as a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011). Soil texture is 63% sand, 28% silt and 9% clay. Climate at the site is classified as Hyperoceanic with a mean annual temperature throughout 2005- 2008 of 11.5 °C and an annual rainfall of 1034 mm.

The experiment plots were located on two fields, a former agricultural field, and former agricultural pasture which had been used for small scale forestry experiments for the previous eight years. The experiment comprised of eight octagonal plots 8 m in diameter, four ambient and four CO_2 enriched, in a 2 × 4 factorial block design. We used three tree species (*Alnus glutinosa* [L.] Gaertner, *Betula pendula* Roth. and *Fagus sylvatica* L.) that were selected due to their contrasting shade tolerance, successional chronology and to represent a range of taxonomic, physiological and ecological types. Each plot was divided into seven planting compartments and planted in a pattern creating areas of one, two and three species. However, for simplicity this experiment utilised three single species subplots

of *B. pendula, A. glutinosa* and *F. sylvatica*, in addition to a fourth subplot which contained a mixture of all three species. The experimental plots were surrounded by a 10 m buffer of the same species planted at the same density. The remaining field was planted with a mixture of species at 10000 stems ha^{-1}. The four FACE plots were enriched to 580 μmol mol^{-1} using pure CO_2 depleted in ^{13}C (-39 ‰ PDB). CO_2 was supplied during the photosynthetically active part of the day through laser drilled holes in horizontal pipes. Two layers of pipes were suspended from eight masts and positioned mid canopy (Miglietta *et al.* 2001). Elevated [CO_2] was measured at one minute intervals and was within 30% of the target concentration for 75-79% of the time during 2005-2008.

5.2.2 Root biomass

Belowground biomass and vertical root distribution was determined by auger coring. Sampling was conducted from January 2005 through 2009 using randomly located positions equidistant from the three nearest trees at two random locations. In total eight cores were taken from each plot, two cores from each of the three single species sub plots and two from the three species sub plot at three depths, 0-10 cm, 10-20 cm and 20-30 cm (192 cores during each sampling). Roots cores were transported back to the laboratory on the day of field collection and stored at 4 °C before being washed free of soil, and separated into two size classes, fine (≤2 mm), coarse (>2 mm) and necromass. Necromass determination was based on black or dark brown colour and a decaying fragmented appearance. Live fine roots were scanned using an Epson 4990 scanner at a resolution of 300 dpi. Images were analysed with WinRhizo (version 2005c Regent Instruments Inc, Quebec, Canada) to determine specific root length, specific root area, root area index, root length density, size class distribution and root length density. Finally, roots mass was determined after drying at 80 °C for 72 hours.

Coarse root biomass was assessed during November 2007 by excavating 30 × 30 × 30 cm pits on the perimeter of each single species subplot and three species mixture subplot (32 pits in total). Each pit was excavated in 10 cm horizons, roots and stones where removed by sieving to 8 mm in the field, finer roots were carefully hand picked from the sieved soil. Roots were transported back to the laboratory on the day of field collection and stored at 4 °C before being washed free of soil, and separated into two size classes, fine (≤2 mm), coarse (>2 mm) and necromass. Root mass was again determined after drying at 80 °C for 72 hours.

5.2.3 *Aboveground biomass*

Tree height and stem diameter at 22.5 cm were measured after tree establishment in March 2005 and then during February of each following year during CO_2 enrichment (2006-2009). Tree measurements were taken during the winter dormant phase, tree height was determined using a telescopic pole, and two measurements of diameter were taken perpendicular to each other using digital vernier callipers. Previously determined species specific allometric relationships (Chapter 3) were used to convert stem diameter measurements to aboveground biomass.

5.2.4 *Leaf measurements*

Following observations of leaf senescence, litter was collected on a weekly basis from four litter baskets 0.11 m^2 until all leaves had abscised (October to December). A litter basket was located in each of the three single species subplots and the three species mixture. Litter was returned to the laboratory, washed and sorted into individual species then dried at 80 °C for 24 hours, and weighed. Measurements of leaf area were made with a LI 3000A portable area meter (LI-COR, Lincoln, NE, USA) from fresh leaves collected during 2006. Immediately following area measurement leaves were dried at 80 °C for 24 hours, and weighed to determine specific leaf area according to Equation 1. Leaf area index was then calculated according to Equation 2 (McCarthy *et al.* 2007). Juvenile *Fagus sylvatica* was excluded from the calculations as a consequence of retaining senesced litter until bud burst the following season.

$$specific\,leaf\ area = \frac{leaf\ area}{leaf\ weight} \qquad \text{(Eqn. 1)}$$

$$leaf\ area\ index = fallen\ litter\ mass \times specific\ leaf\ area \qquad \text{(Eqn. 2)}$$

5.2.5 *Theoretical mixture*

To determine the effect of growing species in mixture the average measured biomass from the three species mixture plots was compared to a theoretical mixture calculated from each of the species contributing to the mixture growing in monoculture.

Equation 3 shows the theoretical mixture biomass calculation based on the relative mixture yield index of Wilson, (1988), where $B_{Species}$ is the biomass component contributing to the mixture. Here, the effect of competition (ratio of mixture and monoculture yield) is expressed graphically.

$$B_{mixture} = \left(\frac{1}{3} \times B_{Alnus}\right) + \left(\frac{1}{3} \times B_{Betula}\right) + \left(\frac{1}{3} \times B_{Fagus}\right) \qquad \text{(Eqn. 3)}$$

The theoretical leaf area index was calculated using only *A. glutinosa* and *B. pendula* due to the litter retention of *F. sylvatica* which prevented accurate determination of fallen litter mass. Within the monoculture stand, three trees planted in an equidistant triangular pattern contributed to the litter collected in fallen litter baskets. While in the mixed species plots a single tree of both *A. glutinosa* and *B. pendula* contributed to the collected fallen litter. Consequently predicted leaf area index of a theoretical mixture was calculated from monoculture plots as Equation 4.

$$LAI_{mixture} = \frac{LAI_{Alnus}}{3} + \frac{LAI_{Betula}}{3} \qquad \text{(Eqn. 4)}$$

5.2.6 Soil physiochemical characteristics

Soil samples were collected during root core sampling using an 8 cm auger corer (described above). On return to the laboratory soil samples were homogenised and sieved to pass 2 mm prior to analysis. Soil pH was measured in water according to Smith and Doran, (1996). Potassium (K), Calcium (Ca) and Sodium (Na) were extracted using ammonium acetate pH 7.0 (Lavkulich, 1981) and determined by Atomic Emission Spectroscopy (Sherwood Flame Photometer 410). Plant available P was extracted in 0.5M sodium bicarbonate ($NaHCO_3$) pH 8.5 (Olsen *et al.* 1954) and determined colourimetrically as molybdate reactive P (Murphy and Riley, 1962). Soil dissolved organic carbon (DOC) and total dissolved N (TDN) was determined using a Shimadzu TOC-V-TN analyzer (Shimadzu Corp., Kyoto, Japan) following extraction in 0.5 M K_2SO_4 (Jones and Willett, 2006) (Table 5.1).

Table 5.1 Average soil physical and chemical properties for Bangor during 2008 used in the experiment, two ambient and two elevated plots are located in each field.

	P mg kg^{-1}	K mg kg^{-1}	Ca mg kg^{-1}	Na mg kg^{-1}	pH	Moisture (%)	OM (%)
Ambient	$153.2^{\pm 13.0}$	$68.5^{\pm 4.4}$	$741.0^{\pm 50.9}$	$10.9^{\pm 0.4}$	$4.9^{\pm 0.1}$	$22.0^{\pm 0.4}$	$6.0^{\pm 0.1}$
Elevated	$82.6^{\pm 5.0}$	$64.3^{\pm 3.9}$	$695.9^{\pm 23.2}$	$14.1^{\pm 0.7}$	$4.7^{\pm 0.0}$	$21.4^{\pm 0.3}$	$5.3^{\pm 0.1}$

5.3 Results

5.3.1 Leaf Area Index

Leaf area index (LAI) measured within the mixed species plots was consistently lower under elevated CO_2 throughout the experiment, conversely predicted LAI was consistently greater in elevated CO_2 than in ambient (P>0.05; Figure 5.1). Average LAI for the duration of the experiment was 3.4 ± 0.3 m^2 m^{-2} and 3.1 ± 0.3 m^2 m^{-2} in ambient and elevated CO_2 respectively. Whereas LAI in mixture predicted from species grown in monoculture was 1.3 ± 0.1 m^2 m^{-2} in ambient and 1.4 ± 0.1 m^2 m^{-2} in elevated CO_2 plots. Therefore, growing species in mixture increased the leaf area index by 38% in ambient and 45% in elevated CO_2, when compared to the leaf area index predicted from species grown in monoculture. Repeated measures ANOVA showed a significant interaction between predicted or measured mixture × year in both ambient and elevated CO_2 plots (P<0.001). The contribution of *A. glutinosa* and *B. pendula* grown in monoculture to predicted LAI was 60:40 respectively in both ambient and elevated CO_2. Whereas the *A. glutinosa* and *B. pendula* species contribution to measured LAI in polyculture was 60:40 in ambient and 50:50 in elevated CO_2.

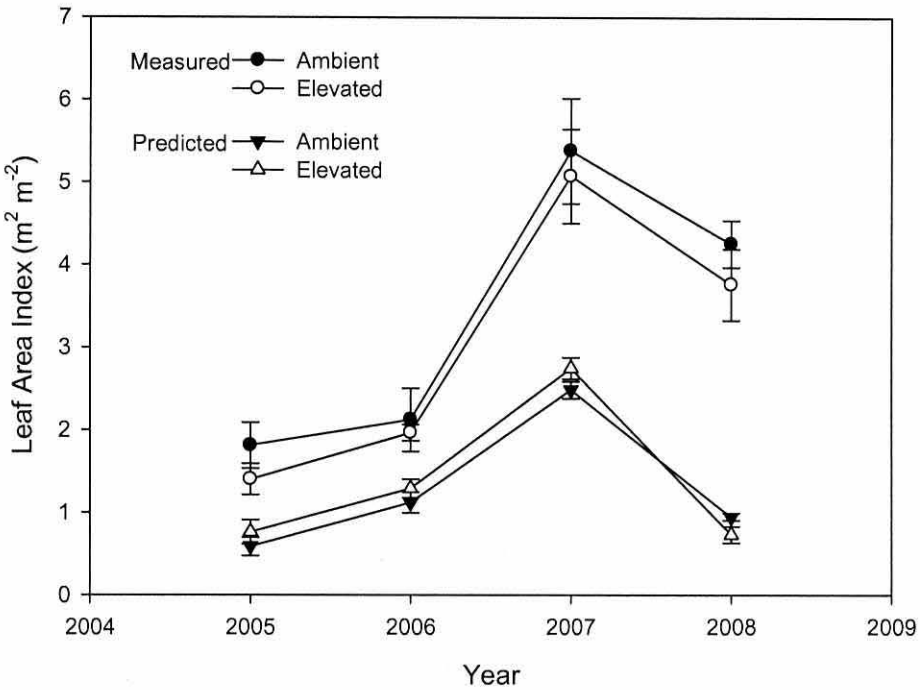

Figure 5.1 Mean ± SE measured and predicted leaf area index (m^2 m^{-2}) for *A. glutinosa* and *B. pendula* grown in mixture. *F. sylvatica* was excluded from the calculations due to retention of litter during autumn impeding litter collection. LAI was calculated as fallen leaf mass × specific leaf area (McCarthy *et al.* 2007). Measured LAI was calculated as (*A. glutinosa* + *B. pendula*) from fallen litter of species in the mixed species plots. Predicted LAI was calculated as (*A. glutinosa* ÷ 3) + (*B. pendula* ÷ 3) from litter collected within the single species plots.

5.3.2 Aboveground Biomass

Following four years of atmospheric CO_2 enrichment aboveground biomass was, *A. glutinosa* (8560 ± 635 g m^{-2}), *F. sylvatica* (598 ± 28 g m^{-2}) and *B. pendula* (10191 ± 322 g m^{-2}) a stimulation of 32%, 9% and 9% for, *A. glutinosa, F. sylvatica* and *B. pendula* respectively. When these three species where grown in mixture under elevated CO_2 6072 g m^{-2} of aboveground biomass accrued, whereas the predicted mixture biomass from species grown in monoculture was 4567 g m^{-2}, an overyielding effect of 33%. However, within the ambient CO_2 plots the above ground overyielding effect exceeded that of elevated CO_2 plots by 14% (predicted 3869 g m^{-2}; measured 5674 g m^{-2}; $P<0.01$) (Figure 5.2). The overyielding response was consistently greater in the ambient CO_2 plots and increased with magnitude throughout the four years of enrichment. Repeated measures ANOVA revealed a highly significant year × mixture interaction in both the ambient and elevated CO_2 plots ($P<0.001$). Elevated CO_2

significantly interacted with sampling year ($P<0.01$) within the predicted mixture plots but there was no FACE interaction in the measured plots ($P=0.553$).

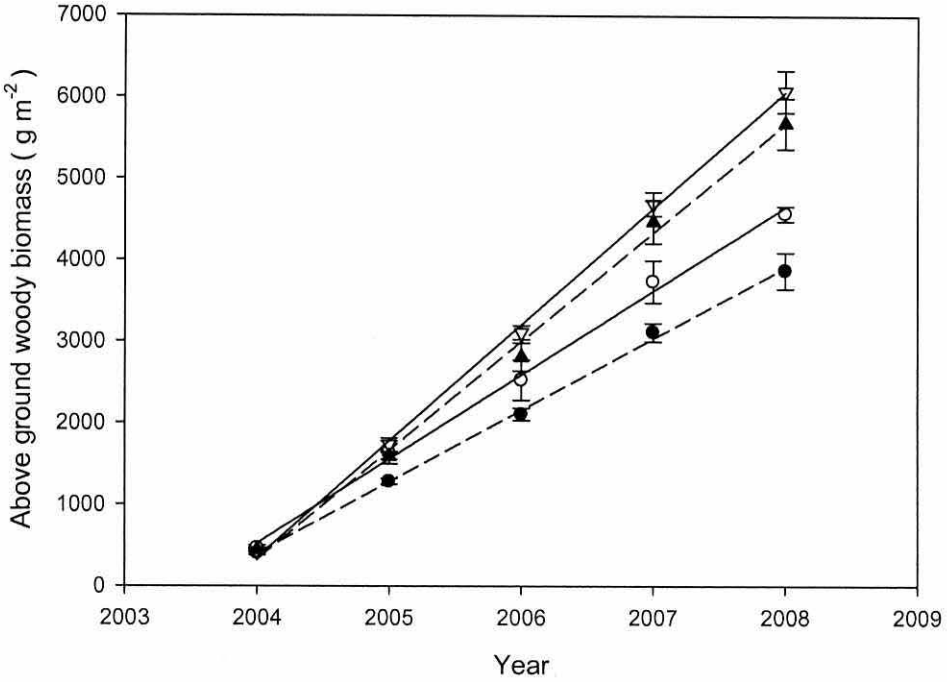

Figure 5.2 Mean ± SE measured and hypothetical aboveground biomass in the three species polyculture plots in ambient and elevated atmospheric CO_2 ($n=4$). Hypothetical biomass was calculated as (0.3×*A. glutinosa* + 0.3×*B. pendula* + 0.3×*F. sylvatica*) from data obtained when species were grown in monoculture. Measured biomass is denoted by triangular symbols △ elevated CO_2 ▼ ambient CO_2 and hypothetical biomass by circular symbols ○ elevated CO_2 ● ambient CO_2. Broken lines linear regression ambient CO_2 and solid linear regression lines elevated CO_2.

5.3.3 Fine Root Biomass

In contrast to aboveground biomass, elevated CO_2 fine root biomass response was larger in the elevated atmospheric CO_2 mixed species plots. In elevated CO_2, fine roots produced 141 ± 23 g m^{-2} of biomass within the top 30 cm of soil, whereas the predicted biomass from species grown singularly was 105 ± 18 g m^{-2}, an overyielding effect of 34% ($P<0.001$; Figure 5.2). In ambient atmospheric CO_2 conditions, measured fine root biomass within the mixed species plots was 104 ± 21 g m^{-2} yet predicted as 81 ± 7 g m^{-2} from species grown in monoculture, an overyielding effect of 28% ($P<0.001$; Figure 5.2). Predicted fine root biomass was consistently lower than measured biomass in both ambient and elevated CO_2 with a mean stimulation over the four years of enrichment, of 39% in ambient and 73% in elevated CO_2, however repeated measures ANOVA did not reveal any significant interactions.

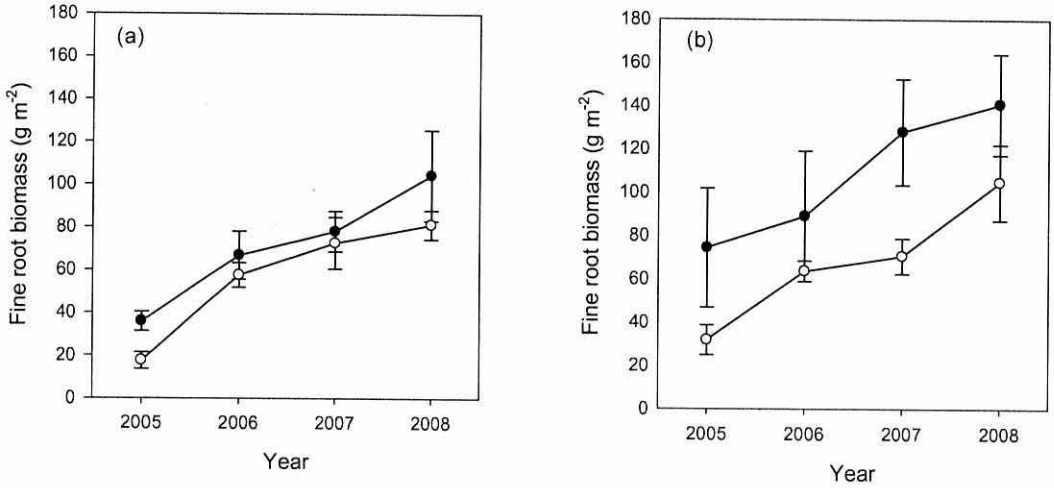

Figure 5.3 Mean ± SE measured and hypothetical fine root biomass throughout the (a) ambient CO_2 (b) elevated CO_2 hypothetical biomass was calculated as (0.3×*A. glutinosa* + 0.3×*B. pendula* + 0.3×*F. sylvatica*) from data obtained when species were grown in monoculture. Measured biomass is denoted by ● symbols and hypothetical biomass by O symbols ($n = 4$)

5.3.4 Root Characteristics

To investigate if root structure and distribution may have altered throughout the soil profile we examined root morphological indices deduced from high resolution images. Root area index (RAI) although larger in the elevated CO_2 plots was not significantly altered (ambient 1.2 ± 0.3 m^2 m^{-2}; elevated 1.7 ± 0.2 m^2 m^{-2}; P=0.120) across all species. Additionally, specific root length (ratio of length to mass) and specific root area (ratio of surface area to mass) were also not significantly altered by elevated CO_2. However, root length density (RLD; root length per soil volume), in the mixed species FACE plots exceeded the controls plots by 21, 56 and 55% at 10, 20 and 30 cm depth respectively. In the single species plots, *B. pendula* was the only species to exhibit a weak significant trend in RLD throughout the soil strata maximising at 20 cm by +65% (0.7 ± 0.1 cm cm^{-3}; P=0.081). Both early successional species (*B. pendula* and *A. glutinosa*) produced most root in the top 10 cm, with a strong reduction with depth in both alder and birch to 0.3 ± 0.1 cm cm^{-3} at 30 cm. Vertical root distribution of beech roots was remarkably consistent throughout the soil profile with 23% of total root being in the bottom 30 cm compared to 13 and 17% for birch and alder. The most pronounced difference in root morphology observed in the final year of the study was a mean 39% (P=0.019) increase in root tip number across

all single species in response to elevated CO_2. However, in the mixed species plots we observed a non significant 23% increase in response to elevated CO_2. Whilst in all single species plots, the number of root tips increased with depth while in the mixed species plots root tips abundance decreased with depth (data not shown).

5.3.5 Coarse Root Biomass

Coarse root biomass was largest in the hypothetical mixed species plots under elevated CO_2, measured coarse root biomass was 280 ± 41 g m^{-2} within the top 30 cm of soil, whereas the predicted biomass from species grown singularly was 294 ± 19 g m^{-2} ($P<0.05$; Figure 5.3). In ambient atmospheric CO_2 conditions, measured coarse root biomass in the hypothetical mixed species plots was 186 ± 44 g m^{-2}, yet predicted as 125 ± 92 g m^{-2} from species grown in monoculture ($P>0.05$; Figure 5.2). Repeated measures ANOVA identified a significant interaction between predicted or measured mixture × year in both ambient and elevated CO_2 plots ($P<0.05$), and a significant interaction between year and predicted or measured mixture ($P<0.05$).

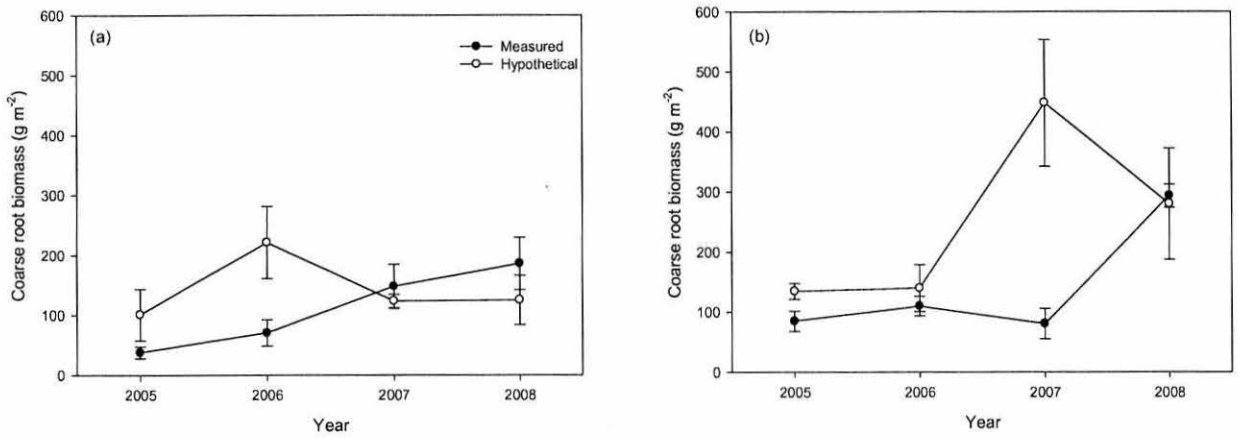

Figure 5.4 Mean ± SE measured and hypothetical coarse root biomass throughout the (a) ambient CO_2 (b) elevated CO_2 hypothetical biomass was calculated as (0.3×*A. glutinosa* + 0.3×*B. pendula* + 0.3×*F. sylvatica*) from data obtained when species were grown in monoculture. Measured biomass is denoted by ● symbols and hypothetical biomass by O symbols ($n = 4$).

5.3.6 *Aboveground Mixture Effect*

The differential effect of growing species in mixture compared to monoculture is shown in Figure 5.4. The species that benefited most from being grown in polyculture was *A. glutinosa* which consistently increased its facilitative mixture effect throughout the four year experiment by an average of 35% in ambient and 13% in elevated CO_2. Furthermore, the facilitative effect appeared to be increasing in magnitude throughout the experiment with a greater difference between treatments eventually attaining 64% and 35% stimulation under ambient and elevated CO_2 respectively. When *B. pendula* was grown in polyculture the facilitative mixture effect increased during the first two growing seasons to approximately 35% and appeared to stabilise. Elevated CO_2 response was initially reduced in comparison to ambient however, the treatment response declined with time to a final mixture effect of 40% in ambient and 38% in elevated CO_2. The third slower growing species *F. sylvatica* initially benefitted from being in polyculture. However, the mixture effect rapidly declined as the canopy developed suppressing this species compared to growth in monoculture. Following canopy closure the effect of being in mixture reduced growth by 35% in ambient conditions and 45% in elevated CO_2. Throughout the four year experiment elevated CO_2 had a suppressive trend effect on all species compared to ambient.

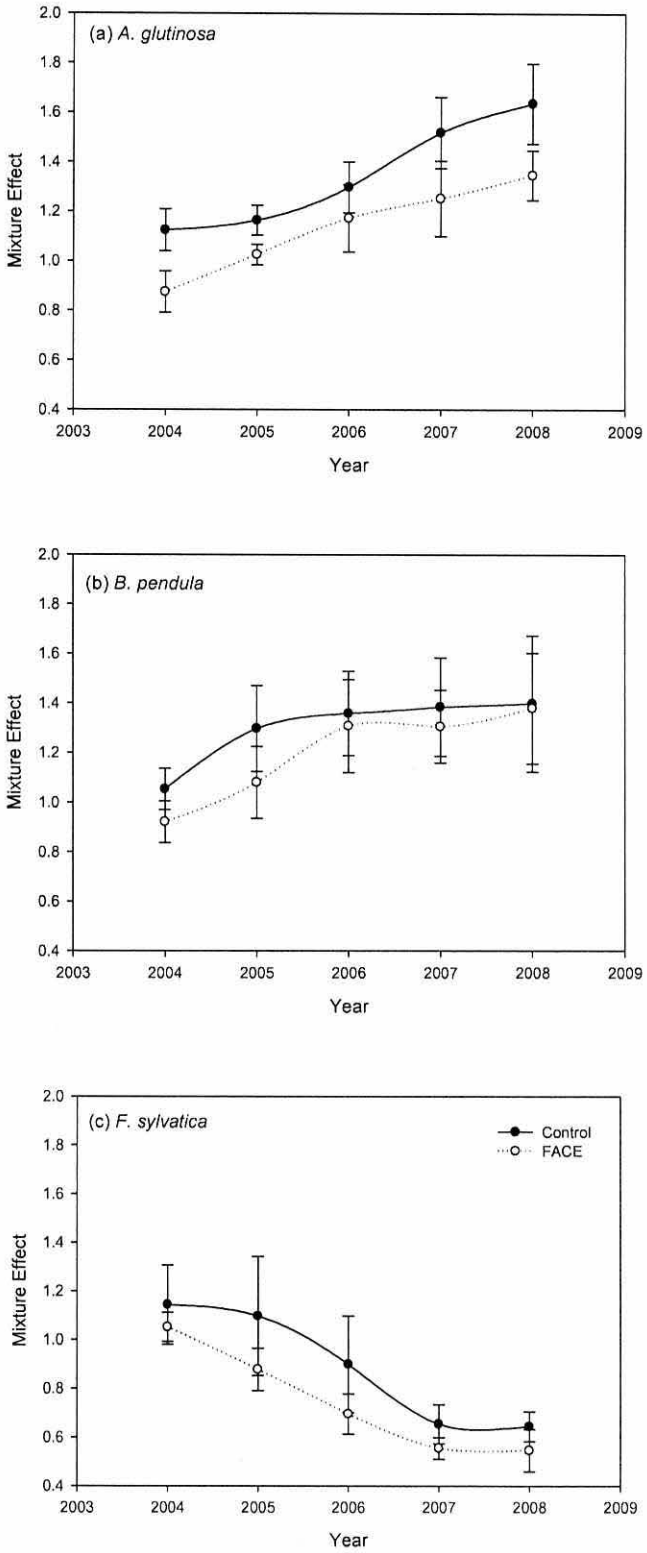

Figure 5.5 Mean ± SE mixture effect $\left(\frac{biomass\ in\ polyculture}{biomass\ in\ monoculture}\right)$ observed on aboveground biomass throughout the four years of atmospheric CO_2 enrichment at BangorFACE. Panels identify individual species (a) *A. glutinosa* (b) *B. pendula* (c) *F. sylvatica.* Data gathered from control plots are denoted by ● symbols whilst data from elevated CO_2 plots are denoted by O symbols ($n = 4$).

5.3.7 *Overall Mixture Effect on C*

The overall overyielding effect of elevated CO_2 on above and belowground C allocation and growth at BangorFACE is shown in Figure 5.6. The influence of growing species in polyculture had a greater effect on above ground biomass accumulation than elevated CO_2. Measured fine root biomass was significantly greater in the mixed species plots, in both ambient and elevated CO_2. However, elevated CO_2 mediated a significantly greater allocation of biomass belowground when species were grown in polyculture.

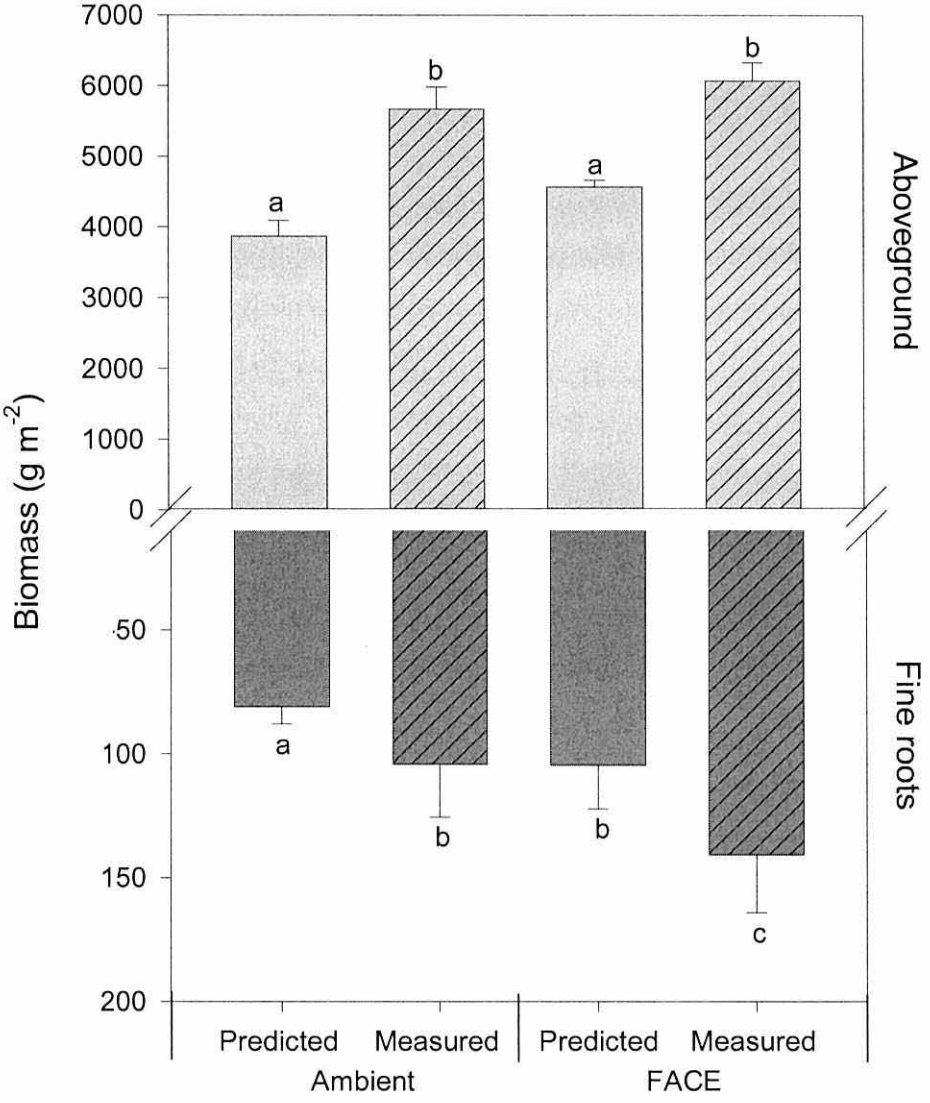

Figure 5.6 Mean ± SE measured and hypothetical aboveground biomass in the three species polyculture plots in ambient and elevated atmospheric CO_2 ($n=4$). Hatched bars ▨ are measured biomass and solid bars ■ hypothetical biomass calculated as (0.3×*A. glutinosa* + 0.3×*B. pendula* + 0.3×*F. sylvatica*) from data obtained when species were grown in monoculture. Significant ($P<0.001$) differences are indicated by letters.

5.4 Discussion

Species diverse forests are declining globally, whilst the extent of plantation forest created for the provision of timber products is increasing (FAO, 2009). Future atmospheric CO_2 levels have the potential to alter tree growth and influence forest community competition (Kozovits *et al.* 2005; Zak *et al.* 2007). Mixed species forests have been shown to have higher rates of biomass production and C sequestration than that of monoculture forest stands (Piotto, 2008; Pretzsch and Schütze, 2009). However, despite decades of research on the physiological effects of elevated CO_2 on plants, and the C sequestration potential of mixed species forests, little is known about how they will respond to future atmospheric CO_2 concentrations. This study has for the first time, definitively shown that overyielding of forests grown at higher diversity levels results in a greater allocation of C belowground when ground under elevated atmospheric CO_2. These finding suggest that maintaining forest species diversity is fundamental in maximising the C sequestration potential of forests. Furthermore, anthropogenic induced loss of forests biodiversity may exacerbate alterations in the global C balance.

5.4.1 Aboveground mixture response

The positive relationship between forest yield and productivity of monospecific and multispecies stands has been a matter of conjecture amongst silviculturists and forest managers for several centuries (Pretzsch, 2005). In the present study, overyielding of mixed species plots increased aboveground biomass by 47% in ambient, and 33% in elevated CO_2. Productivity increases of this magnitude are consistent with recent forest research, which has provided evidence that mixed species plantations can, on average, be improved by 10-20% over that of those grown in monoculture, and in extreme cases, may increase by up to 50% (Pretzsch, 2005). Furthermore, in a recent meta-analysis of 14 studies and 46 tree species, Piotto (2008) showed a 31% mixed species effect on bole diameter, that increased to 60% when non N_2 fixing species where grown in combination with N_2 fixing species. However, the benefit of mixed species forests has often yielded conflicting results in the literature with authors reporting no effect or even negative effects (Vila *et al.* 2003; Meinen *et al.* 2009). These conflicting results have been attributed to the selection of species with antagonistic ecological niche interactions, or site specific characteristics limiting

productivity (Piotto, 2008). Kelty, (1992) examined combinations of light demanding and shade tolerant species that consistently showed increased yield when grown together. In support of these studies, Pretzsch (2005) confirmed that by combining light demanding pioneer species, with shade tolerant late successional species, resource utilisation could be improved by almost 30%, in comparison to species grown in monoculture. The greater productivity demonstrated with these species combinations were explained through differential tree species canopy structure, increasing total light interception, and canopy light use efficiency. Guariguata *et al.* (1995) also found that mixed species stands exhibit greater light use efficiency because of the combination of different crown architectures.

Ecological theory suggests that mixtures may outyield monocultures when the examined species utilise contrasting ecological niches and resource use complementarities, which may manifest themselves in improved resource acquisition through (i) differences in height or canopy structure improving photosynthetic efficiency, (ii) differences in phenology (iii) differences in root structure allowing greater access to available resources. For example, the form of *A. glutinosa* grown in monoculture is typically comprised of straight bole and round crown, whereas when grown in admixture with species of differing ontogeny, light penetration significantly alters crown architecture and branching patterns to form a vertically structured canopy (Claessens *et al.* 2010). As plant C gain primarily depends on a plants ability to intercept light for photosynthesis, a structured canopy comprised of species with contrasting ecological niches should result in a larger leaf area with greater potential for C assimilation. Conformant with this theory we observed a greater measured leaf area index that was between 2.2 (elevated CO_2) and 2.6 times (ambient CO_2) higher in mixed species plots than that predicted from monoculture. In contrast to our results, Norby *et al.* (2003) suggested that elevated CO_2 would lead to a lower photosynthetic light compensation point enabling a positive leaf C balance deeper in the canopy, thereby sustaining a higher LAI. Although early CO_2 experiments failed to observe a significant increase in LAI, more recently a 14% increase in LAI has been observed in both coniferous and broadleaved stands (McCarthy *et al.* 2007; Taylor *et al.* 2008). Differences in observed LAI may be attributed to the quantity and quality of light penetrating the canopy, which is governed by species specific leaf characteristics such as pigmentation and morphology affecting light absorption and transmission spectra (Lintunen and Kaitaniemi, 2010). Foliage in the lower crown levels of a monoculture

or shade-adapted species survives at a very low net photosynthetic rate, at or near the light compensation point, whereas early successional pioneer species typically achieve greater photosynthetic and respiration rates than late successional species. Although it is possible that elevated CO_2 may have decreased the quality or quantity of light throughout the canopy increasing the cost of leaf maintenance. We suggest that the observed 14% reduction in aboveground overyielding under elevated CO_2 relative to the ambient CO_2 is attributable to (i) canopy stratification altering interspecies competition for light, and (ii) a switch in C allocation to belowground organs such as roots and mycorrhizal symbionts. In the present study we observed that in monoculture, the contribution of *A. glutinosa* and *B. pendula* to LAI was 60:40 respectively in both ambient and elevated CO_2. Whereas, when *A. glutinosa* and *B. pendula* were grown in polyculture, their contribution to LAI was 60:40 in ambient and 50:50 in elevated CO_2. Competitive advantage is possibly determined by *B. pendula* observed delayed autumnal senescence (discussed in Chapter 10) allowing greater annual C assimilation. The exact processes that affect species competition are unknown (Lintunen and Kaitaniemi, 2010). However, several mechanisms, predominantly competition for light drive species interactions aboveground, and are known to influence crown architecture (Cavard *et al.* 2010). A shift towards *B. pendula* may increase the overall productivity of stand canopy as a greater amount of light is permitted to enter the canopy. Nevertheless, in this instance aboveground overyielding was reduced in elevated compared to ambient CO_2 suggesting above ground biomass facilitation in mixtures cannot be maintained at the same rate as in ambient conditions without increased allocation belowground to forage for nutrients. Indeed, in recent work with *B. pendula* canopy light inception was correlated with enhanced root production (Matjaž and Primož, 2010) supporting our observation of greater belowground productivity with a shift in canopy dominance towards *B. pendula*.

5.4.2 Belowground mixture response

Differences in fine root production between single and mixed species is poorly understood. Recent studies have started to provide insight into the mechanisms leading to increased production in polyculture. For example, Brassard *et al.* (2010) showed that five species grown in monoculture and admixture across two sites in

Canada produced significant increases in fine root productivity when grown in mixture. Similarly, Wang *et al.* (2002) showed that *Tsuga heterophylla* in mixture with *Thuja plicata* almost doubled root biomass compared to when species were grown in monoculture. Conversely, Meinen *et al.* (2009) examined below ground productivity in deciduous mixed species forests plots of increasing species diversity under ambient conditions, and found no evidence of belowground overyielding in response to species richness. Consensus within the literature indicates that the magnitude of productivity responses to species growth in mixtures is dependant upon contrasting root traits, and the availability of space and nutrients within the soil profile (Brassard *et al.* 2010).

To our knowledge, there are no published studies describing the overyielding effect on fine root productivity when species are grown in polyculture and under the influence of elevated CO_2. In the present study, we observed a fine root overyielding response of 28% in ambient conditions, which was further increased to 34% under elevated CO_2. The only FACE experiment to investigate mixed species, field afforested broadleaved forest communities, was conducted at the AspenFACE facility, Rhinelander, Wisconsin. In agreement with most elevated CO_2 studies, fine root biomass was found to be 45% larger in monoculture under elevated CO_2, whereas root biomass was increased by 64% and 29% in aspen-birch, and aspen-maple mixed species communities under elevated CO_2, when averaged across six years (King *et al.* 2005). The overyielding effect was not calculated by King *et al.* (2005), however, it is apparent that growing species in mixture had both a positive and negative effect on root biomass. Elevated CO_2 has generally been shown to have a positive effect on root growth. Tingey *et al.* (2000) found a median 54% increase in the root biomass of woody plants when grown in a CO_2 enriched atmosphere of 700 $\mu mol\ mol^{-1}$ CO_2. Furthermore, Iverson *et al.* (2010) showed that the positive effect of elevated CO_2 increases with depth (also see Chapter 4 for similar results found at BangorFACE). However, trees enriched with CO_2 at the alpine treeline showed no root growth response (Handa, 2008).

Increased root growth observed when trees are grown in an elevated CO_2 atmosphere has been attributed to two mechanisms (i) increased C allocation belowground to maintain plant source-sink relationships, and provide the carbohydrate sink required to maintain enhanced rates of photosynthesis (Arp, 1991; Stitt, 1991) and, (ii) greater demand for nutrients, predominantly N, required to

maintain increases in net primary production (Luo *et al.* 2004). Nitrogen is often the most limiting plant nutrient and its availability is believed to be one of the major drivers of persistent fine root response to elevated CO_2 in forest ecosystems (Johnson, 2006). P acquisition is also known to result increase root production and mycorrhization. At BangorFACE the plant available P pool in the ambient plots was 153 $\pm$13 mg P kg^{-1} dwt soil and 83 $\pm$5 mg P kg^{-1} dwt in the elevated plots a 44% decrease (P<0.001) indicating a strong demand for P in elevated CO_2 plots (Chapter 8). In a recent review, Richards *et al.* (2010) found that N and P use efficiencies were significantly altered in 65% of studies examining trees grown in mono and polyculture. These shifts were attributed to changes in photosynthetic capacity, as a function of light, and subsequent changes in C allocation to roots and foliage.

Ostonen *et al.* (2007) postulated two strategies for the adaptation of tree fine roots to nutrient supply in a competitive environment, firstly an enhancement of root length and biomass through greater below ground allocation, and secondly morphological changes to enhance SRA and increase nutrient uptake efficiency. In both cases, these mechanisms would lead to greater C allocation below ground. Furthermore, Coomes and Grubb (2000) suggested that root systems may respond to competing species by utilising morphological plasticity, altering spatial distribution and exploiting niche complementarities leading to greater resource use efficiency. Spatial compartmentalisation may also reduce inter and intra-specific root competition and lead to complementary use of water and nutrients in soil. On the other hand, if species are selected with similar ecological niches competition may result in a negative effect (Bolte and Villanueva, 2006).

Meinen *et al.* (2009) described how mixed species forests comprised of early and late successional species may benefit from different rooting patterns inherent in their survivorship strategy, potentially leading to increased productivity. The use of *F. sylvatica* (late successional) in admixture with two early successional species (*A. glutinosa* and *B. pendula*) in the present study, potentially exploits contrasting ecological niches. The survivorship strategy of late successional species such as beech is to produce more roots throughout the soil strata enabling rapid exploitation of canopy gaps when they occur. Indeed, several authors have reported a vertical stratification of beech roots in admixes with other species allowing greater access to resources (Rust and Savill, 2000; Leuschner *et al.* 2001). For example, Bolte and

Villanueva (2006) showed how beech admixed with spruce tended to display a foraging strategy to increase soil exploration of deeper soils to exploit unoccupied space. Congruent with these observations Schmid and Kazda (2002) found different vertical fine root distributions in mono-specific and mixed spruce stands, which was attributed to avoidance of competition and differential vertical stratification of root systems of species rich stands.

The species used in this study utilise complementary mechanisms for the acquisition of N. *A. glutinosa* is capable of fixing 48-185 kg N ha^{-1} yr^{-1} (Daniere *et al.* 1986). Admixing *A. glutinosa* with non N fixing species creates potential for reduced N competition and facilitation as N fixing species acquire their N from the atmosphere, and contribute to soil N availability through the turnover and bioperturbation of roots and litter fall (Jose *et al.* 2006; discussed in Chapter 11; Millet *et al. submitted*).

5.4.3 C Sequestration Potential

In comparison to other tree biomass components, fine roots have a relatively short lifespan, and fast turnover rate (Pregitzer, 2003). Therefore, the inherent location of roots within the soil profile, and their ephemeral existence increases the probability that decomposition and bioturbation will result in translocation of root derived C to the soil organic carbon (SOC) pool (Rasse *et al.* 2005). Consequently, any increase in belowground biomass may increase the sequestration potential of forest ecosystems (Iverson *et al.* 2008; Gale *et al.* 2000). This is in agreement with a modelling study of forest inventory data conducted by Caspersen and Pacala (2001), who reported that stands with high successional diversity were correlated with greater productivity, and consequently have greater potential to sequester C than stands of low diversity. Experimental data from BangorFACE also indicates that when species of contrasting successional characteristics are selected, belowground C allocation is enhanced through the overyielding of fine roots. However, belowground allocation is then further enhanced through growth interactions with elevated CO_2. If our goal is to sequester C by afforestation then approximately an extra 0.3t CO_2 equivalents ha^{-1} yr^{-1} may be sequestered by increasing species richness and selecting species with contrasting root morphology. In the context of Common Agricultural Policy this would be equivalent to converting croplands to grassland (Dawson and Smith, 2007)

5.5 Conclusion

We have, for the first time, shown that species diversity positively influences belowground forest productivity at the cost of aboveground production when tree species of contrasting ecological characteristics are grown together under an elevated CO_2 atmosphere. In agreement with our first hypothesis, we showed that above ground biomass of tree species with contrasting ecological and successional characteristics was greater when grown in polyculture, indicating a positive interactive effect of species richness on productivity. Surprisingly, in trees grown under elevated atmospheric CO_2 above ground overyielding was reduced compared to ambient CO_2. Supporting our second hypothesis, fine root biomass was positively affected by species richness in both ambient and elevated CO_2 plots. However, the magnitude of the response was greater in response to elevated CO_2 enrichment. Data from BangorFACE clearly demonstrates increases in tree productivity when species are grown in polyculture, which in combination with, a shift in C allocation from above to below-ground biomass pools under elevated atmospheric CO_2 has major implications for the global C balance.

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6 Elevated CO_2 Induces Temporal Limitation of Low Molecular Weight Substrate Mineralisation in Temperate Forest.

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Contributions:

Andrew Smith wrote the manuscript, collected data, performed analysis and maintained the FACE facility during 2006-2009. Dr. Martin Lukac designed, built and maintained the experiment during 2004-2006. Prof. Davey Jones and Dr. Paul Hill provided ^{14}C isotopes and guidance on experimental design. Prof. Douglas Godbold is the project principle investigator who designed and constructed the experiment. He also provided assistance with data interpretation, analysis and manuscript preparation.

6.1 Introduction

During the last 250 years atmospheric [CO_2] has increased by 36%, and continues to rise at a rate of 1.4 ppm yr^{-1} (IPCC, 2007). Such a dramatic shift in atmospheric [CO_2] is likely to induce dramatic changes in global biogeochemical cycling of C (Zak *et al.* 2000). Almost half of the C in terrestrial ecosystems is stored within forests and their soils (Schlesing and Lichter, 2001). Therefore, temperate forests are a vast potential C sink responsible for the largest flux of C belowground of all ecosystems (Millard *et al.* 2007). There remains much uncertainty regarding belowground C fluxes, turnover and the potential for long term C sequestration of forests exposed to a changing climate (Hoosbeek *et al.* 2006). Determining microbially driven C processing rates remains critical to understanding ecosystem C fluxes.

Studies investigating the effects of rising atmospheric CO_2 concentration on terrestrial ecosystems during the past few decades have tended to focus on above ground physiological responses and interactions (Saxe *et al.* 1998; Curtis and Wang, 1998; Norby *et al.* 1999). Forest ecosystem responses to elevated atmospheric CO_2 include: a median 23% stimulation of net primary production across a broad gradient of productivity (Norby *et al.* 2005), increases in above ground biomass (Calfapietra *et al.*2003), leaf area index (Liberloo *et al.* 2005), altered plant chemistry such as higher C:N ratio, sugars, carbohydrates and C rich secondary compounds (Ainsworth and Long, 2005). Changes in C allocation have been shown to result in increases root production and turnover (Lukac *et al.* 2003). Roots grown under elevated CO_2 have also been shown to explore deeper to maintain the nutrient demand of enhanced productivity (Rogers *et al.* 1994; Iverson, 2010). In addition to allocating more C to root biomass, plants roots also exude a wide array of C rich compounds such as amino acids, carbohydrates, fatty acids, organic acids and enzymes (van Hees *et al.* 2005). This rhizodeposition has been shown to be enhanced under elevated atmospheric CO_2 and is likely to impact on microbially influenced C dynamics (Grayston *et al.* 1997; van Veen *et al.* 1991; Cheng, 1999; Phillips *et al.* 2006).

The soil microbial community is an important SOC pool that regulates key below ground C and nutrient processes which is pivotal in the response of ecosystems to global

environmental change (Austin *et al.* 2009; Nguyen and Henry, 2002). However, elevated CO_2 mediated responses of soil microbial community biomass has been highly variable. For example, studies have demonstrated increases (Grayston *et al.* 1998), decreases (Schortemeyer *et al.* 1996) and no net change (Randlett *et al.* 1996). Microbial activity and growth in soil is most often limited by the availability of C (Wardle, 1992). Subsequently, an increased flux of labile C as rhizodeposition is likely to significantly impact upon C and nutrient cycling of the microbial community through changes in both structure and function.

An increased flux of labile compounds to soil may retard or increased the rate of soil organic matter (SOM) mineralisation through microbial competition (Fontaine *et al.* 2003) or co-metabolism of old and new C by microbial growth and increased enzyme production (Kuzyakov *et al.* 2002). Enhanced decomposition of soil organic C (SOC) by the microbial community following deposition of recently assimilated organic C, termed the *priming effect* (Bingeman *et al.* 1953), has been hypothesised to be responsible for these observations (Kuzyakov *et al.* 2002; Fontaine *et al.* 2003).

The primary aims of this study were to determine: (a) if elevated atmospheric CO_2 impacts on LMW C kinetics, turnover and pool partitioning, (b) if increases in labile C flux after bud burst increases turnover of LMW C under elevated atmospheric CO_2 and, (c) to determine if turnover and pool partitioning are affected by species composition under elevated CO_2.

6.2 Materials and Methods

6.2.1 Site description and experimental design.

The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2 ° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Soils are fine loamy brown earth over gravel (Rheidol series) and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002), and as a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011). Soil texture is 63% sand, 28% silt

and 9% clay. Climate at the site is classified as Hyperoceanic with a mean annual temperature during 2008 of 13.8 °C and an annual rainfall of 1077 mm.

Bangor University established a Free Air Carbon Enrichment experiment in March 2004 at Henfaes Research Station, Abergwyngregyn (53°14'N, 4°01'W). The experiment covered an area of 2.36 ha and was located on two fields, a former agricultural field, and former agricultural pasture which had been used for small scale forestry experiments for the previous eight years. The experiments comprised of eight octagonal plots 8 m in diameter, four ambient and four CO_2 enriched, in a 2 × 4 factorial block design. We used three tree species (*Alnus glutinosa* [L.] Gaertner, *Betula pendula* Roth. and *Fagus sylvatica* L.), which were selected due to their contrasting shade tolerance, successional chronology and to represent a range of taxonomic, physiological and ecological types. Each plot was divided into seven planting compartments and planted in a pattern creating areas of one, two and three species. However, for simplicity this experiment utilised three single species subplots of *B. pendula*, *A. glutinosa* and *F. sylvatica*, in addition to a fourth subplot which contained a mixture of all three species. The experimental plots were surrounded by a 10 m buffer of the same species planted at the same density. The remaining field was planted with a mixture of species at 10000 stems ha^{-1}. The four FACE plots were enriched to 580 ppm using pure CO_2 depleted in ^{13}C (-39‰ PDB). CO_2 was supplied during the photosynthetically active part of the day through laser drilled holes in horizontal pipes suspended from eight masts and positioned mid canopy (Miglietta *et al.* 2001). Elevated [CO_2] was measured at one minute intervals and was within 30% of the target concentration for 75-79% of the time during 2005-2008.

6.2.2 Soil Sampling

Soil samples were collected with a 2 cm diameter auger corer during April 2008, immediately prior to bud burst, and late June 2008. Samples were obtained in 10 cm increments to a depth of 30 cm within each of three single species subplots (*A. glutinosa*, *B. pendula*, *F. sylvatica*) and a three species mixture subplot from the elevated and ambient experimental plots (n=4) providing 96 soil samples for April and June (192 total). Soil was collected directly into polythene bags and stored at 5 °C in the field. On

return to the laboratory soil samples were homogenised and sieved to pass 2 mm prior to analysis.

6.2.3 Soil Chemical and Biological Analysis

Microbial biomass was measured following the $CHCl_3$ fumigation-extraction method of Vance *et al.* (1987). Nitrate was determined colorimetrically by the Cu-Zn-hydrazine reduction method of Downes (1978) and ammonium by the salicylate-hypochlorite method of Mulvaney (1996). Soil pH was measured in water according to Smith and Doran (1996). Potassium (K), Calcium (Ca) and Sodium (Na) were extracted using ammonium acetate pH 7.0 (Lavkulich, 1981) and determined by Atomic Emission Spectroscopy (Sherwood Flame Photometer 410). Plant available P was extracted in 0.5 M sodium bicarbonate ($NaHCO_3$) pH 8.5 (Olsen *et al.* 1954) and determined colourimetrically as molybdate-reactive P (Murphy and Riley, 1962). Soil dissolved organic carbon (DOC) and total dissolved N (TDN) was determined using a Shimadzu TOC-V-TN analyzer (Shimadzu Corp., Kyoto, Japan) following extraction (1:5 w/v) in 0.5 M K_2SO_4 (Jones and Willett, 2006). Soil chemical and physical properties are shown in Table 6.1, while K_2SO_4 extractable organic C, N and inorganic N are shown in Table 6.2.

6.2.4 ^{14}C Substrate Induced Respiration

Soil solution was collected from three depths throughout the soil profile (0-10, 10-20 and 20-30 cm) from soil sampled using a 2 cm auger corer. Soil solution was extracted from the collected samples according to Giesler and Lundström (1993). Briefly, soil samples were centrifuged (4000*g*, 15 min, 20 °C) to obtain soil solution, and the collected solutions passed through Whatman 42 filter paper before freezing at -20°C. An aliquot of the extracted soil was then enhanced with six ^{14}C labelled substrates. These included 50 mM glucose, 5 mM fructose, 5 mM sucrose, 10 mM citrate, 5 mM malate and 2 mM succinate (total C concentration 120 μmol ml^{-1}, 4 kBq ml^{-1}). Substrate concentrations were chosen to represent the typical composition of root sap that would enter the soil upon root cell lysis occurring during cell sloughing, mucilage production and root turnover (Jones *et al.* 2003).

Table 6.1 Mean ± SE soil physical and chemical properties for the experimental site. Two fields used in the experiment, two ambient and two elevated plots are located in each field. Significance is denoted by ns (not significant), $^{*}P<0.1$, $^{**}P<0.05$, $^{***}P<0.01$

Treatment	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Na (mg kg^{-1})	pH (H_2O)	EC (µS cm^{-1})	Moisture (%)	Organic Matter (%)	%C	%N
Ambient CO_2	153.2 ± 13.0	68.5 ± 4.4	741.0 ± 50.9	10.9 ± 0.4	4.9 ± 0.1	46.3 ± 2.3	22.0 ± 0.4	6.0 ± 0.1	2.8 ± 0.1	0.3 ± 0.0
Elevated CO_2	82.6 ± 5.0	64.3 ± 3.9	695.9 ± 23.2	14.1 ± 0.7	4.7 ± 0.0	34.8 ± 1.2	21.4 ± 0.3	5.3± 0.1	2.3 ± 0.1	0.3 ± 0.0
Significance	***	ns	ns	**	ns	**	ns	**	ns	ns

Table 6.2 Mean ± SE K_2SO_4 extractable carbon, total dissolved, organic and inorganic nitrogen at the two sampling times. Significance is denoted by ns (not significant), $^{*}P<0.1$, $^{**}P<0.05$, $^{***}P<0.01$

Treatment	April					June				
	DOC (mg C l^{-1})	DON (mg N l^{-1})	TDN (mg N l^{-1})	NO_3^- (mg N l^{-1})	NH_4^+ (mg N l^{-1})	DOC (mg C l^{-1})	DON (mg N l^{-1})	TDN (mg N l^{-1})	NO_3^- (mg N l^{-1})	NH_4^+ (mg N l^{-1})
Ambient CO_2	136.7 ± 3.2	18.7 ± 2.2	27.2 ± 2.7	3.3 ± 0.2	5.2 ± 0.5	118.0 ± 6.5	15.6 ± 1.3	28.3 ± 1.6	10.1 ± 0.1	2.6 ± 0.1
Elevated CO_2	118.0 ± 5.6	23.3 ± 0.6	30.5 ± 1.0	3.5 ± 0.2	3.7 ± 0.2	105.3 ± 2.7	12.2 ± 0.7	24.2 ± 1.0	10.3 ± 0.1	1.8 ± 0.1
Significance	ns	ns	ns	ns	ns	ns	ns	ns	ns	***

To trace the fate of the exudate cocktail of sugars and organic acids, 500 µl (2 kBq) of the ^{14}C labelled substrates was evenly applied to the soil surface of a 50 cm^3 polypropylene vessel containing 5 g of 2 mm sieved soil. Following addition of the substrate a 1 M NaOH trap (1 ml) was placed inside the tube to trap evolved $^{14}CO_2$ and the tube hermetically sealed at the top. The NaOH trap was suspended above the soil to allow free air passage from the soil surface and replaced following 1, 3, 6, 9, 24, 48, 120, 192, 360, 864, 2160 hours of incubation at room temperature (21 °C). Following the removal of each NaOH trap $^{14}CO_2$ absorbed was determined by liquid scintillation counting using a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK) with automatic quench correction and Optiphase 3® alkali compatible scintillation fluid (Wallac EG&G). Immediately after the final incubation sampling time, soils were extracted with 0.5 M K_2SO_4 to quantify remaining unabsorbed ^{14}C. Briefly, 25 ml of 0.5 M K_2SO_4 was added to 5 g soil and shaken at 200 revs min^{-1} for 30 minutes, a 2 ml aliquot of the supernatant was subsequently transferred to a 2 ml eppendorf tube and centrifuged at 15000 rev min^{-1} for 10 minutes, and 1 ml of supernatant transferred to a scintillation vessel for ^{14}C determination as above.

The kinetics of microbial $^{14}CO_2$ evolution can be described by fitting first order exponential decay models to experimental data. Kinetics have been described by many authors using exponential decay models with varying numbers of terms usually relating to the length of experiment and complexity of substrates utilised (Kuzyakov and Demin, 1998; Nguyen and Guckert, 2001; Boddy *et al.* 2007; Boddy *et al.* 2008). However, as proposed by Kuzyakov and Demin (1998), we found that a triple term first order decay model best fitted our data and related to biological and physiochemical processes (Equation 1). Using this model f describes the amount of ^{14}C-labelled substrate or metabolite remaining in the soil at time t, and the exponential coefficient k_1, describes the initial rapid uptake and respiration of ^{14}C labelled substrate by the microbial community immediately following application. k_2 describes a slower secondary mineralisation phase which we ascribe to immobilisation in microbial biomass and transformation of organic metabolites (microbial turnover), and k_3 the mineralisation of ^{14}C labelled material sorbed to unavailable soil complexes or metabolites partitioned into less available recalcitrant

material. The terms a_1, a_2 and a_3 relate to the proportion of ^{14}C labelled substrate that is associated with each exponential coefficient at time (t).

$$f = a_1 e^{-k_1 t} + a_2 e^{-k_2 t} + a_3 e^{-k_3 t} \qquad \text{(Eqn. 1)}$$

The mean residence time or substrate half life ($t_{1/2}$) can be calculated according to Equation 2.

$$t_{1/2} = \frac{\ln(2)}{k_n} \qquad \text{(Eqn. 2)}$$

Turnover of each pool can then be calculated as the inverse of the mean residence time (1/MRT). In support of the description of three C pools, separated by distinct biogeochemical processes, and described by a triple exponential decay model. Correlations between microbial biomass and pool turnover becomes weaker as C moves through each pool (Figure 6.1; a_1 r^2=0.32 P<0.001; a_2 r^2=0.28, P<0.001; a_3 r^2=0.10 P<0.05), which suggests that reliance on the microbial community is reduced as C is immobilised in the biomass (a_2) and sorbed to the solid phase (a_3).

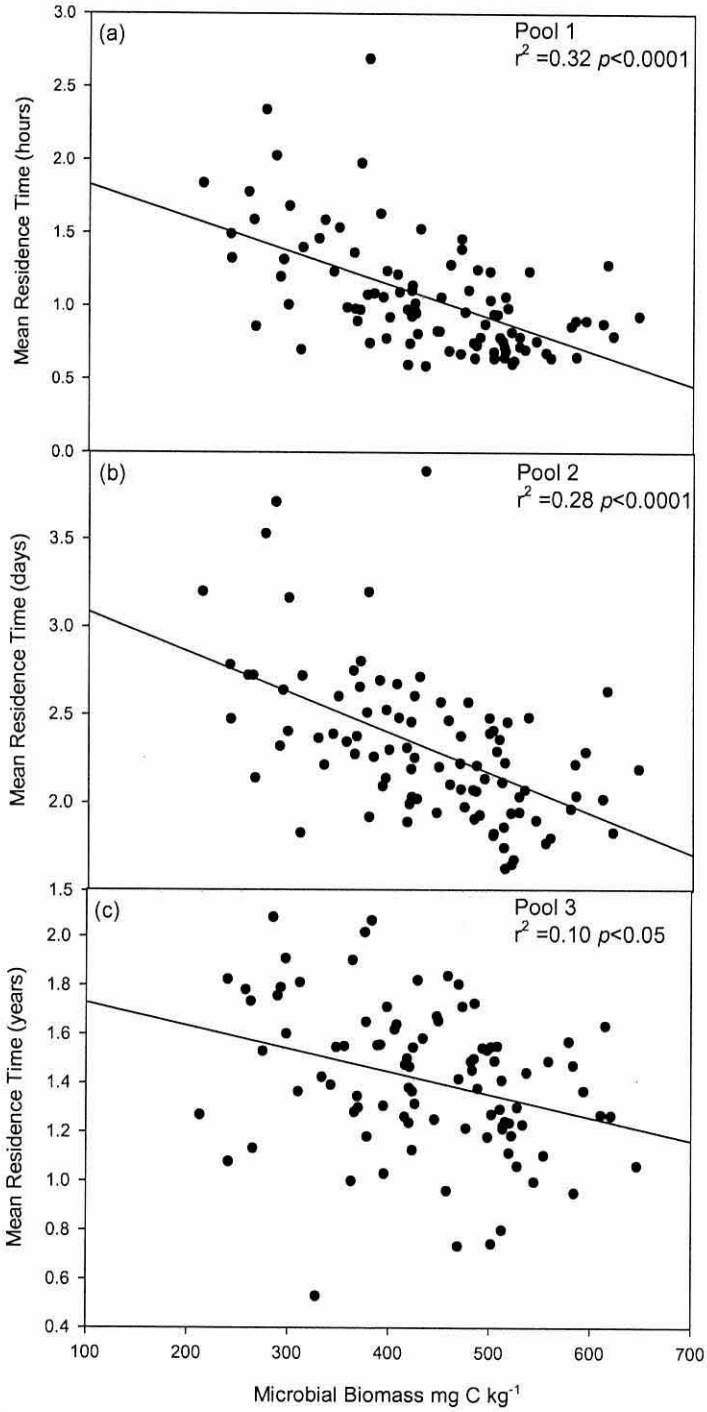

Figure 6.1 The relationship between mean residence time ($\ln(2)/k_n$) and microbial biomass for all three pools described in Equation 1. The decreasing relationship between residence time and biomass supports the biological description of carbon movement through pools.

6.2.5 Statistical Analysis

The exponential decay regression was conducted using SigmaPlot v11.0 (Systat Software Inc, Chicago, IL.) and statistical procedures were undertaken with SPSS 17.0 (SPSS Inc., Chicago, IL) with $P<0.05$ used as the limit for statistical significance. Data were subjected to repeated measures factorial ANOVA, and Multivariate General Linear Model. Half lives, rate constants and pool sizes where used as dependants with treatment, species and depth as factors. Data were log transformed and tested for normality using Shapiro-Wilk's test. Homogeneity of variance was determined using Levene's test.

6.3 Results

6.3.1 Mineralisation kinetics of a simulated root exudate.

Following the application of a simulated ^{14}C labelled root exudate cocktail to soil evolution of $^{14}CO_2$ over the 13 weeks experiment best fitted a triple term first order exponential decay regression model ($r^2=0.99 \pm 0.02\times10^{-3}$; Equation 1; Figure 6.2). The rate constants and pool sizes are shown in Table 6.3.

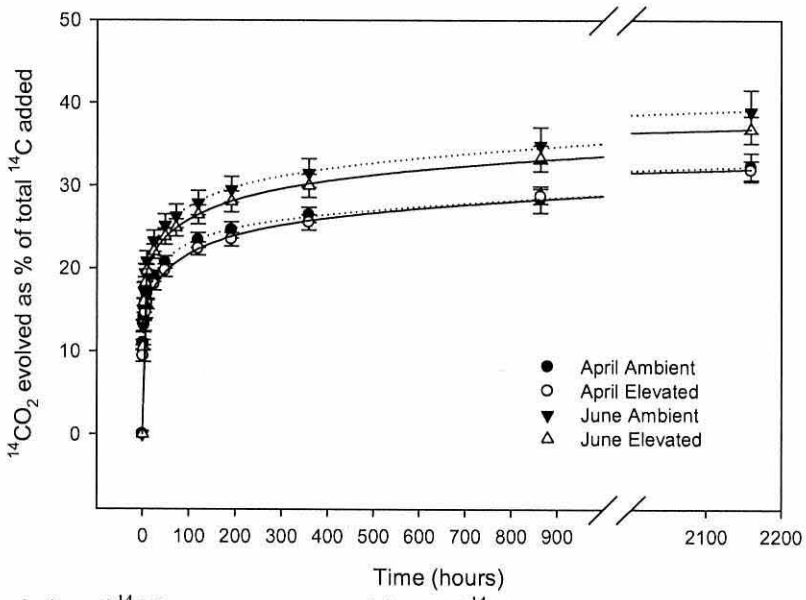

Figure 6.2 Evolution of $^{14}CO_2$ as a percentage of the total ^{14}C added to soil as a simulated root exudate sugar cocktail. Soil was collected at a depth of 0-10 cm under *Betula pendula* exposed to elevated and ambient atmospheric CO_2 treatments before (April) and after (June) bud burst. Values represent mean ± SE, (n=4).

6.3.2 Seasonal effects of ^{14}C labelled C partitioning.

The mean exudate cocktail C partitioning across treatment, species and depth was 13.7 ± 0.2 % to pool a_1, 10.1 ± 0.5% to pool a_2, and 76.2 ± 0.6% into pool a_3. There were no significant differences between treatments or sampling month. However, soil depth significantly altered allocation in both April and June. During April *B. pendula* significantly allocated 1.6% more C to pool a_2 at 30 cm under elevated CO_2 whilst conversely *F. sylvatica* significantly allocated 1.5% less C to pool a_2 at 30 cm during June. No other species significantly altered C partitioning during either month in response to treatment (Table 6.2). Between the two months there was a significant reduction in the allocation to the fast turnover pool in elevated CO_2 at 20 cm (Figure 6.3) but no significant changes with species composition.

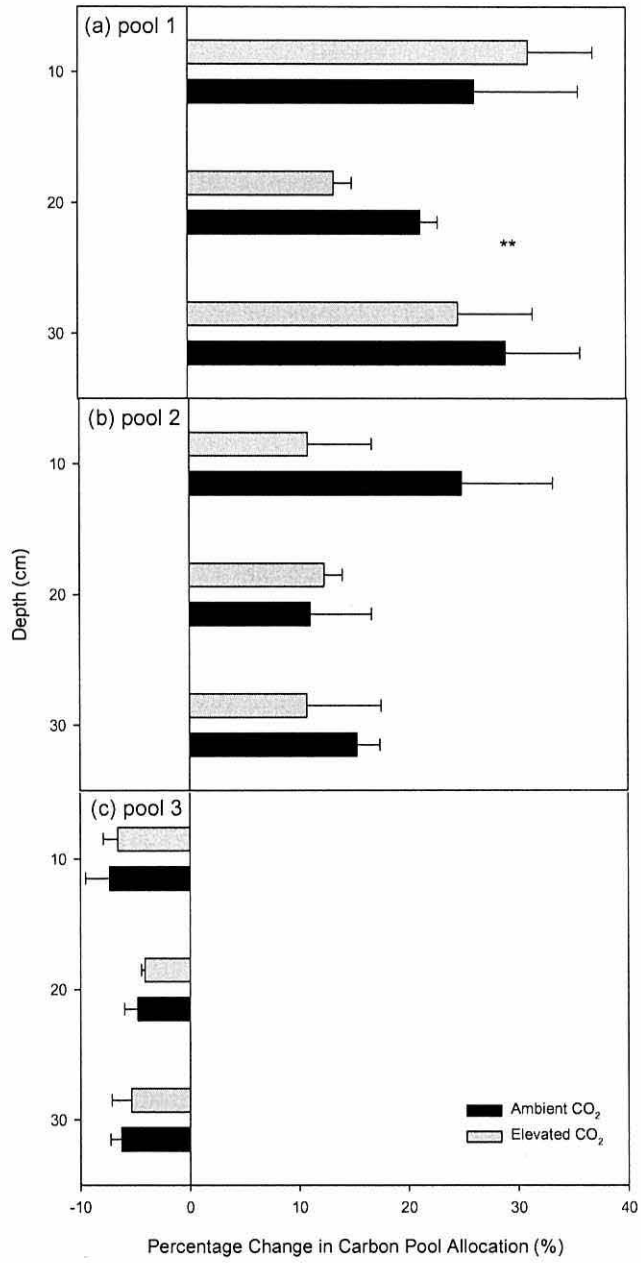

Figure 6.3 Percentage change in carbon partitioning by depth between the April and June sampling, determined by fitting a triple term first order exponential decay model where panels a) 1st pool a_1 attributable to LMW carbon in soil solution, b) 2nd pool a_2 attributable to C held within the microbial biomass c) 3rd pool a_3 attributable to C partitioned into unavailable recalcitrant soil complexes or recalcitrant metabolites. Values represent means ± SEM (n=6) Significance is denoted by $^{*}P<0.1$, $^{**}P<0.05$.

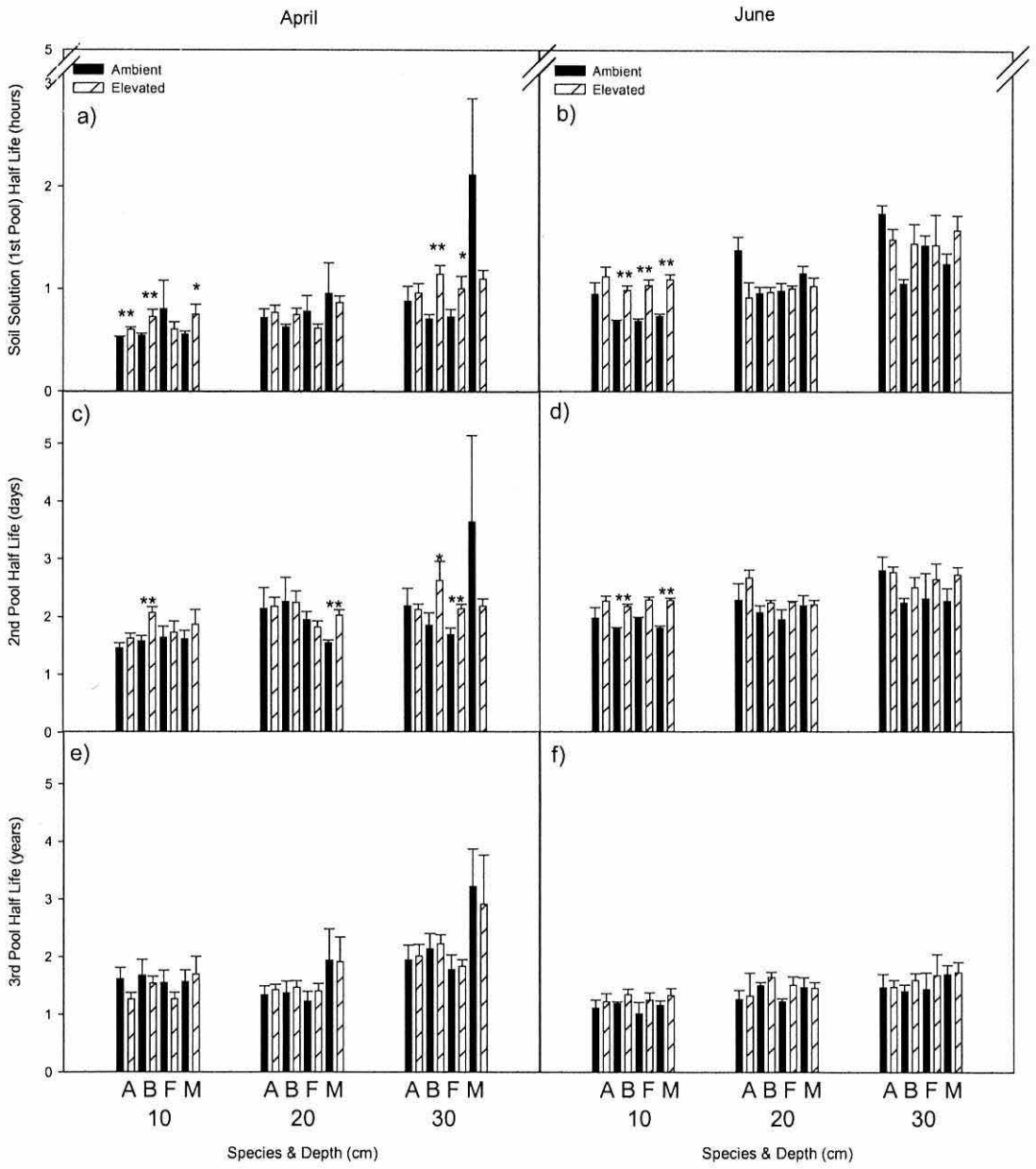

Figure 6.4 Exudate cocktail mean residence time (half life) factored by species and depth. Species are denoted by abbreviations as A, *A. glutinosa*; B, *B. pendula*; F, *F. sylvatica*; M, three species mixture. The left hand panel shows half lives during April while the right hand panel shows half lives for June. Elevated CO_2 are denoted by white bars with diagonal lines while ambient CO_2 are denoted by filled bars. Values depicted are means ± SEM (n=4) Significance is denoted by $^{*}P<0.1$, $^{**}P<0.05$.

6.3.3 Substrate turnover as altered by species and depth

Figure 6.4 shows that during April, before bud burst, and within the top 0-10 cm of the soil profile the soil solution half life calculated from the first phase rate coefficient (k_1) (Equation 6.2) was significantly 13.6% slower under *A. glutinosa* (0.603 ± 0.003 h; $P<0.05$) and 25.2% slower under *B. pendula* (0.728 ± 0.028 h; $P<0.05$) relative to the ambient CO_2 control plots (Figure 6.4). The change in primary phase half life increased with soil depth under *B. pendula* (20-30 cm; 1.145 ± 0.033 h; $P<0.05$) and *F. sylvatica,* whilst decreasing under *A. glutinosa* and the species mixture (Table 6.4). There were no statistically significant effects on the half life of ^{14}C substrates within 10-20 cm of the soil profile, and no treatment × species interactions ($P=0.092$) were observed. However, prior to bud burst the turnover of ^{14}C labelled substrates for each species within the elevated CO_2 plots followed the order *A. glutinosa* (0.603 ± 0.003 h) < *F. sylvatica* (0.607 ± 0.026 h) < *B. pendula* (0.728 ± 0.028 h) < *mixture* (0.752 ± 0.050 h). Following bud burst (June) primary phase half lives of the ^{14}C labelled substrates significantly decreased within the elevated CO_2 plots (Figure 6.4; Table 6.5) and the species rate of cycling switched to the following order *B. pendula* (0.928 ± 0.026 h) < *F. sylvatica* (0.970 ± 0.027 h) < *mixture* (1.024 ± 0.030 h) < *A. glutinosa* (1.051 ± 0.011 h). The magnitude of the primary phase half life change for *B. pendula, F. sylvatica* and the species mixture was approximately double that of *A. glutinosa* within 0-10 cm of the soil profile (Table 6.4).

Repeated measures multivariate analysis showed that month (season) was the factor that explained the greatest amount of variance for both the primary and tertiary phase half life. There was a significant interaction between month × treatment during the secondary phase of substrate mineralisation, and a month × species × treatment interaction in half life was significant during both the primary and secondary mineralisation phase. A significant month × species interaction was observed for the mean residence time of all coefficients. However, month × depth explained a greater variance in the third coefficient of the fitted model (Table 6.6).

6.3.4 Extractable C, Nitrogen and Microbial Biomass

Table 6.3 Microbial C and N determined by the fumigation-extraction method of Vance *et al.* (1985). Values are mean ± SE (n=4). Significance is denoted by ns (not significant), $^{*}P<0.1$, $^{**}P<0.05$, $^{***}P<0.01$

Treatment	April			June		
	Microbial C (mg C kg^{-1})	Microbial N (mg N kg^{-1})	Microbial C/N	Microbial C (mg C kg^{-1})	Microbial N (mg N kg^{-1})	Microbial C/N
Ambient CO_2	484.6 ± 32.3	31.8 ± 0.8	21.3 ± 2.2	447.3 ± 24.5	39.0 ± 3.2	12.2 ± 1.1
Elevated CO_2	460.3 ± 44.9	25.5 ± 5.7	23.5 ± 4.1	421.5 ± 21.2	38.0 ± 3.8	11.5 ± 0.4
Significance	ns	ns	ns	ns	ns	ns

Table 6.4 First-order kinetic parameters describing the mineralization of a simulated ^{14}C-labeled root exudate cocktail for soils collected before (April) and after (June) bud burst under three species in monoculture (*A. glutinosa, B. pendula, F.* sylvatica) and a mixture of all three species exposed to elevated and ambient atmospheric CO_2. The kinetic parameters a_1, a_2 and a_3 represent the size of rapid uptake pool (a_1), microbial immobilisation and turnover (a_2) and sorption to the solid phase (a_3) whilst k_1, k_2 and k_3 are the constants describing the rate of turnover of these pools. Values depicted are means ± SEM (n=4)

Treatment	Species	Depth (cm)	April						June					
			a_1	k_1	a_2	$k_2\times10^2$	a_3	$k_3\times10^5$	a_1	k_1	a_2	$k_2\times10^2$	a_3	$k_3\times10^5$
Ambient CO_2	*Alnus*	0-10	14.2±0.8	1.3±0.1	9.1±0.5	2.0±1.3	76.6±1.3	5.1±1.1	17.8±1.3	0.7±0.3	10.4±3.3	1.3±0.2	71.6±4.6	6.4±4.5
		10-20	14.5±1.5	1.0±0.2	10.3±1.0	1.5±1.6	75.1±1.6	6.2±0.7	16.6±1.0	0.9±0.3	13.6±1.1	1.1±0.2	69.6±1.9	6.0±0.6
		20-30	13.5±1.2	0.8±0.2	11.0±0.7	1.4±1.5	75.4±1.5	4.4±0.8	16.3±1.4	0.5±0.1	12.4±1.6	1.0±0.2	70.8±2.9	5.6±1.3
	Betula	0-10	14.7±1.0	1.3±0.1	9.9±1.0	1.9±1.8	75.4±1.8	5.2±0.8	18.0±2.1	0.8±0.0	10.5±1.0	1.3±0.1	71.3±3.1	5.8±2.1
		10-20	14.6±1.1	1.1±0.1	10.0±1.2	1.4±2.1	75.3±2.0	6.2±0.9	16.2±1.8	0.8±0.2	11.1±0.7	1.3±0.1	72.4±2.3	4.8±0.9
		20-30	11.9±0.9	1.0±0.1	9.0±0.6	1.6±0.8	79.1±1.0	3.9±0.5	15.7±0.8	0.5±0.1	12.4±1.0	1.1±0.1	71.5±1.7	5.0±0.6
	Fagus	0-10	13.5±1.2	1.1±0.5	9.2±0.9	1.8±1.7	77.2±1.2	5.4±1.2	18.4±1.4	0.7±0.1	10.7±1.1	1.3±0.3	70.7±2.4	6.3±2.0
		10-20	14.7±1.3	1.0±0.3	9.6±1.0	1.5±1.9	75.6±2.0	6.9±1.1	16.6±1.0	0.7±0.2	11.3±0.4	1.3±0.1	71.9±0.9	5.2±2.5
		20-30	12.7±1.8	1.0±0.2	10.0±1.3	1.7±1.8	77.2±1.3	4.7±0.6	16.6±2.0	0.6±0.3	11.8±1.8	1.1±0.3	71.2±3.7	4.9±1.7
	Mixture	0-10	14.7±1.9	1.3±0.1	9.8±1.0	1.8±2.7	75.5±1.4	5.3±1.3	17.9±5.0	0.7±0.1	10.7±3.2	1.3±0.1	71.1±3.2	6.0±1.0
		10-20	14.0±1.4	0.9±0.4	11.7±4.0	1.5±4.9	74.0±2.2	4.9±1.5	16.6±2.0	0.7±0.2	11.4±1.1	1.3±0.2	71.8±2.7	5.4±1.4
		20-30	12.2±0.7	0.5±0.4	11.0±2.6	0.8±3.7	76.3±0.9	2.7±1.2	15.0±1.7	0.6±0.3	11.3±0.6	1.1±0.2	73.3±2.3	4.7±0.7
Elevated CO_2	*Alnus*	0-10	13.4±0.8	1.2±0.1	9.1±0.3	1.8±0.2	77.4±0.8	6.4±1.1	17.4±1.7	0.9±0.1	12.0±1.0	1.5±0.2	70.3±2.5	8.2±0.7
		10-20	15.3±1.5	0.9±0.2	10.7±0.7	1.4±0.2	73.9±2.2	5.6±0.7	17.6±2.6	0.6±0.3	11.8±4.1	1.3±0.3	70.2±3.5	6.2±1.2
		20-30	13.6±1.3	0.7±0.1	11.5±1.2	1.4±0.1	74.8±2.5	4.1±0.8	16.6±2.2	0.5±0.1	12.5±1.2	1.0±0.1	70.3±3.5	5.5±1.6
	Betula	0-10	14.2±1.0	1.0±0.2	10.0±0.5	1.4±0.1	75.8±1.4	5.2±0.8	18.6±1.4	1.1±0.1	10.9±1.3	1.6±0.1	70.3±2.8	7.0±0.7
		10-20	14.5±0.8	0.9±0.2	10.9±0.9	1.3±0.2	74.5±1.4	5.5±0.9	16.7±1.2	0.8±0.1	11.0±0.6	1.4±0.1	72.2±1.6	5.3±0.5
		20-30	11.7±1.0	0.6±0.1	10.5±0.5	1.1±0.3	77.8±1.2	3.6±0.5	16.4±1.9	0.7±0.2	11.6±1.2	1.3±0.1	71.8±3.0	5.6±0.7
	Fagus	0-10	13.7±2.1	1.2±0.3	9.0±0.2	1.7±0.4	77.2±2.1	6.4±1.2	19.1±0.6	1.1±0.1	11.4±0.7	1.5±0.1	69.4±1.3	8.0±0.7
		10-20	14.2±2.1	1.1±0.1	10.4±0.3	1.6±0.2	75.3±2.4	5.8±1.1	18.1±0.3	0.8±0.0	10.9±0.7	1.5±0.2	70.8±1.0	6.9±0.5
		20-30	13.7±2.2	0.7±0.2	11.3±0.4	1.4±0.1	74.8±2.4	4.4±0.6	16.3±2.7	0.7±0.3	11.3±1.1	1.3±0.3	72.2±3.5	5.8±1.4
	Mixture	0-10	14.4±1.1	1.0±0.2	10.6±1.2	1.6±0.4	75.0±2.3	5.0±1.3	15.8±1.3	1.0±0.1	12.3±0.6	1.6±0.1	71.7±1.8	6.8±1.2
		10-20	14.7±0.9	0.8±0.1	10.4±0.7	1.4±0.1	74.8±1.6	4.6±1.5	16.9±1.4	0.7±0.1	11.1±1.2	1.3±0.1	71.7±2.6	5.5±0.6
		20-30	12.6±0.7	0.6±0.1	10.3±0.4	1.3±0.2	76.9±1.0	3.2±1.2	14.8±1.4	0.7±0.1	11.1±1.4	1.3±0.1	73.9±2.8	4.7±1.1

Table 6.5 ANOVA F-values of kinetic parameters a_1, a_2 and a_3 and the respective rate constants describing the rate of turnover of these pools (k_1, k_2 and k_3) before (April) and after (June) bud burst. Significance is denoted by $^{*}P<0.1$, $^{**}P<0.05$, $^{***}P<0.01$

		April						June					
Species	Depth	a_1	k_1	a_2	k_2	a_3	k_3	a_1	k_1	a_2	k_2	a_3	k_3
Alnus	0-10	1.974	8.687**	0.001	1.806	1.069	2.126	0.090	1.699	0.822	2.333	0.231	0.090
	10-20	0.583	0.323	0.334	0.209	0.868	0.452	0.540	1.557	0.727	0.747	0.090	0.053
	20-30	0.022	0.589	0.509	0.018	0.215	0.134	0.058	0.723	0.025	0.000	0.047	0.617
Betula	0-10	0.576	9.788**	0.028	11.698**	0.120	0.000	0.252	94.206***	0.260	17.559*	0.216	1.033
	10-20	0.007	3.046	1.321	0.111	0.459	0.365	0.174	0.191	0.111	2.564	0.040	1.138
	20-30	0.048	24.224**	17.550**	4.622*	5.078*	0.273	0.436	3.441	1.023	2.404	0.026	2.212
Fagus	0-10	0.035	0.101	0.115	0.119	0.001	1.489	0.831	47.931***	1.071	2.018	0.870	2.784
	10-20	0.128	1.189	2.505	0.503	0.042	0.930	7.887**	0.520	0.809	3.945*	2.695	1.728
	20-30	0.502	4.402*	3.563	8.319**	2.571	0.282	0.042	0.109	0.265	0.538	0.152	0.654
Mixture	0-10	0.066	5.818*	0.818	0.525	0.081	0.101	0.626	34.524***	0.906	10.780**	0.108	1.112
	10-20	0.677	0.201	0.429	0.003	0.084	0.050	0.064	0.084	0.104	0.053	0.001	0.042
	20-30	0.657	0.331	0.314	2.410	0.120	0.500	0.026	1.396	0.055	3.253	0.080	0.000

Table 6.6 Repeated Measures ANOVA F- values of kinetic parameters a_1, a_2 and a_3 and the respective rate constants describing the rate of turnover of these pools (k_1, k_2 and k_3). Significance is denoted by $^{*}P<0.1$, $^{**}P<0.05$, $^{***}P<0.01$

Interaction	a_1	k_1	a_2	k_2	a_3	k_3
month	145.822***	110.925***	33.395***	43.073***	123.670***	13.246***
month × species	1.264	3.515**	1.159	3.082**	1.333	0.396
month × depth	2.565*	2.578*	0.488	3.408**	1.888	3.736**
month × treatment	0.361	0.007	0.640	0.358	0.558	2.817*
month × species × treatment	0.246	2.893**	0.853	1.748	0.293	0.250
month × depth × treatment	0.917	2.119	1.257	0.066	0.001	1.617
month × depth × species × treatment	0.254	2.130*	0.548	1.308	0.023	0.000

6.4 Discussion

We determined LMW-C residence times at two temporal stages. The first was collected prior to bud burst when we would expect a low efflux of LMW-C from roots and mycorrhizae, the second immediately followed bud burst when young leaves were fully flushed providing an opportunity for a high rate of photosynthesis to drive rhizodeposition of labile C compounds to soil (Kaiser *et al.* 2010). Many studies have shown that plants respond to elevated atmospheric [CO_2] by partitioning a greater proportion of photosynthate belowground. This C flux is mediated through increases in litter input, root production and turnover (Norby *et al.* 2004; Lukac *et al.* 2003) and exudation (Philips *et al.* 2009; Johansson *et al.* 2009). Elevated atmospheric [CO_2] induced changes in the composition and quality of C inputs into the rhizosphere are likely to influence microbial community structure and activity to efficiently metabolise exudates and cellular debris (Couteaux *et al.* 1999; Körner, 2000; Rillig *et al.* 2001).

6.4.1 The effect of elevated CO_2 on the half life of LMW C

It well known that LMW compounds such as sugars and organic acids possess short half lives in soil (van Hees, *et al.* 2006); these are typically attributed to rapid substrate depletion from the dissolved organic carbon (DOC) pool by soil microbe catabolic processes (Boddy *et al.* 2008). Using the first exponential coefficient (k_1) of our model we calculated DOC pool residence time between 0.47 and 1.64 hours in the top soil at BangorFACE. These values are consistent with reported values between 0.59 and 1.68 hours in a tundra forest (Kuzyakov and Demin, 1998) and between 1 and 6 hours in boreal forest soils (van Hees *et al.* 2003).

In contrast to our expectation the residence time of the DOC pool was greatest in the elevated CO_2 treatment plots, an effect that became greater as the growing season progressed. This may indicate that the supply of LMW-C to the DOC pool is exceeding the uptake capacity of the microbial population, most likely due to nutrient resource limitation. Soil microorganisms are often C limited and rely on a labile source of C to maintain metabolic processes and growth (Drigo *et al.* 2008). As plants exposed to

elevated CO_2 produce greater quantities of labile rhizodeposition with a high C:N ratio (Phillips *et al.* 2009). C availability may cease to limit microbial growth and activity as depletion of other resources such as N or phosphate becomes increasingly important. Brown *et al.* (2009) showed a primary N and secondary P limitation to microbial activity in a scrub oak ecosystem exposed to elevated CO_2 by measuring O_2 consumption. Other studies have reported an interaction between C and N to stimulate microbial activity but not P (Vance and Chapin, 2001). Lagomarisino *et al.* (2007) also demonstrated a decrease of microbial catabolic activity in N limited soils in contrast to enhanced microbial substrate utilisation in N fertilised soil under *P. alba* and *P. nigra*. Furthermore, van Veen *et al.* (1991) suggested that microbial utilisation of C compounds may be limited by the availability of mineral N or poor N supply. This is further supported by the fact that plants tend to reduce the exudation of N rich metabolites in response to elevated CO_2 (Drigo *et al.* 2008).

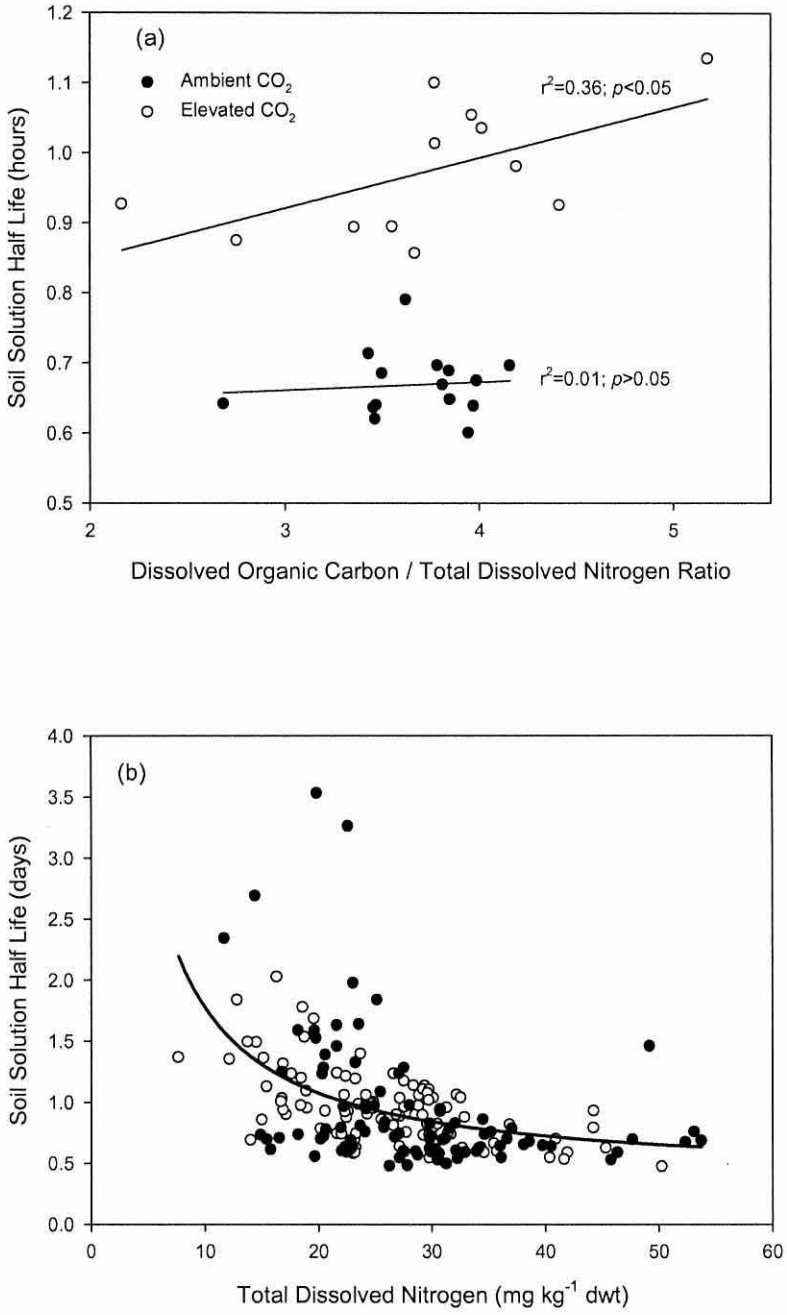

Figure 6.5 Panel (a) Shows the relationship between soil solution half life and the K_2SO_4 extractable ratio of dissolved C:N. A significant relationship (r^2=0.36; P<0.05) suggests N limitation in elevated CO_2 but not in ambient plots. Panel (b) shows the relationship between soil solution half life and K_2SO_4 extractable total dissolved nitrogen elucidating the impact of available N on soil solution half life.

The mineralisation kinetics of soil collected from *A. glutinosa* did not produce an increase in primary phase LMW-C mineralisation residence time observed in other species. This may be associated with the N fixing status of *A. glutinosa*, where enhanced N input through fixation and decomposition of N rich plant detritus may alleviate limitation of N. This hypothesis is supported by data showing elevated CO_2 enhanced N fixation of the vine *Galactia elliotii* in an Oak scrubland (Hungate *et al.* 1999). The mechanisms driving this change were postulated to be reduced mineral N inhibition of biological N fixation, resulting in production of root nodules with greater mass and abundance (Hu *et al.* 2006). However, using ^{15}N natural abundance it has been demonstrated that *A. glutinosa* at BangorFACE only significantly fixed more N when in grown in admixture with *B. pendula* and *F. sylvatica* (Chapter 11; Millet *et al. submitted*) which we attributed to niche competition or facilitation.

The second mineralisation phase explained by the exponential coefficient (k_2) has been attributed to the turnover of C immobilised in the microbial biomass as metabolites and structural compounds (Kuzyakov and Demin, 1998; Boddy *et al.* 2007). Mean residence time of ^{14}C labelled C in this pool was between 1.3 and 6.6 days which is consistent with glucose mineralisation times between 1.8 and 3 days described by Kuzyakov and Demin (1998) in forest soils. However, Scheiswig *et al.* (2003) reported microbial turnover times between 0.7 and 1.8 days approximately twice as fast as reported here. During their study a two term exponential decay model was used and evolved $^{14}CO_2$ assessed on a daily basis. In their study, a low sampling resolution and use of a two term decay model may have underestimated microbial C residence time as there is greater linkage between pools using a two term model. Interestingly, the impact of elevated CO_2 on half-lives in the second pool was small with significant changes evident within birch and the three species mixture subplots during both sampling periods. These data in combination with a small non-significant temporal increase in microbial biomass suggests that (a) microbial activity was unable to increase and (b) C availability is not limiting microbial growth. However, a large shift in the C:N ratio of the microbial community occurred during this time. The most likely explanation for this change is a shift in microbial community structure, with an increase in microbial C: N ratio indicating a shift towards a fungal dominated community (Paul and Clark, 1989; Hu *et al.*

2001). Furthermore, early elevated CO_2 work with the *Betula* genus indicated that mycorrhizal abundance and composition was altered by elevated CO_2 to provide a stronger C sink and improve nutrient availability (Rey and Jarvis 1997; Godbold *et al.* 1997).

The exponential coefficient (k_3) describes the residence time of C that is less available and is associated with molecules sorbed to unavailable soil complexes or metabolites partitioned into recalcitrant material. MRT of this pool was between 1.1 and 2.3 years. Three pool models are infrequently used in LMW-C studies, and subsequently information on this pool is scarce. In one of the few studies using a three term model, Kuzyakov and Demin (1998) reported a MRT of 0.49 and 0.45 years for glucose and glycine respectively. We detected no treatment effect on the residence time of C within this pool, indicating that elevated CO_2 was not affecting the residence time of LMW C partitioned into long term storage pools.

6.4.2 Soil Properties and LMW C Residence Time.

Table 6.7 Correlations with the mean residence time of LMW substrates of the dissolved organic carbon pool and background variables measured at BangorFACE (**$P<0.05$).

	RLD	Olsen P	DOC	TDN	Ca	K	Na	pH	OM	Moisture	Microbial C/N
CNTL	-.493**	-.479**	-.524**	-.612**	.506**	-.495**	.242	-.570**	-.350	-.260	.006
FACE	-.326	-.534**	-.190	-.545**	-0.27	-.213	-0.22	.190	-.545**	-.472**	.474**

The effect of soil properties on LMW C mean residence time was assessed by linear regression. In the ambient plots significant ($P<0.001$) negative relationships were observed between $NaHCO_3$ extractable phosphorus (P), potassium (K), pH, dissolved C & N and root length density. Temperate forest ecosystems are often deficient in P, and phosphate is not readily available for utilisation by microbes or plants (Cardon, 1996). Availability is controlled by the secretion of phosphatases from roots, mycorrhizae or bacteria which breakdown ester bonds between P and SOM. The strong relationship between P and LMW-C mean residence time in the primary mineralisation pool of this study suggests that catabolic processes have high P metabolic requirements possibly due to ATP demand or utilisation of P rich enzymes (Bradford *et al.* 2008). The significant negative relationship between SOM and mean residence time alludes to the utilisation of

SOM as a source of nutrients and nutrient limitation during mineralisation. Soil moisture affects mobility of substrates and microbes through the soil which would account for a correlation between substrate residence time and soil moisture.

6.4.3 Microbial Biomass

A temporal doubling of microbial C: N occurred in this ecosystem as plant productivity increased between April and June, however this was observed in both the ambient and FACE plots. Hu *et al.* (2001) showed that in grasslands rising levels of CO_2 lead to higher microbial C:N ratios which were attributed to an increase in the fungal: bacterial ratio or decreased availability of N to the microbial biomass. By comparing fungal extramatrical hyphal length to microbial C, Lipson *et al.* (2005) also alluded to an increase in fungal dominance of the microbial community when a stand dominated by the woody shrub *Adenostrorna fasciculatum* was exposed to 8 years of CO_2 enrichment. As fungal biomass has a higher C to N ratio, and availability of N tends to be negatively correlated to the abundance of fungi (van Groenigen *et al.* 2007) it is likely that these changes can be explained by a shift towards fungal composition of the microbial community.

6.4.4 Low Molecular Weight C pool partitioning

Studies elucidating the partitioning of low molecular weight C are scarce. We only observed a significant temporal reduction in C partitioned to pool a_1 (catabolic respiration) in the elevated CO_2 plots at 20 cm depth in *B. penulda* (Figure 6.3). This observation correlates well with an increase in rooting depth often observed in FACE experiments (Rogers *et al.* 1994; Norby *et al.* 2004; Iverson *et al.* 2008; Iverson, 2010). Increased root production and exploration enables the delivery of labile C deeper in the soil profile though exudation and bioturbation. Typically deep soil C is comprised of organo-mineral complexes which are recalcitrant to microbial mineralisation limiting microbial growth and activity. Additionally, resource limitation such as a lack of oxygen may confound microbial decomposition of available C substrates. Deeper rooting provides an opportunity for O_2 passage and a supply of labile C to supplement microbial

enzyme production. With the growth constraining conditions satisfied, it is possible that the microbial community co-metabolises old SOM (Fontaine *et al.* 2007) resulting in a lower utilisation of fresh C in pool a_1. Kuzyakov *et al.* (2000) previously postulated that stimulation of decomposer micro-organisms may result in the mineralisation of old SOM under elevated CO_2, or priming, that would release unlabelled CO_2 resulting in unaccountable microbial activity in our experiment (Kuzyakov *et al.* 2002). Unfortunately as we did not quantify basal respiration we are not able to confirm that SOM priming occurred in this instance. However, if microbial activity was partially supported by the mineralisation of old SOM it may mediate the use of alternative metabolites to supply nutrients and transform C to storage in the microbial biomass. If substrate uptake was reduced due to resource limitation we might expect to see larger quantities of K_2SO_4 extractable substrate at the end of the experiment. However, we determined that only 0.37-0.65% of the total substrate remained extractable at the end of the experiment. Therefore, we believe that the change in partitioning can be explained by a reduction of microbial catabolic processes and a switch towards microbial uptake, storage and immobilisation.

6.5 Conclusion

The primary aim of this study was to determine the impact of elevated atmospheric CO_2 enrichment on LMW C, kinetics, turnover and C pool partitioning. The application of a cocktail of LMW C substrates to soil collected under three tree species grown under elevated CO_2 in monoculture and a three species mixture revealed differential substrate utilisation kinetics. LMW C substrate mineralisation kinetics were explained using a three pool exponential decay regression model, with each of three pools linked to biogeochemical processes, (a_1) microbial uptake and respiration, (a_2) microbial storage and turnover, (a_3) sorption to soil complexes.

In our study, season had a large effect on LMW C mineralisation kinetics, whilst elevated CO_2 strongly affected the kinetics and catabolic processes of the soil solution pool (a_1). Prior to bud burst, C turnover was fastest in pool a_1 with a greater impact of elevated CO_2 with depth. Surprisingly, following bud burst, the turnover of LMW C in

the soil solution pool (a_1) decreased within the elevated CO_2 plots. Season and tree species had a differential effect on the LMW C mineralisation kinetics suggesting that a greater labile C input was switching the microbial population from C to nutrient limitation. C partitioning was only affected by elevated CO_2 at a depth of 20 cm in *B. pendula*, whereas season had a large and significant effect. Following bud burst a greater percentage of C was partitioned to pools a_1 and a_2, whilst there was a reduction in allocation to pool a_3. This study has demonstrated that not only are microbial processes being differentially affected by tree species, but that microbial processes are also affected by temporal dynamics and nutrient limitation under elevated CO_2.

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7 Species Diversity Decreases Low Molecular Weight Substrate Turnover in Temperate Forest Enriched by Elevated Atmospheric CO_2.

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Contributions:

Andrew Smith wrote the manuscript, collected data, performed analysis and maintained the FACE facility during 2006-2009. Dr. Mark Farrell assisted during the ^{14}C laboratory experiment. Dr. Daniel Murphy and Prof. Davey Jones provided radioisotopes and equipment. Prof. Douglas Godbold is the project principle investigator; he also provided assistance with data interpretation, analysis and manuscript preparation

7.1 Introduction

Atmospheric CO_2 concentrations are increasing as a consequence of anthropogenic land use change and burning of fossil fuels (IPCC, 2007). These changes to the global environment have the potential to alter the biogeochemical cycling of carbon (C) in terrestrial ecosystems with serious implications for C sequestration. (Zak *et al.* 2000). The largest C pool of the terrestrial biosphere is the soil, containing an estimated 1600 Pt of C in the top 1 m of soil worldwide (Jobbagy and Jackson, 2000). Forests are an important component of the terrestrial C pool as half of the C in terrestrial ecosystem, and most of the 60 Pg C yr^{-1} allocated belowground by plants can be attributed to forest ecosystems and their soils (Schlesing and Lichter, 2001; Millard *et al.* 2007). Therefore, understanding the mechanistic processes governing soil C fluxes in forest ecosystems is paramount to determining the susceptibility of terrestrial ecosystems to global change.

The physiological stimulation of plants by elevated atmospheric CO_2 has been thoroughly investigated over recent years revealing that plant enrichment with elevated atmospheric CO_2 results in a greater allocation of assimilated C belowground through increased litter input, root production, turnover and exudation (Ceulemans and Mousseau, 1994; Curtis and Wang, 1998; Saxe *et al.* 1998; Long *et al.* 2004; Ainsworth and Long, 2005; Johansson *et al.* 2009). However, there remains much uncertainty surrounding belowground C pool fluxes, and long-term C storage (Hoosbeek *et al.* 2006; Lichter *et al.* 2005). Increases in C allocation belowground are a well known response to elevated atmospheric CO_2 fertilisation (Rogers *et al.* 1994; Iverson *et al.* 2008). For example, coniferous trees enriched with CO_2 and grown in field conditions produce 23% more root biomass (Tingey *et al.* 2000) and three decidous *Populus* species grown at EuroFACE increased fine root biomass by 35-84% (Lukac *et al.* 2003). In addition to enhanced root production and turnover, deeper exploration throughout the soil profile has also been reported (Norby *et al.* 2004; Iverson, 2010). Root exudation has not only been shown to increase in proportion to root biomass under elevated CO_2, but also to positively correlate with root system productivity (Garcia *et al.* 2001). Tight coupling between stimulated photosynthetic assimilation rates under elevated CO_2 and root exudation suggests a substantial increased flux of labile C to soil (Dilkes *et al.* 2004). In a review of ^{14}C plant

labelling Farrar *et al.* (2003) calculated that exudation accounted for approximately 0.5-5% of net fixed C. Other studies are less conservative suggesting that plants may exude 10% of assimilated C below ground, equating to a substantial 6 mol C m^{-2} yr^{-1} in ambient conditions (Grayston *et al.* 1996; van Hees *et al.* 2005). Several authors have reported increases in rhizodeposition in response to elevated atmospheric CO_2 (van Veen *et al.* 1997; Jones *et al.* 1998; Cheng, 1999). In a study exposing *Pinus sylvestris* seedlings to 700 μmol mol^{-1} CO_2 Johansson *et al.* (2009) identified a 120-160% increase in LMW organic acids, and 250% increase in amino acids exudation compared to ambient conditions. Furthermore, sustained stimulation of photosynthesis in elevated atmospheric CO_2 is also understood to alter the quality and composition or C inputs to soil through changes in the relative availability of C and N resources to plants (de Graaff *et al.* 2007; Leakey *et al.* 2009). That may manifest itself through the production of metabolites with reduced N content and the enhanced production of C based compounds, such as non-structural carbohydrates, polyphenols, lignin or tannins (Cotrufo *et al.* 1998; Drigo *et al.* 2008). Microbial growth in soil is generally constrained by available C, therefore qualitative and quantitative changes in rhizodeposition are likely to alter the metabolism of heterotrophic microorganisms (Zak *et al.* 2000; Phillips *et al.* 2006). In addition, an increased flux of C compounds to the soil, may retard or increase the rate of soil organic matter mineralisation. Faster mineralisation may lead to the greater respiratory losses through microbial co-metabolism of old and new C and increased enzyme production, known as the *priming effect* (Kuzyakov *et al.* 2000; Fontaine *et al.* 2003). As a consequence, production of metabolically expensive enzymes better able to degrade soil organic matter may improve nutrient acquisition and promote productivity. On the other hand faster mineralisation may lead to nutrient immobilisation in the microbial biomass constraining productivity.

Since soil microorganisms have a seminal role in controlling the availability of nutrients via mineralisation of soil organic matter and mineral weathering, improving our understanding of how microbial ecosystem function is altered by global change is important (Grayston *et al.* 1997). Microbial catabolic diversity of a soil is directly related to the C decomposition function within a soil and potentially provides a sensitive and ecologically relevant measure of microbial community structure (Garland and Mills,

1991). Subsequently, multiple substrate-induced respiration assays have been used to produce community level physiological profiles (CLPP) and produce fingerprints of microbial function.

Three approaches for measuring CLPP in soil are reported in the literature (Garland and Mills, 1991; Degens and Harris, 1997; Campbell *et al.* 2003) all of these methods are based on measuring CO_2 respired during metabolism of a range organic compounds that vary in size, charge and structural complexity. The first approach Biolog MicroPlate™ (Biolog) assesses the catabolic diversity of soil organisms in a microtitre plate by incubating a soil culture in the presence of nutrients and 95 different C substrates; evolved CO_2 reduces tetrazolium violet salt resulting in a change in colour which may be quantified colourimetrically (Garland and Mills, 1991). However, this approach has been criticized for biasing towards faster growing organisms that thrive in culture (Preston-Mafham *et al.* 2002). In response to criticisms of the Biolog method, Degens and Harris (1997) developed a method based on substrate-induced respiration (SIR) where individual substrates are added to intact soil and evolved headspace CO_2 sampled and measured. Finally, Campbell *et al.* (2003) combined aspects of both the previous methods (MicroResp™) where substrate addition to whole soil is measured colourimetrically using a cresol red indicator dye in a microtitre plate format. In the present study we determined the catabolic utilisation profile, turnover and pool allocation of LMW C by utilising a selection of ^{14}C organic C substrates in a multiple SIR assay on whole soil. This has the advantage of enabling the attribution of the respired CO_2 to metabolism of the radiolabelled substrates, and enabling the study of microbial uptake kinetics and turnover using substrates varying in structural complexity and recalcitrance.

The objectives of this manuscript were to determine; (a) if elevated CO_2 stimulated microbial catabolic processes, (b) if tree species grown in mono and polyculture and enriched with elevated atmospheric CO_2 altered microbial catabolic utilisation profiles, and (c) if elevated CO_2 driven root exploration increased microbial mineralisation throughout the soil profile.

7.2 Materials and Methods

7.2.1 Site description and experimental design.

The BangorFACE experiment was established in 2004 at the Bangor University research farm, Henfaes, Abergwyngregyn (53°14'N, 4°01'W). The facility is comprised of eight octagonal plots, four ambient and four CO_2 enriched, creating a 2 × 4 factorial block design across two former agricultural fields. Each plot encompasses an area approximately 50 m^2 and is divided into four, the planting arrangement comprises three singles species subplots of birch (*Betula pendula* Roth.), alder (*Alnus glutinosa* (L.) Gaertner) and beech (*Fagus sylvatica* L.), and a fourth subplot contained a mixture of all three species.

The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Climate at the site is classified as Hyperoceanic with a mean annual temperature during 2005-2008 of 11.5 °C and rainfall of 1034 mm. Soils are fine loamy brown earth over gravel (Rheidol series) and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002)), and as a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011). Soil texture is 63% sand, 28% silt and 9% clay. Soil chemical and physical properties are shown in Table 7.1.

Soil samples were collected with a 2 cm diameter auger corer during July 2008 in 10 cm increments to a depth of 30 cm within each of the three single species sub-plots and the three species mixture sub-plot of each experimental plot. Soil was collected directly into polythene bags and stored at 5 °C in the field. On return to the laboratory soil samples were homogenised and sieved to pass 2 mm prior to analysis. Soil pH was measured in water according to Smith and Doran (1996). Plant available phosphorous (P), Potassium (K), Calcium (Ca) and Sodium (Na) were extracted using Mehlich 3 procedure (200 mM CH_3COOH, 250 mM NH_4NO_3, 15 mM NH_4F, 13 mM HNO_3, and 1 mM ethylene diamine tetraacetic acid (EDTA)) (Mehlich, 1984) and phosphate was determined colourimetrically as molybdate-reactive P (Murphy and Riley, 1962) using a VERSAmax micro plate reader (Molecular Devices), and cations determined by Atomic

Emission Spectroscopy (Sherwood Flame Photometer 410). Total elemental C and N were determined by CHN-2000 analyser (LECO Corp, St Joseph, MI, USA).

Table 7.1 Average soil physical and chemical properties sampled during 2008 and used in subsequent experiments. Values represent mean ± SE.

Treatment	P mg kg^{-1}	K mg kg^{-1}	Ca mg kg^{-1}	Na mg kg^{-1}	pH ($CaCl_2$)	Moisture (%)	OM (%)
Ambient	153.2± 13.0	68.5 ± 4.4	741.0 ± 50.9	10.9 ± 0.4	4.9±0.1	22.0±0.4	6.0±0.1
Elevated	82.6 ± 5.0	64.3 ± 3.9	695.9 ± 23.2	14.1± 0.7	4.7±0.0	21.4±0.3	5.3±0.1

7.2.2 ^{14}C Substrate-induced respiration

To trace the fate of sixteen organic compounds 500 µl of each ^{14}C-labelled substrate (50 mM), shown in Table 7.2 was evenly applied to the soil surface of a 50 cm^3 polypropylene vessel containing 5 g of 2 mm sieved soil. Following addition of the substrate a 1 M NaOH trap (1 ml) was placed inside the tube to trap evolved $^{14}CO_2$ and the tube hermetically sealed at the top. The NaOH trap was suspended above the soil to allow free air passage from the soil surface and replaced following 1, 2, 4, 8, 12, 24, 48, 72, 96, 192, 504, 847, 1176, 1514, 1877, 2328 hours of incubation at room temperature (21 °C). Following the removal of each NaOH trap, its $H^{14}CO_3$ content was determined by liquid scintillation counting using a Wallac 1404 scintillation counter (Wallac EGandG, Milton Keynes, UK) with automatic quench correction and Optiphase 3® alkali compatible scintillation fluid (Wallac EGandG). Immediately after the final incubation sampling time, soils were extracted with 0.5 M K_2SO_4 to quantify ^{14}C remaining available in the soil. Briefly, 25 ml of 0.5 M K_2SO_4 was added to 5 g soil and shaken at 200 rev min^{-1} for 30 minutes. Subsequently a 2 ml aliquot of the supernatant was transferred to a 2 ml eppendorf tube and centrifuged at 15000 rpm for 10 minutes, 1 ml of supernatant was then transferred to a scintillation vessel for ^{14}C determination as above.

Table 7.2 Organic compounds and there activity used in community level physiological profiling.

Functional Group	Substrate	Formulae	Activity (kBq ml^{-1})
Carbohydrates	Fructose	$C_6H_{12}O_6$	1.25
	Glucose	$C_6H_{12}O_6$	0.75
	Starch	$C_6H_{16}O_5$	0.73
	Sucrose	$C_{12}H_{22}O_{11}$	0.48
Amino acids/amides	Arginine	$C_6H_{14}N_4O_2$	0.92
	Aspartic acid	$C_4H_7NO_4$	0.55
	Glycine	$C_2H_5NO_2$	0.94
	Lysine	$C_6H_{14}N_2O_2$	0.89
	Phenlylalanine	$C_9H_{11}NO_2$	0.55
	Valine	$C_5H_{11}NO_2$	0.58
Amino sugar	Glucosamine	$C_6H_{13}NO_5$	1.34
Aromatic compounds	Salicylic	$C_7H_6O_3$	0.94
Aliphatic carboxylic acids	Acetate	$C_2H_3O_2$	1.12
	Malate	$C_4H_6O_5$	1.16
	Oxalic	$C_2H_2O_4$	0.96
	Succinic	$C_4H_6O_4$	1.30

7.2.3 Mineralisation kinetics

Substrate mineralisation typically follows at least a two compartment model (Paul and Clark, 1989). Authors have previously described mineralisation using various models (Saggar *et al.* 1996; Nguyen and Guckert, 2001; Boddy *et al.* 2007), however in this instance we utilise a double first order decay model (Equation 1) where f is the ^{14}C-label remaining in the soil at an instant in time, and k_1 is the rate constant describing the primary mineralisation phase of injected ^{14}C labelled substrates by the microbial community, k_2 is the exponential rate constant describing the slower secondary mineralisation phase, a_1 and a_2 describe the proportion of ^{14}C associated with two pools and their exponential coefficients k_1, k_2 and t time.

$$f = a_1 e^{-k_1 t} + a_2 e^{-k_2 t} \qquad \text{(Eqn. 1)}$$

In the case of substrates that are immobilised by sorption to mineral surfaces eg. oxalic acid and the formation of calcium oxalate it was necessary to include an asymptote (a_0) to account for ^{14}C that was permanently immobilised on mineral surfaces (Equation. 2).

$$f = a_0 + a_1 e^{-k_1 t} + a_2 e^{-k_2 t} \qquad \text{(Eqn. 2)}$$

The first term (k_1) of the model is attributable to the initial mineralisation of the injected ^{14}C labelled substrates in catabolic processes (Saggar *et al.* 1996) and expansion of the microbial pool which relate to the depletion rate of substrates from the soil solution. The second term of the model (k_2) is thought to be associated with the microbial uptake of ^{14}C labelled metabolites and turnover of dead cells resulting in the production of $^{14}CO_2$ (Paul and Clark, 1989; Boddy *et al.* 2007). The half-life ($t_{½}$) or Mean Resident Time (MRT) of pools a_1 and a_2 can be calculated as in Equation. 3.

$$t_{½} = \frac{\ln(2)}{k_1} \qquad \text{(Eqn. 3)}$$

Microbial yield, or C use efficiency of substrate conversion to biomass (Paul and Clark, 1989) is defined in (Equation 4) where *yield* is the proportion of the ^{14}C substrate added which is immobilised in microbial biomass (a_2) as a percentage of the total ^{14}C utilised for the production of microbial material ($a_1 + a_2$).

$$yield = \frac{a_2}{a_1 + a_2} \times 100 \qquad \text{(Eqn. 4)}$$

7.2.4 *Statistical Analysis*

The BangorFACE experiment was designed as a replicated split-plot design with eight whole plots (4 FACE and 4 ambient plots) in four blocks. Each plot is split into seven sub-plots which contain one, two and three species (Figure 1). For this research however, the three single species plots and the three species mixture where studied. Data was analysed using principle component analysis (PCA) using a correlation matrix to standardise values across all ecological data. Statistical significance was tested using a univariate general linear model (GLM) in SPSS 17.0 for windows (SPSS, Inc. Chicago, IL) effects were considered significant at $P<0.05$ but are also reported at $P<0.10$.

Regression coefficients were generated using SigmaPlot 11 (Systat Software, Inc. Chicago, IL).

7.3 Results

The mineralisation of each substrate was best described by fitting a double first order exponential decay model without an asymptote (mean $r^2 = 0.985 \pm 0.002$) across all substrates and treatments with the exception of two compounds. Possibly as a result of substrate immobilisation on mineral surfaces, oxalic acid and salicylic acid best fitted a double exponential decay model with an asymptote ($r^2 = 0.988 \pm 0.004$). Fitted model parameters for all species, depth and treatment combinations are shown in Tables 7.5a and 7.5b.

7.3.1 Top soil solution mineralisation

Changes in soil solution half-life in the top 0-10 cm of the soil profile, and relative to ambient atmospheric CO_2 are shown in Figure 7.1. In general, the response to elevated atmospheric CO_2 resulted in soil solution half-life decreasing for the single species sub plots by an average of -12.2%, -8.2%, -12.6% for *A. glutinosa*, *B. pendula* and *F. sylvatica* respectively. Conversely, in the three species mixture subplots, soil solution half-life increased by a mean of 8.9% relative to ambient CO_2. Examining each individual substrate revealed that elevated atmospheric CO_2 had the effect of significantly decreasing the soil solution half-life of acetate and valine by 22% (11.9 ± 0.8 h; P=0.008) and 26% (89.5 ± 6.0 h; P=0.003) respectively within the *A. glutinosa* subplots. While in the *B. pendula* subplots the soil solution half-life of valine decreased by 22% (96.6 ± 3.3 h; P=0.016). Glucose soil solution half-life was the only significant change, a decrease of 27% (37.8 ± 3.0 h; P=0.035) within the *F. sylvatica* subplots. Within the three species mixture subplots an apparent increased trend in soil solution half-life was observed in all substrates (Figure 7.1), with the exception of a 22% decrease in oxalic acid catabolism (37.8 ± 3.0 h; P=0.055). However, there were no statistically significant changes by any of the substrates used within the three species mixture subplots at the 5% level.

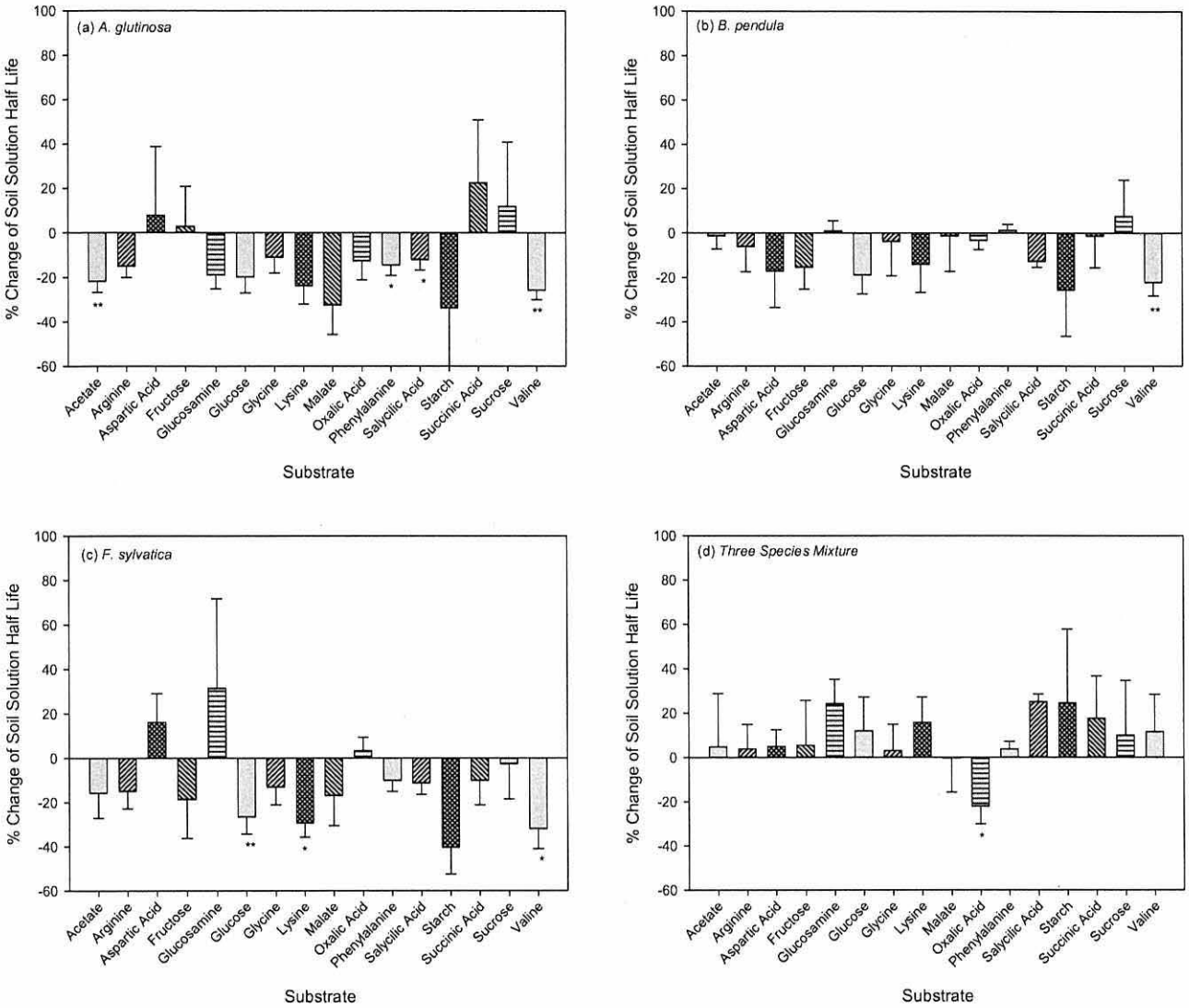
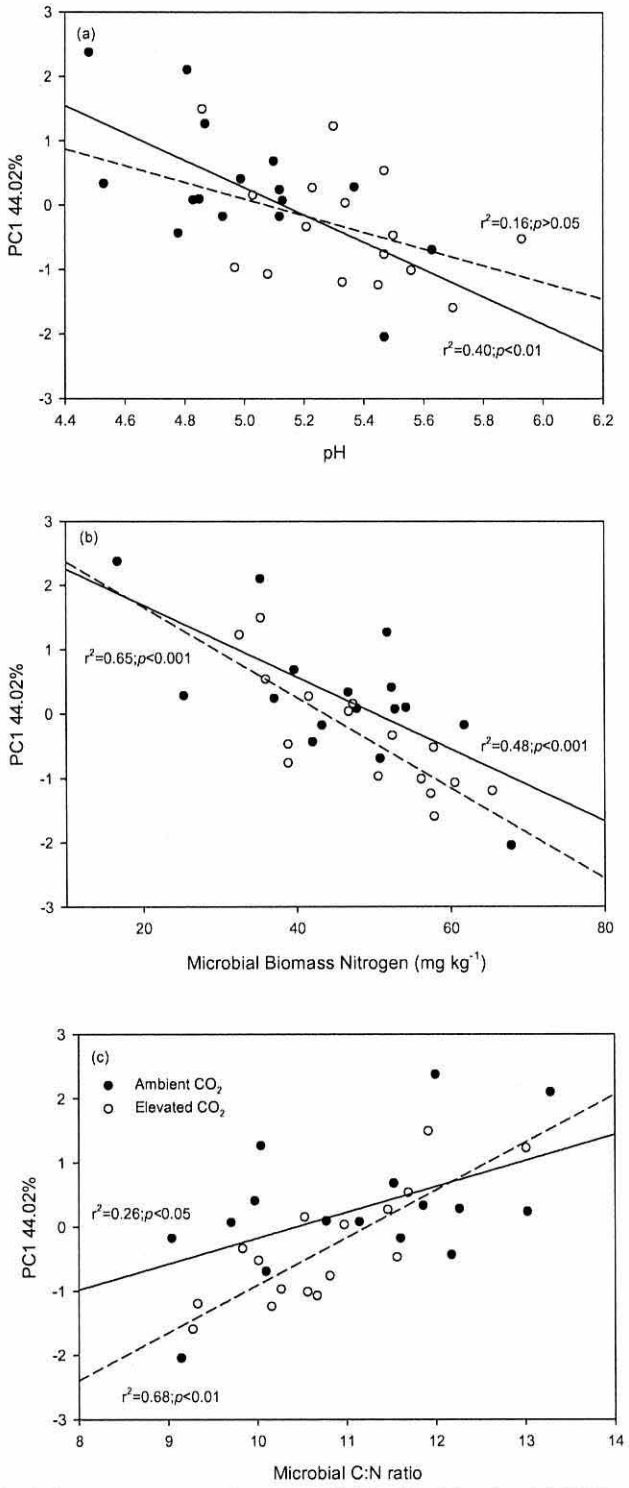


Figure 7.1 Percentage change in soil solution half life relative to ambient atmospheric CO_2 plots. Panel (a) *A. glutinosa* (b) *B. pendula* (c) *F. sylvatica* (d) three species mixture within the 0-10 cm soil horizon, error bars signify one standard error. Statistical significance is denoted by $*P<0.10$, $**P<0.05$.

Correlations performed against principle component (PC1; 44.02%) axis one (Table 7.4) that explained 44.02% of variation in all substrates soil solution half-lives tended to reveal positive correlations between N and substrate turnover in ambient CO_2 and conversely negative correlations in elevated CO_2 plots. Furthermore, fine root biomass and necromass correlated strongly within the elevated CO_2 plots. Negative correlations were observed between pH, microbial N and PC1. Whereas conversely a positive correlation between microbial C: N ratio and PC1 was observed (Figure 7.2).



ıre 7.2 Correlations between principle component axis one and (a) pH (b) microbial biomass (c) microbial C: N ratio. Principle ıponent Analysis was used to reduce the dimensionality of all substrates to a single variable explaining 44.02% of the variation. :d circles indicate ambient CO_2 and hollow circles elevated CO_2. The solid line shows the linear regression of ambient CO_2 data and ıed line linear regression of elevated CO_2 data, correlation coefficients and significance is shown inset in each figure.

7.3.2 *Top soil microbial half-life*

The mean change in microbial half-life across all substrates relative to the ambient CO_2 plots was -9.9%, -1.6%, -7.5%, -1.2% for *A. glutinosa, B. pendula, F. sylvatica* and three species mixture subplots respectively. The substrates with the largest magnitude change within the *A. glutinosa* subplots was glucosamine and sucrose which decreased microbial half-life by -27.3% (P=0.059) and -26.8% (P=0.114) respectively. Within the *B. pendula* subplots there was relatively little change (mean -1.63%) in microbial half-life for all substrates with the exception of salicylic acid (-14.5%; P=0.101) although not statistically significant. However, within the *F. sylvatica* subplots lysine significantly decreased its half-life by -16.5% (P=0.010), and glucosamine and phenylalanine produced the largest magnitude of change -18.9% (P=0.540) and -14.9% (P=0.060) respectively. The largest observed change of microbial half-life in response to elevated CO_2 was observed with oxalic acid within the three species mixture -29.3% (P=0.048).

7.3.3 *Top soil microbial yield*

The mean change in allocation of soil solution substrates to the microbial biomass or structural C pool was 5.1%, 1.0%, 3.0%, and 1.0% for *A. glutinosa, B. pendula, F. sylvatica* and the three species mixture respectively. The partitioning of valine to the immobilised microbial pool (Figure 7.7) was significantly increased by 24.3% (P=0.045) and 29.6% (P=0.023) for *A. glutinosa* and *F. sylvatica.* Whilst in the three species mixture subplots a 7.0% (P=0.028) increase in the partitioning of malate to the immobilised microbial pool was observed. In the *B. pendula* subplots there were no statistically significant changes however, a 5.1% increase in the partitioning of fructose was noted (P=0.059).

7.3.4 *The effect of depth on soil solution half-life*

Examining the effect of depth within the three species subplots (Figure 7.4) showed a mean soil solution half-life response for all substrates of 7.7%, 12.5% and 28.1% at 10, 20 and 30 cm respectively. Principle component analysis was used to reduce the dimensionality of the data to two components explaining 55.7% of the variation.

Subsequent multivariate analysis of variance conducted on the principle component axis one revealed a significant treatment and depth affect ($P<0.05$) although there was no depth × treatment interaction. Acetate soil solution half-life in the elevated atmospheric CO_2 plots was 13.2 ± 1.9 h at 0-10 cm within the soil profile, 19.1 ± 1.0 h at 10-20 cm ($P=0.044$) and 25.1 ± 2.1 h at 20-30 cm an increase of 4.8%, 30.1% and 45.3% respectively relative to the ambient CO_2 plots. Additionally, glycine, starch and succinic acid appeared to switch from an increase in soil solution half-life of 2.9, 19.7, and 15.0% within 0-10 cm of the soil profile, corresponding to turnover times of 25.7 ± 2.9 h, 2.9 ± 1.3 h, 33.0 ± 3.4 h to a decrease within 20-30 cm of the soil profile of -9.2, -20.9, -24.5% with respective turnover times of 29.3 ± 1.7 h, 1.2 ± 0.1 h, 30.3 ± 5.0 h (Figure 7.4). In contrast to the general increase in the soil solution, the half-life of oxalic acid consistently decreased by 22, 20 and 29% respectively at all depths (10 cm 37.8 ± 2.9 h; 20 cm 51.4 ± 8.4 h; 30 cm 58.9 ± 10.3 h) although this was not significant at the 5% level. Principle component analysis was performed with all substrates and measured environmental drivers to reduce the dimensionality of the data. Data was then plotted to reveal the degree of separation and influence of environmental drivers on component loadings (Figure 7.3). A second PCA analysis was performed to reduce substrate half lives to one dimension accounting for 63.69% of the variation. Correlations between principle component axis one (PC1; 63.69%) and environmental characteristics factored by depth and CO_2 treatment are detailed in Table 7.3. Furthermore, significant negative correlations irrespective of depth and treatment were found between PC1 and soil organic matter ($r^2=0.18$), soil moisture ($r^2=0.57$) dissolved organic N ($r^2=0.19$), microbial biomass ($r^2=0.69$), total soil N ($r^2=0.37$) and total soil C ($r^2=0.42$) (Figure 7.5)

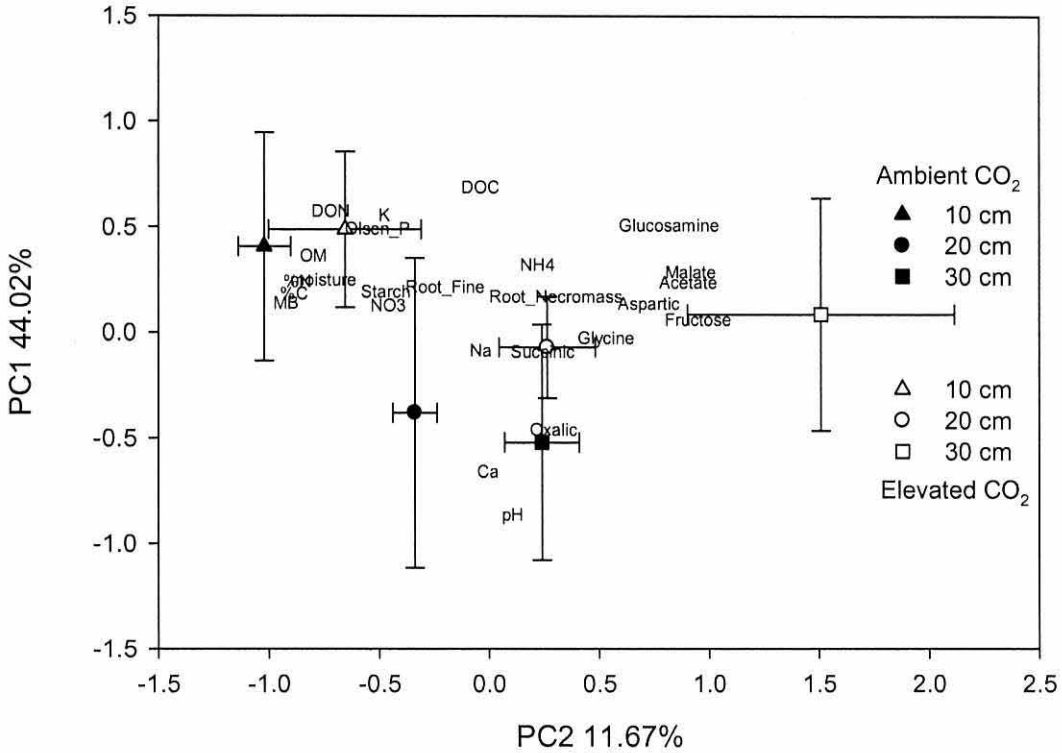


Figure 7.3 Principle component analysis of soil solution half life ($ln(2)/k_1$) for the three species mixture at three depths within the soil profile. Triangular symbols indicate 0-10 cm, circular symbols 10-20 cm and square symbols 20-30 cm. Filled symbols depict ambient atmospheric CO_2 and hollow symbols elevated atmospheric CO_2 values are means of eigenvalues ± SEM (n=4). The influence of selected substrates and environmental drivers are overlaid.

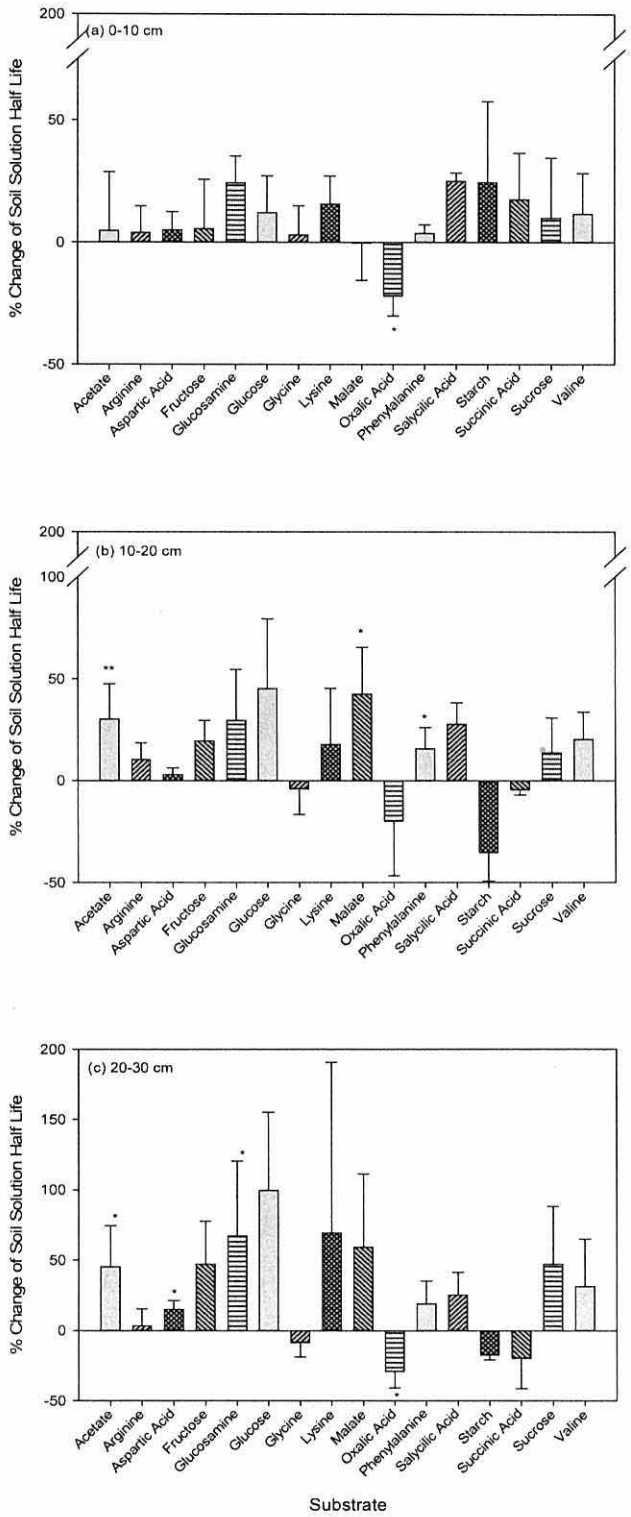


Figure 7.4 Percentage change in soil solution half-life relative to ambient atmospheric CO_2 plots for the three species mixture, panels (a) 0-10 cm (b) 10-20 cm (c) 20-30 cm. Error bars signify one standard error. Statistical significance is denoted by $*P<0.10$, $**P<0.05$.

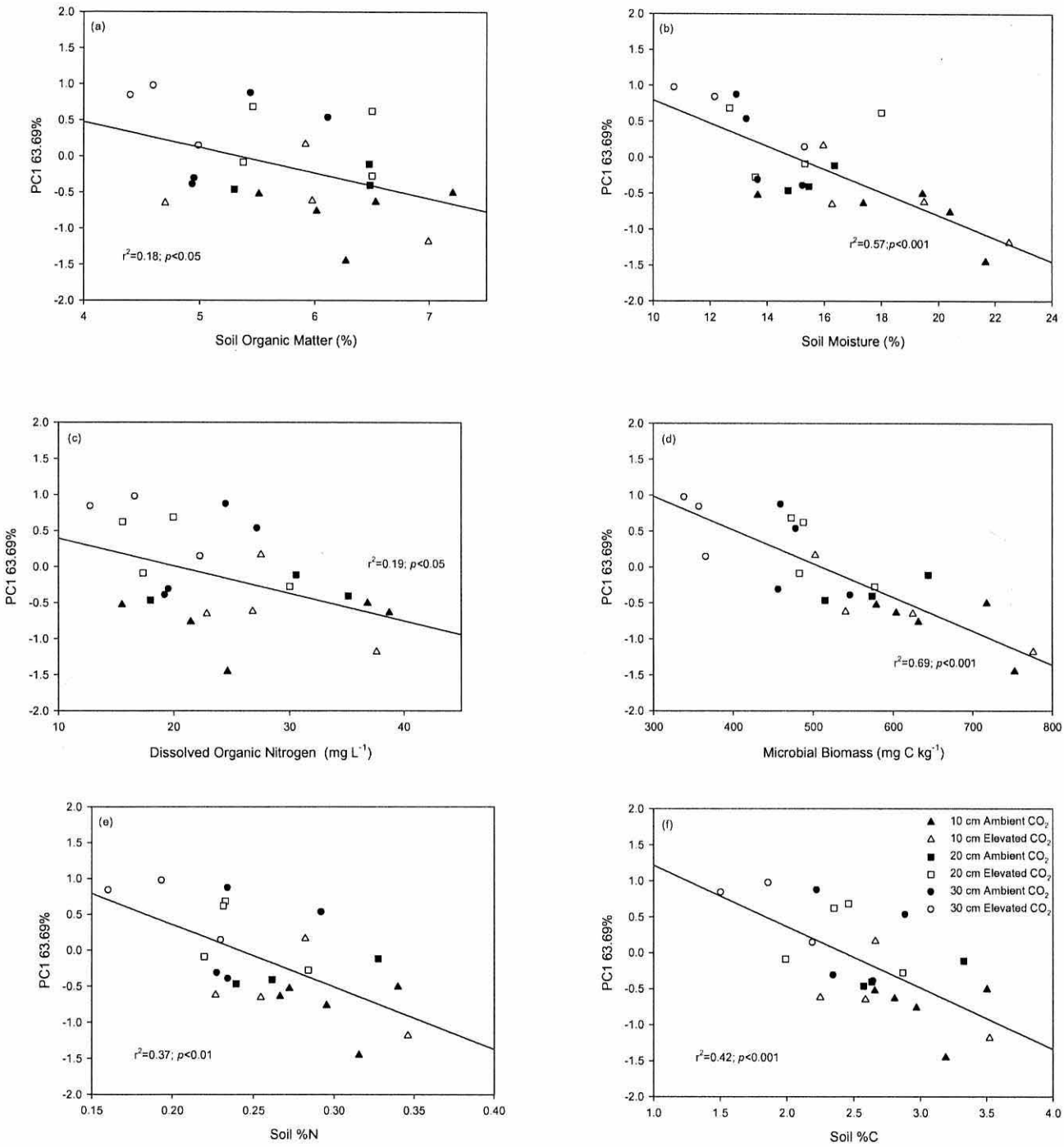


Figure 7.5 Correlations between principle component axis and (a) soil organic matter (b) soil moisture (c) dissolved organic nitrogen (d) microbial biomass (e) soil %N (f) soil %C following principle component analysis of soil solution half lives in the mixed species plots at three soil depths 10,20 and 30 cm. Triangles symbols indicat 0-10 cm, square symbols 10-20 cm and circle symbols (20-30 cm) Filled symbols indicate ambient CO_2 and hollow symbols elevated CO_2. The solid line shows the linear regression of all combined data , correlation coefficient and significance is shown inset in each figure.

7.3.5 The effect of depth on microbial biomass half-life

The mean turnover rate of the microbial biomass in response to oxalic addition was significantly reduced by -13.8% (P=0.048) with 0-10 cm of the soil profile. While lower within the soil profile (10-20 cm) the addition of acetate and salicylic acid resulted in changes of -11.6% (P=0.013) and 20.0% (P=0.009) respectively there was a large decrease in the mean residence time of glucosamine (-27.3%; P=0.065) however this was statistically non-significant. Finally, the deepest sampling (20-30 cm) yielded significant changes in acetate 41.7% (P=0.016), aspartic acid -14.5% (P=0.047) and sucrose 20.2% (P=0.013) shown in (Figure 7.6).

7.3.6 The effect of depth on microbial yield

Overall deeper within the soil profile elevated atmospheric CO_2 reduced the allocation of metabolised substrates to microbial biomass with increasing magnitude and significance (Figure 7.7). Acetate decreased its allocation to the immobilised microbial pool by -6.7% to 41.4 ± 0.0% of total added ^{14}C at 10 cm, whilst at 20 cm there was a -18.8% (37.7 ± 0.0%; P=0.062) reduction in allocation and at 30 cm a -21.7% reduction (32.6 ± 0.0%; P=0.032). However, there was a 7.0% increase in the partitioning of malate to microbial biomass (P=0.028) within 0-10 cm of the soil profile, following the observed general trend in C partitioning this allocation decreased with depth to a -4.9% decrease (P=0.059). The largest magnitude of changes were observed within 20-30 cm of the soil profile, these appeared to be tending to significance with increasing depth, in addition to the previously mentioned substrates there was a decrease of -5.8% (P=0.083) with phenylalanine and a decrease of 6.1% (P=0.030). In contrast to the general trend the allocation of aspartic acid (2.1% ; 0-10 cm, 3.5% ; 20-30 cm; P=0.062, 3.6% ; 20-30 cm; P=0.069) and glycine to the microbial pool increased with depth. However, these changes were small and not significant.

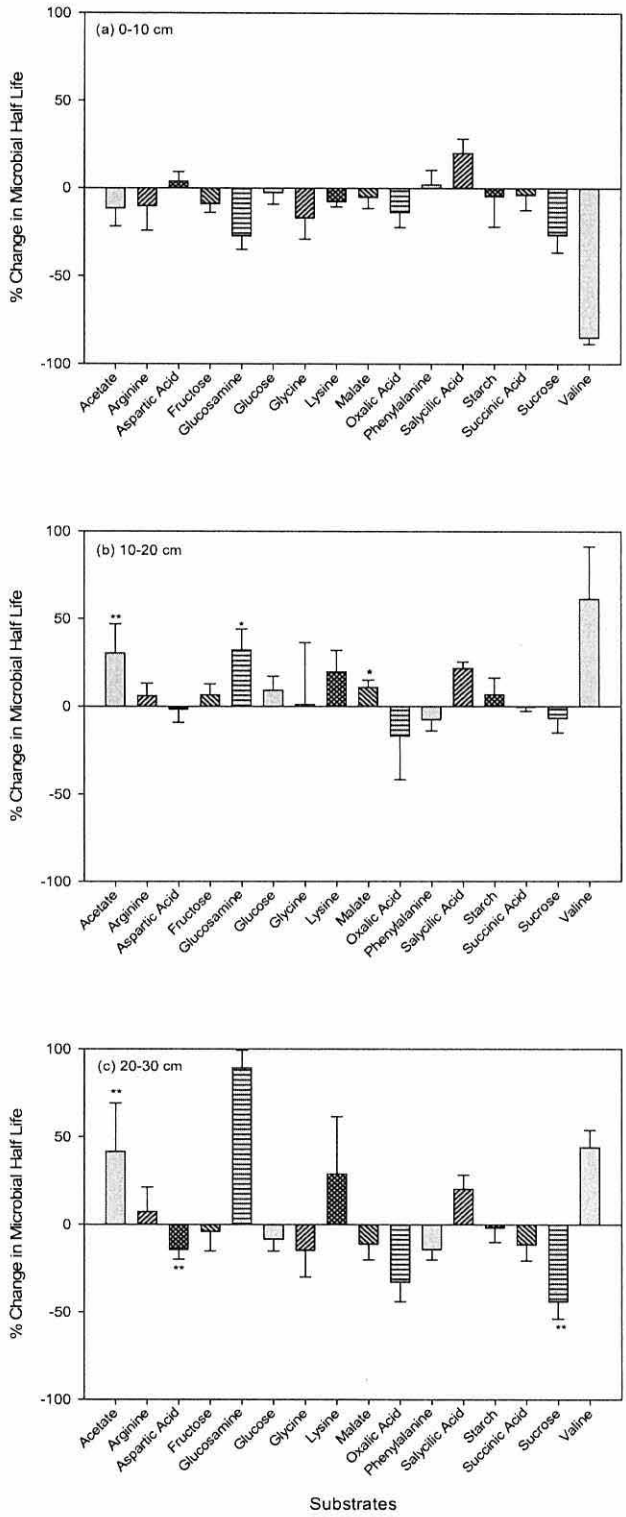


Figure 7.6 Percentage change in microbial half life relative to ambient atmospheric CO_2 plots for the three species mixture, panels (a) 0-10 cm (b) 10-20 cm (c) 20-30 cm. Error bars signify one standard error. Statistical significance is denoted by $*P<0.10$, $**P<0.05$.

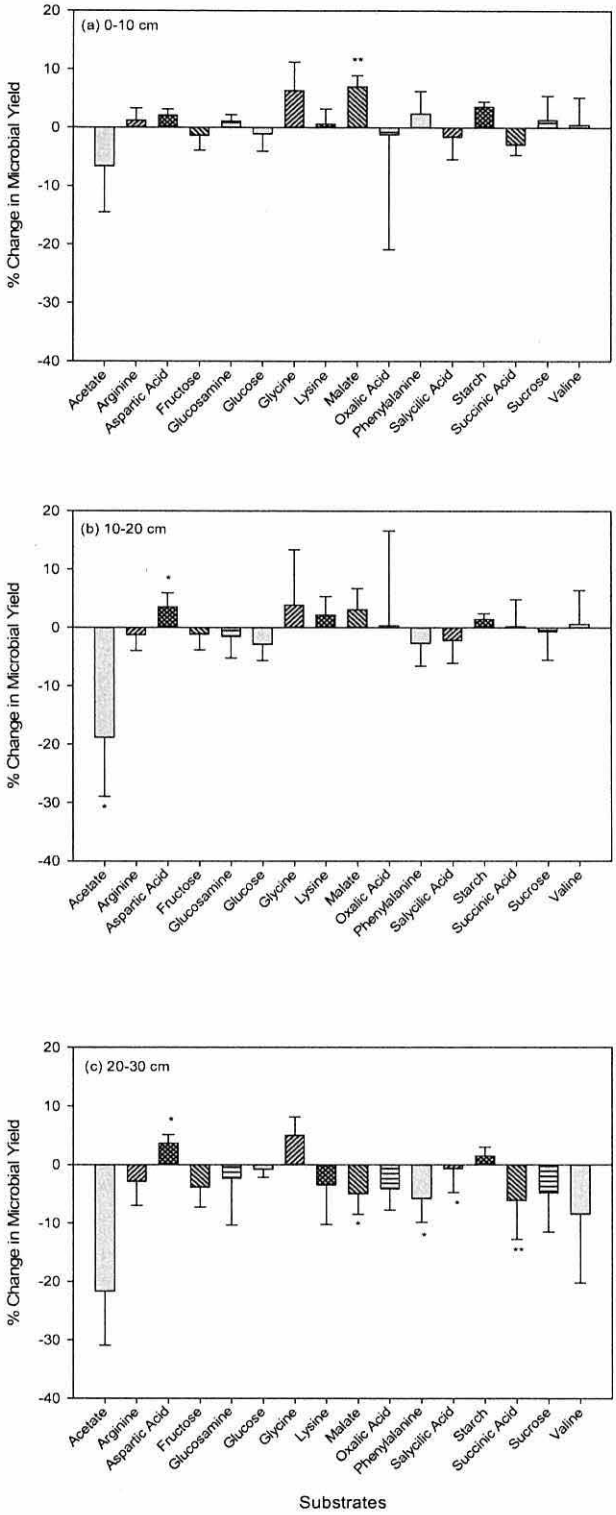


Figure 7.7 Percentage change in microbial yield relative to ambient atmospheric CO_2 plots for the three species mixture, panels (a) 0-10 cm (b) 10-20 cm (c) 20-30 cm. Error bars signify one standard error. Statistical significance is denoted by $*P<0.10$, $**P<0.05$.

Table 7.3 Pearson correlation coefficients determined between principle component analysis axis explaining 63.69% of the variation in the soil solution half lives of all substrates applied to the soil sampled from the three species mixture plots at three depths (0-10, 10-20 and 20-30 cm). Significant correlations are highlighted in bold. The level of statistical significance is denoted by $^{*}P<0.05$; $^{**}P<0.01$.

Depth	Treatment	SOM	Soil moisture	%N	DON	NO_3^-	NH_4^+	DOC	%C	pH	Microbial biomass	Fine root	K	Ca	Na
10	Ambient	-.126	-.557	-.470	.096	-.802	.659	-.203	-.455	-.040	-.870	.934	.519	-.215	-.151
	Elevated	-.693	-.938	-.704	-.849	**-.963***	**-.974***	.438	-.801	-.122	-.930	.560	.739	.990	.724
20	Ambient	**-.985***	-.699	-.542	-.945	.367	-.119	-.695	-.470	**.978***	-.678	**-.997****	-.827	**1.000****	.730
	Elevated	-.495	.178	-.825	-.822	.665	.54	.39	-.551	.922	-.944	.878	-.362	.709	-.876
30	Ambient	.461	-.777	.086	.649	-.636	-.634	**.977***	-.431	-.730	-.557	-.792	.481	-.354	.113
	Elevated	**-.997****	-.416	-.950	-.868	-.452	.831	.884	**-.952***	.132	**-.964***	.372	.229	.359	-.924

Table 7.4 Pearson correlation coefficients determined between principle component analysis axis explaining 44.02% of the variation in the soil solution half lives of all substrates applied to the soil sampled from each of the single species subplots at 0-10 cm. Significant correlations are highlighted in bold. The level of statistical significance is denoted by $^{*}P<0.05$; $^{**}P<0.01$.

Treatment	Species	SOM	Soil moisture	%N	Microbial biomass	DON	NO_3^-	NH_4^+	DOC	%C	Fine root	Dead root	K	Ca	Na	P
Ambient	*A. glutinosa*	-.057	-.820	**.974***	-.783	.767	**.985***	**.972***	.551	**.997****	.711	.622	.131	-.460	-.761	.451
	B. pendula	-.328	-.260	.125	-.255	.169	.096	.696	.366	.213	-.516	.325	.560	.500	.707	-.843
	F. sylvatica	-.800	.253	.493	-.592	.839	-.096	-.688	.637	.369	-.326	-.408	.157	-.317	-.577	.881
	Mixture	.606	-.785	-.076	-.822	.697	-.347	.520	.550	.052	.421	-.884	.943	-.740	-.512	.718
Elevated	*A. glutinosa*	.375	-.510	**-.972***	**-.965***	.155	.024	-.809	-.281	-.735	**.964***	.317	-.665	.818	.831	.195
	B. pendula	.500	.069	.325	-.167	.714	-.718	.263	-.169	.160	-.695	-.910	-.382	-.556	.202	.725
	F. sylvatica	.952	-.507	-.969	-.989	.974	.577	-.908	.196	-.946	-.945	.715	.311	-.783	-.620	-.458
	Mixture	-.251	-.760	-.205	-.769	-.402	-.634	-.696	.358	-.371	.328	**.999***	.940	-.347	**.990***	.480

Table 7.5a First-order kinetic parameters describing the mineralization of ^{14}C-labelled LMW substrates under *A. glutinosa* and *B. pendula* grown in monoculture exposed to elevated and ambient atmospheric CO_2. The kinetic parameters a_1 and a_2 represent the size of the carbon catabolism pool (a_1), and microbial immobilisation and turnover (a_2) whilst k_1 and k_2 are rate constants describing the of turnover of these pools.

Treatment	Substrate	*A. glutinosa*				*B. pendula*			
		a_1 (%)	k_1	a_2 (%)	$k_2 \times 10^3$	a_1 (%)	k_1	a_2 (%)	$k_2 \times 10^3$
Ambient CO_2	Fructose	**36.87** ± 1.05	**0.05** ± 0.00	**63.42** ± 1.94	**0.17** ± 0.00	**37.51** ± 2.17	**0.04** ± 0.01	**62.61** ± 1.94	**0.17** ± 0.00
	Glucose	**27.83** ± 1.31	**0.10** ± 0.01	**70.04** ± 1.08	**0.15** ± 0.01	**26.61** ± 1.22	**0.08** ± 0.00	**70.78** ± 1.06	**0.16** ± 0.01
	Starch	**21.23** ± 0.10	**0.38** ± 0.09	**76.49** ± 0.56	**0.13** ± 0.00	**20.01** ± 1.05	**0.24** ± 0.09	**77.24** ± 1.11	**0.12** ± 0.01
	Sucrose	**36.86** ± 1.57	**0.03** ± 0.00	**63.68** ± 1.35	**0.14** ± 0.02	**40.31** ± 1.35	**0.02** ± 0.00	**60.35** ± 1.16	**0.15** ± 0.01
	Arginine	**57.63** ± 0.56	**0.03** ± 0.00	**42.76** ± 0.62	**0.16** ± 0.02	**56.78** ± 1.51	**0.03** ± 0.00	**43.36** ± 1.37	**0.14** ± 0.02
	Aspartic acid	**30.36** ± 0.57	**0.75** ± 0.11	**68.76** ± 0.53	**0.09** ± 0.00	**29.70** ± 0.26	**0.81** ± 0.07	**69.54** ± 0.25	**0.09** ± 0.00
	Glycine	**65.32** ± 1.76	**0.03** ±0.00	**37.16** ± 1.59	**0.11** ± 0.01	**66.05** ± 2.00	**0.03** ± 0.00	**35.92** ± 1.74	**0.10** ± 0.01
	Lysine	**40.65** ± 4.45	**0.01** ± 0.00	**55.19** ± 4.00	**0.12** ± 0.01	**44.43** ± 1.71	**0.01** ± 0.00	**51.08** ± 1.56	**0.11** ± 0.00
	Phenlylalanine	**46.51** ± 0.92	**0.01** ± 0.00	**52.37** ± 0.94	**0.13** ± 0.01	**46.21** ± 0.57	**0.01** ± 0.00	**52.37** ± 0.52	**0.13** ± 0.01
	Valine	**60.16** ± 1.46	**0.01** ± 0.00	**41.86** ± 1.36	**0.07** ± 0.02	**66.69** ± 3.18	**0.01** ± 0.00	**35.59** ± 3.09	**0.04** ± 0.01
	Glucosamine	**56.14** ± 0.43	**0.01** ± 0.00	**46.43** ± 0.45	**0.08** ± 0.01	**57.09** ± 1.37	**0.01** ± 0.00	**45.62** ± 1.23	**0.08** ± 0.01
	Salicylic	**63.87** ± 9.37	**0.02** ± 0.00	**43.46** ± 9.64	**12.79** ± 4.32	**63.62** ± 9.60	**0.01** ± 0.00	**43.29** ± 9.94	**11.02** ± 3.68
	Acetate	**55.55** ± 2.99	**0.05** ± 0.00	**46.85** ± 2.64	**0.17** ± 0.01	**60.17** ± 1.57	**0.04** ± 0.00	**42.33** ± 1.34	**0.18** ± 0.01
	Malate	**35.23** ± 1.66	**0.12** ± 0.02	**63.93** ± 1.71	**0.16** ± 0.01	**34.05** ± 0.56	**0.09** ± 0.02	**64.49** ± 0.41	**0.15** ± 0.01
	Oxalic	**42.61** ± 1.14	**0.02** ± 0.00	**42.08** ± 1.14	**18.11** ± 1.28	**42.93** ± 1.58	**0.02** ± 0.00	**42.42** ± 1.55	**16.52** ± 1.23
	Succinic	**15.27** ± 0.73	**0.03** ± 0.00	**83.49** ± 0.73	**0.12** ± 0.01	**17.40** ± 4.17	**0.02** ± 0.00	**81.46** ± 3.91	**0.12** ± 0.01
Elevated CO_2	Fructose	**33.61** ± 0.91	**0.06** ± 0.00	**66.49** ± 0.79	**0.18** ± 0.00	**35.28** ± 2.04	**0.05** ± 0.01	**64.89** ± 1.88	**0.19** ± 0.00
	Glucose	**26.29** ± 0.67	**0.12** ± 0.02	**71.68** ± 0.67	**0.17** ± 0.00	**28.17** ± 0.94	**0.12** ± 0.01	**69.91** ± 1.11	**0.17** ± 0.00
	Starch	**20.95** ± 0.80	**0.43** ± 0.01	**77.47** ± 0.89	**0.13** ± 0.00	**18.82** ± 0.53	**0.31** ± 0.07	**78.84** ± 0.64	**0.12** ± 0.01
	Sucrose	**36.76** ± 2.45	**0.03** ± 0.00	**63.33** ± 2.32	**0.13** ± 0.01	**36.69** ± 2.70	**0.02** ± 0.00	**63.37** ± 2.50	**0.15** ± 0.01
	Arginine	**56.52** ± 0.66	**0.03** ± 0.00	**44.03** ± 0.49	**0.15** ±0.01	**57.28** ± 1.15	**0.03** ± 0.00	**43.47** ± 1.09	**0.15** ± 0.01
	Aspartic acid	**29.99** ± 0.99	**0.83** ± 0.04	**69.30** ± 1.03	**0.09** ± 0.00	**29.68** ± 0.59	**0.71** ± 0.08	**69.49** ± 0.53	**0.09** ± 0.01
	Glycine	**66.16** ± 1.41	**0.03** ± 0.00	**36.29** ± 1.26	**0.11** ± 0.00	**67.16** ± 2.26	**0.03** ± 0.00	**35.50** ± 1.74	**0.11** ± 0.01
	Lysine	**44.25** ± 0.41	**0.01** ± 0.00	**52.41** ± 0.78	**0.11** ± 0.00	**44.79** ± 1.16	**0.01** ± 0.00	**51.28** ± 1.00	**0.13** ± 0.00
	Phenlylalanine	**46.06** ± 0.61	**0.01** ± 0.00	**52.61** ± 0.65	**0.14** ± 0.01	**46.64** ± 1.62	**0.01** ± 0.00	**51.77** ± 1.29	**0.15** ± 0.01
	Valine	**59.40** ± 0.68	**0.01** ± 0.00	**42.75** ± 0.66	**0.07** ± 0.01	**56.11** ± 1.61	**0.01** ± 0.00	**46.12** ± 1.50	**0.07** ± 0.01
	Glucosamine	**56.77** ± 1.19	**0.01** ± 0.00	**45.86** ± 2.45	**0.09** ± 0.02	**54.67** ± 2.83	**0.10** ± 0.00	**47.66** ± 2.45	**0.10** ± 0.02
	Salicylic	**54.54** ± 0.24	**0.02** ± 0.00	**52.79** ± 0.04	**17.81** ± 0.99	**54.13** ± 0.22	**0.02** ± 0.00	**53.08** ± 0.07	**15.59** ± 0.99
	Acetate	**56.61** ± 3.24	**0.05** ± 0.00	**45.69** ± 2.82	**0.16** ± 0.01	**60.90** ± 1.64	**0.05** ± 0.00	**41.56** ± 1.54	**0.19** ± 0.01
	Malate	**32.59** ± 0.90	**0.13** ± 0.00	**66.81** ± 0.78	**0.15** ± 0.01	**33.72** ± 0.91	**0.11** ± 0.02	**65.30** ± 1.70	**0.15** ± 0.01
	Oxalic	**42.69** ± 1.05	**0.02** ± 0.00	**42.01** ± 1.02	**18.14** ± 0.45	**42.82** ± 1.43	**0.02** ± 0.00	**42.09** ± 1.37	**16.07** ± 1.06
	Succinic	**13.60** ± 0.61	**0.03** ± 0.00	**85.09** ± 0.50	**0.12** ± 0.01	**13.86** ± 0.90	**0.02** ± 0.00	**84.91** ± 0.70	**0.13** ± 0.01

Table 7.5b First-order kinetic parameters describing the mineralization of ^{14}C-labelled LMW substrates under *F. sylvatica* grown in monoculture and a mixture of three species exposed to elevated and ambient atmospheric CO_2. The kinetic parameters a_1 and a_2 represent the size of the carbon catabolism pool (a_1), and microbial immobilisation and turnover (a_2) whilst k_1 and k_2 are rate constants describing the of turnover of these pools.

Treatment	Substrate	*F. sylvatica*				*Mixture*			
		a_1 (%)	k_1	a_2 (%)	$k_2 \times 10^3$	a_1 (%)	k_1	a_2 (%)	$k_2 \times 10^3$
Ambient CO_2	Fructose	**35.99** ± 0.81	**0.06** ± 0.00	**64.38** ± 0.74	**0.18** ± 0.00	**33.81** ± 1.19	**0.06** ± 0.01	**66.45** ± 1.11	**0.17** ± 0.00
	Glucose	**28.50** ± 1.78	**0.10** ± 0.01	**69.29** ± 1.78	**0.16** ± 0.01	**26.31** ± 0.66	**0.13** ± 0.01	**71.89** ± 0.46	**0.16** ± 0.00
	Starch	**19.76** ± 1.37	**0.34** ± 0.10	**77.21** ± 1.01	**0.12** ± 0.01	**20.83** ± 1.38	**0.31** ± 0.04	**76.54** ± 1.42	**0.13** ± 0.01
	Sucrose	**38.88** ± 4.04	**0.02** ± 0.00	**61.86** ± 3.67	**0.13** ± 0.01	**35.81** ± 2.19	**0.03** ± 0.00	**64.55** ± 2.09	**0.14** ± 0.01
	Arginine	**59.66** ± 1.74	**0.03** ± 0.00	**41.11** ± 1.52	0.16 ± 0.02	**59.96** ± 0.83	**0.03** ± 0.00	**43.41** ± 0.64	**0.16** ± 0.01
	Aspartic acid	**28.86** ± 0.74	**0.77** ± 0.11	**70.12** ± 0.54	**0.09** ± 0.00	**30.46** ± 0.93	**0.84** ± 0.05	**68.83** ± 0.91	**0.09** ± 0.00
	Glycine	**68.81** ± 3.68	**0.02** ± 0.00	**33.42** ± 3.31	**0.09** ± 0.01	**66.71** ± 1.14	**0.03** ± 0.00	**35.17** ± 1.08	**0.11** ± 0.00
	Lysine	**44.15** ± 1.34	**0.01** ± 0.00	**51.39** ± 1.28	**0.10** ± 0.00	**42.73** ± 1.08	**0.01** ± 0.00	**53.27** ± 0.88	**0.10** ± 0.00
	Phenlylalanine	**46.77** ± 1.12	**0.01** ± 0.00	**51.71** ± 1.02	**0.14** ± 0.01	**46.80** ± 1.47	**0.01** ± 0.00	**51.66** ± 1.50	**0.14** ± 0.01
	Valine	**63.55** ± 0.71	**0.01** ± 0.00	**38.08** ± 0.71	**0.04** ± 0.00	**60.66** ± 1.83	**0.01** ± 0.00	**41.60** ± 1.77	**0.06** ± 0.01
	Glucosamine	**55.27** ± 1.89	**0.01** ± 0.00	**47.24** ± 1.74	**0.07** ± 0.00	**54.82** ± 1.14	**0.01** ± 0.00	**47.66** ± 1.05	**0.08** ± 0.01
	Salicylic	**54.10** ± 0.14	**0.02** ± 0.00	**52.92** ± 0.08	**15.58** ± 0.79	**54.19** ± 0.35	**0.02** ± 0.00	**52.82** ± 0.22	**18.97** ± 1.74
	Acetate	**61.36** ± 3.59	**0.05** ± 0.00	**41.64** ± 3.10	**0.17** ± 0.01	**57.04** ± 2.04	**0.06** ± 0.01	**45.47** ± 2.02	**0.19** ± 0.01
	Malate	**36.37** ± 1.80	**0.09** ± 0.02	**62.47** ± 1.70	**0.16** ± 0.01	**36.82** ± 0.45	**0.12** ± 0.02	**62.48** ± 0.55	**0.17** ± 0.01
	Oxalic	**43.34** ± 0.83	**0.02** ± 0.00	**42.85** ± 0.86	**16.05** ± 1.09	**42.53** ± 0.50	**0.01** ± 0.00	**42.16** ± 0.47	**14.39** ± 0.94
	Succinic	**18.21** ± 4.23	**0.02** ± 0.00	**81.04** ± 4.11	**0.13** ± 0.01	**13.98** ± 0.61	**0.03** ± 0.00	**84.69** ± 0.55	**0.12** ± 0.00
Elevated CO_2	Fructose	**34.38** ± 1.08	**0.06** ± 0.01	**65.72** ± 1.11	**0.20** ± 0.00	**34.67** ± 1.03	**0.06** ± 0.01	**65.54** ± 1.10	**0.19** ± 0.00
	Glucose	**26.73** ± 1.06	**0.12** ± 0.01	**71.70** ± 1.05	**0.17** ± 0.01	**27.01** ± 0.89	**0.12** ± 0.01	**71.00** ± 1.15	**0.16** ± 0.01
	Starch	**19.28** ± 1.22	**0.38** ± 0.13	**78.25** ± 0.86	**0.12** ± 0.01	**18.18** ± 1.08	**0.36** ± 0.09	**79.58** ± 0.88	**0.12** ± 0.00
	Sucrose	**35.57** ± 1.01	**0.03** ± 0.00	**64.81** ± 1.10	**0.16** ± 0.01	**34.86** ± 2.35	**0.03** ± 0.00	**65.27** ± 2.33	**0.15** ± 0.01
	Arginine	**58.43** ± 1.07	**0.03** ± 0.00	**42.17** ± 1.06	**0.17**± 0.01	**56.44** ± 2.76	**0.03** ± 0.00	**43.95** ± 2.62	**0.15** ± 0.01
	Aspartic acid	**28.83** ± 0.84	**0.70** ± 0.09	**70.24** ± 0.73	**0.09** ± 0.00	**28.96** ± 1.32	**0.79** ±0.03	**70.22** ± 1.27	**0.09** ± 0.00
	Glycine	**63.95** ± 2.06	**0.03** ± 0.00	**38.46** ± 1.77	**0.11**± 0.01	**64.74** ± 1.03	**0.03** ± 0.00	**37.55** ± 0.92	**0.12** ± 0.02
	Lysine	**46.09** ± 0.26	**0.01** ± 0.00	**49.76** ± 0.37	**0.11**± 0.01	**42.43** ± 0.92	**0.01** ± 0.00	**53.66** ± 0.98	**0.10** ± 0.01
	Phenlylalanine	**44.82** ± 0.97	**0.01** ± 0.00	**54.28** ± 0.98	**0.14** ± 0.00	**45.56** ± 0.99	**0.01** ± 0.00	**52.89** ± 0.90	**0.14** ± 0.01
	Valine	**54.49** ± 3.77	**0.01** ± 0.00	**47.43** ± 3.48	**0.08** ± 0.01	**60.37** ± 0.73	**0.01** ± 0.00	**41.77** ± 0.71	**0.06** ± 0.01
	Glucosamine	**53.45** ± 0.88	**0.01** ± 0.00	**49.03** ± 0.85	**0.94** ± 0.01	**54.28** ± 1.02	**0.01** ± 0.00	**48.17** ± 0.96	**0.07** ± 0.01
	Salicylic	**54.10** ± 0.08	**0.02** ± 0.00	**53.08** ± 0.39	**17.89** ± 1.33	**54.33** ± 0.27	**0.02** ± 0.00	**52.74** ± 0.22	**16.17** ± 1.70
	Acetate	**56.72** ± 2.33	**0.06** ± 0.00	**45.72** ± 2.10	**0.19** ± 0.02	**60.25** ± 2.48	**0.05** ± 0.00	**42.54** ± 2.12	**0.18** ± 0.01
	Malate	**35.95** ± 0.60	**0.12** ± 0.01	**63.58** ± 0.66	**0.17** ± 0.01	**32.45** ± 1.52	**0.12** ± 0.02	**66.82** ± 1.31	**0.15** ± 0.01
	Oxalic	**43.79** ± 1.29	**0.02** ± 0.00	**42.99** ± 1.22	**18.09** ± 0.54	**42.20** ± 0.00	**0.02** ± 0.00	**42.20** ± 1.04	**18.71** ± 1.60
	Succinic	**15.15** ± 1.28	**0.02** ± 0.00	**83.26** ± 1.05	**0.13** ± 0.01	**16.45** ± 2.45	**0.02** ± 0.00	**82.24** ± 2.25	**0.11** ± 0.01

7.4 Discussion

The main objective of this work was to determine how elevated CO_2 impacted upon soil microbial LMW substrate mineralisation kinetics and utilisation profiles in soil supporting three temperate forest species. The planting design implemented at the BangorFACE experiment also allowed inter and intra species competitive interactions of species grown in monoculture and a three species mixture to be examined in response to an enriched CO_2 atmosphere. This was achieved by applying an array of sugars, organic and amino acids to soil harvested from experimental plots exposed to elevated CO_2 treatment for three years.

7.4.1 Species Diversity and LMW Substrate Metabolism in Soil

Our results clearly show a decrease in the residence time (faster turnover) of the dissolved organic C pool under elevated CO_2 when species are grown in monoculture. Conversely, when the three tree species in this study were grown in polyculture a consistent increase in the residence time (slower turnover) of LMW C in the dissolved organic C pool was observed. The residence time of sugars, organic, and amino acids in the top 10 cm of soil in this study ranged between 1.0 and 128.7 hours with a median residence time of 27.8 hours which is similar to previously reported values, and consistent with the slower turnover of amino acids compared to sugars (Boddy *et al.* 2007; Lipson *et al.* 2001; Jones *et al.* 1999). Correlation between principle component factors and measured environmental variables revealed a strong negative relationship between pH and substrate residence time. Fundamental to soil microbial community dynamics is the dominant role of pH and substrate quality (C:N ratio) on soil microbial community composition. At low pH, fungi are favoured over bacteria and vice versa, while fungi prefer higher C:N ratios than bacteria (Högberg *et al.* 2007). In agreement with current theory our observations suggest that substrate catabolism was mediated through substrate-specific microbial populations within the community. An observation that is supported by the significant correlation between microbial C:N ratios and substrate residence time. However, as we were unable to identify a treatment or species specific correlation with these variables our data also indicates that the microbial community structure was not functionally altered by elevated CO_2. Previous studies using biomarkers and molecular techniques have reported a lack of significant change in microbial community composition with FACE

(Lipson *et al.* 2005; Austin *et al.* 2009). In contrast, the AspenFACE forests amended soil with ^{13}C labelled cellulose and *N*-acetyl-glucosamine altered phospholipid fatty acids profiles indicating a shift in fungal community composition and proportional switch from gram positive to gram negative bacteria (Phillips *et al.* 2002).

Trees grown in monoculture and polyculture produced different catabolic substrate utilisation patterns following enrichment with CO_2. These differences are most easily explained by tree species-specific microbial communities. In agreement Grayston *et al.* (1998) showed species specific rhizosphere microbial populations in forests, and attributed the changes to differences chemical composition and quantity of exudates (Grayston *et al.* 1996; Grayston *et al.* 1998). Species diversity has also been shown to alter bacterial diversity in grasslands enriched with elevated atmospheric CO_2, although elevated CO_2 alone did not increase bacterial diversity or richness (Grüter *et al.* 2006). Differences in microbial community composition may be explained by recent evidence showing that plants recognise their neighbour as kin or stranger and modify their root growth pattern and exudation appropriately (Badri and Vivanco, 2009). Although the mechanisms pertaining to inter and intra-species interactions are poorly understood it has been suggested that root exudation and subsequent changes in microbial community may mediate this response (de Kroon, 2007).

An alternative source of LMW C in the present study is root turnover and litter inputs, often evident in elevated CO_2 studies. We found strong positive and negative correlations between LMW C residence time and root inputs under different species suggesting the species specific LMW C inputs have difference impacts on microbial mineralisation. In general, microbial populations are thought to be C limited (Zak *et al.* 2000). On the other hand microbial utilisation of C compounds may be limited by the availability of mineral N or poor N supply especially during the exponential growth phase of forests (van Veen *et al.* 1991). Decreases in microbial catabolic activity was observed in N limited soils under *P. alba* and *P. nigra* whereas enhancement of substrate utilisation was observed in fertilised plots (Lagomarisino *et al.* 2007). However, the lack of impact upon C partitioning and microbial turnover in this study suggests that substrate addition favoured catabolic respiration or *r*-strategist microbes that are immediately response to substrate addition (Stenström *et al.* 1998). As *K*-strategists are usually involved in the metabolism of recalcitrant substrates our

data suggests that priming of recalcitrant soil organic matter is not currently utilised as a mechanism maintaining nutrient supply.

7.4.2 Elevated CO_2 induced functional responses

The functional role of LMW C is often postulated however there are relatively few conclusive studies on their metabolism in soil (Jones *et al.* 2009). Many plant species have been shown to increase the flux of organic acids such as citrate, malate and oxalate in response to P and Fe deficiency (Ae *et al.* 1990). In the data presented here, oxalic acid was the only substrate that increased (22%) its rate of mineralisation within the polyculture subplots. Oxalic acid exudation via roots or mycorrhizae has been shown to directly increase availability of inorganic P through the complexation of Ca, Al and Fe (Marschner, 1995; Graustein *et al.* 1977). Organic acids have a seminal role in the dissolution of inorganic P, their efficiency of dissolution follows the order citrate > oxalate > malate > acetate (Jones *et al.* 1998). The significant increase in oxalic acid turnover in polyculture eludes to greater demand for inorganic P, which is consistent with increased P demand during above and belowground overyielding observed at BangorFACE (Chapter 5).

As forest productivity is tightly coupled to nutrient availability, elevated atmospheric CO_2-induced growth responses require a proportional change in nutrient cycling to the trees increased meet metabolic demand and prevent growth constraint (Oren *et al.* 2001; Millard *et al.* 2007). Rhizodeposition is an essential component of the coupling between plant growth and soil nutrient cycling (Paterson *et al.* 2003). Consequently, in nutrient poor conditions uptake and mobilisation of nutrients may be enhanced by stimulation of organic acid exudation. Organic acids may directly impact upon inorganic nutrient availability or influence microbial mineralisation of soil organic matter (Farrar *et al.* 2003). Zak *et al.* (1993) found an increased microbial biomass and organic matter mineralisation under *Populus grandidentata* seedlings exposed to elevated CO_2 which implied that rapidly aggrading forests may obtain N from the soil during high N demand. Conversely, high C:N ratios have been shown to immobilize N in the microbial biomass rather than increase availability through mineralisation (Diaz *et al.* 1993). In the present study soil N content was contrastingly correlated between elevated and ambient CO_2 plots in both *A. glutinosa* and *F. sylvatica* suggesting that N availability is influencing mineralisation. Supporting this

observation was the large response of malate to CO_2 treatment in these species. Malic acid, an intermediate in the tricarboxylic acid (TCA) cycle is also purported to be a signalling molecule with regulatory roles in plant-microbe interactions (Rudrappa *et al.* 2008). As a preferred substrate of N fixing bacteria it's presence in soil may attract bacteria and facilitate N input through bacterial fixation (Fernie *et al.* 2009). Dakora and Philips, (2002) reported other specific chemoattractants for micro organisms such as amino acids and dicarboxylic acids which can also attract pathogenic microbes, mutualistic fungi and bacteria antagonistic to pathogenic organisms. In agreement with our results, community level physiological profiles of the microbial community under *P. alba, P. nigra* and *P. x euramericanana* examined using MicroResp™ revealed that malic and oxalic acid produced the highest respiration response to elevated CO_2 regardless of fertilisation treatments (Lagomarisino *et al.* 2007).

7.4.3 The impact of depth on LMW C mineralisation

Application of LMW substrates to soil collected from three soil depths unequivocally resulted in reduced substrate residence time of increasing magnitude with depth in the elevated CO_2 plots. With all data pooled correlations revealed that organic matter, moisture, dissolved organic N, microbial biomass and soil total C and N were overarching drivers unaffected by treatment or depth. We believe our treatment observations are easily explained through increased root proliferation at depth, an often observed response to elevated CO_2 enrichment and were supported by PCA (Iverson *et al.* 2008; Iverson *et al.* 2010; Chapter 4). With this in mind, increased oxygenation and labile C input mediated by roots are likely to have stimulated microbial mineralisation. Indeed, during an incubation experiment Fontaine *et al.* (2007) showed that the addition of fresh C to deep soil stimulated microbial catabolic processes, but highlighted with potential to mineralise old C by microbial co-metabolism.

Principle component analysis supported our observation of increased substrate catabolism at depth. Plots of environmental component loadings indicated that soil moisture, organic matter content and Olsen P influence mineralisation at the soil surface, whereas fine root biomass and necromass influenced mineralisation at depth. Interestingly oxalic acid, Ca and pH were grouped together at 30 cm in the ambient plots suggesting that calcium oxalate formation may be involved in the liberation of

phosphate. In the elevated CO_2 plots glucosamine and malic, acetic, and aspartic organic acids correlated well with mineralisation at 30 cm. Glucosamine is a substantial component of fungal cell walls eluding to the presence of saprophytic or mycorrhizal fungi (Joergensen and Wichern, 2008). Trees utilise symbiotic relationships with mycorrhizal fungi for improved nutrient scavenging and acquisition which is often achieved through the exudation of organic acids (Smith and Read, 2008; Jones *et al.* 2009). In a meta-analysis of mycorrhizal response to CO_2 in field studies Treseder (2004) showed a 47% increased in abundance. Supporting the hypothesis that higher nutrient demand of forests in an elevated CO_2 atmosphere is mediated by mycorrhizal fungi in exchange for carbohydrates. Our observations are further supported data obtained at the AspenFACE experiment where Karnosky *et al.* (2003) measured soil enzyme activity and identified increased activity of enzymes involved in plant and fungal cell wall degradation and concluding that soil microbial structure had altered the soil metabolism of plant-derived compounds.

Microbial utilisation patterns were remarkably similar throughout the soil profile suggesting that the microbial community was not altered by depth. While the turnover of microbial biomass and C partitioning between catabolic and anabolic pools was significantly altered by elevated CO_2 the results were not consistent with increases or decreases in microbial turnover.

7.5 Conclusion

Community level physiological profiles were determined from soil collected under three tree species grown in an enriched CO_2 atmosphere in monoculture and a three species polyculture. Soil was amended with sixteen different LMW substrates and CO_2 evolution was monitored at regular intervals enabling the determination of microbial substrate use kinetics and C use efficiency. When species were grown in monoculture in the elevated CO_2 plots the overall response to substrate addition was an increase in catabolic respiration in agreement with our first hypothesis. However, when the studied species were grown in polyculture and exposed to elevated CO_2 a decrease in catabolic respiration was observed relative to ambient conditions suggesting that resource limitation constrained microbial catabolic simulation. In agreement with our second hypothesis was a difference in catabolic utilisation profile

of each species when enriched by elevated CO_2 indicating that elevated CO_2 induced changes in the microbial community structure. We suspect that species-specific biochemical treatment responses and differential LMW compound input to soil, partly to enhance resource acquisition, is responsible for altering the community structure and function. In agreement with our third hypothesis the catabolism of LMW compounds increased with depth in the elevated CO_2 plots, which was attributable to deeper, more prolific rooting, and mycorrhizal mycelium inputs resulting in microbial stimulation at depth. In summary, our data shows that microbial function has been altered by elevated CO_2 treatment which we believe is attributable to root and mycorrhizal perturbation of exudation and detrital inputs which are tightly coupled to stand productivity and nutrient acquisition.

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7.7 References

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8 Temperate forest enriched by elevated atmospheric CO_2 increases phosphorus mineral weathering

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Contributions:

Andrew Smith wrote the manuscript, collected data, performed analysis and maintained the FACE facility during 2006-2009. Dr. Martin Lukac designed, built and maintained the experiment during 2004-2006. Iftekhar Ahmed provided leaf phosphorus data from his MSc in 2006. Prof. Douglas Godbold is the project principle investigator who designed and constructed the experiment. He also provided assistance with data interpretation, analysis and manuscript preparation.

8.1 Introduction

Human activities are increasing atmospheric CO_2 concentrations at an unprecedented rate (IPCC, 2007). Temperate and boreal forests are important sinks for anthropogenic CO_2 emissions, and have been estimated to contain 60.72 Gt C in vegetation alone, and provide a sink of 0.68 Gt C yr^{-1} (Millard *et al.* 2007). In the current atmosphere the carboxylation enzyme ribulose-1,5-bisphosphate is not CO_2 saturated and experiments using elevated CO_2 have demonstrated increases in rates of photosynthesis (Long *et al.* 2004). In forests, a 200 ppm increase in CO_2 concentration has been shown to result in *circa.* 23% increase in biomass across a range of productivity (Norby *et al.* 2005). Nutrient availability exerts a major control over the response of plants and ecosystems which has been shown to constrain the response of forests growing in nutrient poor environments under elevated CO_2 (Oren *et al.* 2001; Campbell & Sage, 2006). After N, P is the most limiting macronutrient for plant growth. P is a significant structural component of plants comprising approximately 0.2% of a plants dry weight. Essential biochemical molecules such as nucleic acids, phospholipids, phosphoproteins, co-enzymes and the biological energy transfer molecule adenosine-5'-triphosphate are dependant on its availability (Tate, 1984; Schachtman *et al.* 1998). Availability of P for plant growth is governed by complex biogeochemical processes (Larson, 1967).

P is present in soil as inorganic minerals and organic residues of vegetation and microbial biomass. These range in availability from highly labile orthophosphates dissolved in soil solution to intermediately available organic P, and highly recalcitrant primary minerals such as apatite or feldspar that replenish soil P pools. As primary mineral weathering is a slow process, the availability of P in forest ecosystems is principally controlled by the mineralisation of organic P derived from root and mycorrhizal turnover and aboveground detritus input (Schlesinger, 1991). Replenishment of available P for plant uptake in forest soils is controlled by the rapid cycling of organic P, which releases inorganic P for incorporation into microbial biomass, or for plant root uptake. Smaller contributions to this pool occur through slow mineral weathering and mineralisation of increasingly recalcitrant organic residues (Coleman *et al.* 1983; Tate, 1984). Plants and microbes may directly influence organic P cycling through the excretion of phosphatases into the rhizosphere that catalyze the mineralisation of organic P and hydrolyse phosphoric acid

monoesters (McGill and Cole, 1981). Both plants and mycorrhizae are able to influence P availability from inorganic sources through the exudation of low molecular weight (LMW) organic acids from fine roots and hyphae. Phosphate ions liberated by mineralisation or weathering are often rapidly combined with Ca^{2+}, Al^{3+} or Fe^{3+} to form relatively insoluble phosphates. Organic acids such as oxalate directly increase the availability of inorganic P by chelating Ca, Al and Fe and releasing inorganic P for plant uptake (Jones *et al.* 2004). Evidence of organic acid exudation on mineral weathering has been demonstrated by Jongmans *et al.* (1997) who found networks of tubular pores 3-10 μm diameter in feldspar of forest soils suggesting that extramatrical mycelium actively mines nutrients from mineral rocks. Furthermore, using particle induced X-ray emission Wallander *et al.* (2002) showed that the ectomycorrhizal species *Rhizopogon* mobilized significant quantity of nutrient ions from apatite via rhizomorphs without the ions entering the soil exchangeable pool suggesting that ectomycorrhizal primary mineral weathering may be a significant source of P to trees.

Elevated CO_2 may directly influence P cycling through strong metabolic demand, which may deplete plant available P pools and limit plant growth. However, in forest ecosystems 20-80% of total P is occluded in organic residues which are relatively rapidly cycled. Elevated CO_2 increases net primary productivity (Norby *et al.* 2005), and subsequently organic matter inputs to soil as litter, root and mycorrhizal turnover thereby creating positive feedback. As speculated by Kelly *et al.* (1998) a relationship is likely to exist between NPP and mineral weathering. Mechanisms known to enhance weathering induced by elevated CO_2 include soil acidification (Oh and Richter, 2004), rhizodeposition (Phillips *et al.* 2006) or organic acid leaching during decomposition of litter detritus which can increase silicate dissolution rates by at least a factor of two (Welch and Ullman, 1993).

Despite forest nutrient limitation in forests and elevated CO_2 receiving considerable attention in the literature (Oren *et al.* 2001; Lukac *et al.* 2010), studies investigating the effects of elevated atmospheric CO_2 on soil P fractions are scarce. In early work with elevated CO_2, Norby *et al.* (1986) using *Quercus alba* grown in pots for 40 weeks demonstrated that increased plant uptake of P was not concomitant with decreases in extractable P. Indicating that demand was being met by mineralisation of organic P or solubilisation of inorganic P. Recently acquired data using a modification of the Hedley P fractionation at POPFACE supports this hypothesis. Khan *et al.*

(2008) showed that after 5 years of elevated CO_2 enrichment NaOH, HCl and HNO_3 P fractions under field grown *Populus* increased, postulating that organic matter mineralisation, weathering and mycorrhizal turnover were responsible for replenishing organic P pools. Conversely, in an investigation of a fire regenerated Florida scrub oak ecosystem spanning 5 years using root simulator anion exchange membranes P availability declined (Johnson *et al.* 2000; Johnson *et al.* 2003b). Whilst no effect on P cycling was observed at the sweetgum FACE site at Oak Ridge following 2 years of CO_2 enrichment (Johnson *et al.* 2004). If P use efficiency in plants is not increased elevated CO_2 induced NPP increases should result in a greater demand and possible depletion of soil P.

We examined the effect of growing three broadleaved species in monoculture and a three species mixture under ambient and elevated CO_2 on soil P fractions for two consecutive years using the Hedley P fractionation procedure. The primary aims of this study were to determine if higher P demand of increased forest productivity under elevated atmospheric CO_2 (a) leads to depletion of the plant available P pool, (b) increases the rate of primary mineral P dissolution to meet demand, and (c) increased biomass inputs increase the organic P soil P pools.

8.2 Materials and methods

8.2.1 Site Characteristics

The BangorFACE experimental site was located at Bangor University research facility, Henfaes, Abergwyngregyn (53°14'N, 4°01'W). The site was comprised of eight octagonal plots each separated by a buffer of deciduous trees planted during the experiment initiation in 2004. Each plot was planted with three single species subplots of (*Betula pendula* Roth.), alder (*Alnus glutinosa* (L.) Gaertner) and beech (*Fagus sylvatica* L.) the fourth subplot contained a three species mixture. Four plots were grown in ambient CO_2 conditions and four CO_2 enriched by 200 ppm above ambient. Control of CO_2 delivery was achieved using equipment designed and software modified from that used at the EuroFACE facility (Miglietta *et al.* 2001).

The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Soils are fine loamy brown earth over gravel (Rheidol series) and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002), and as

a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011). Soil texture is 63% sand, 28% silt and 9% clay determined by laser diffraction (Coulter LS particle size analyzer). Climate at the site is classified as Hyperoceanic. Mean annual temperature collected at hourly intervals throughout 2005-2008 was 11.5 °C with an annual rainfall of 1034 mm (Campbell Scientific Ltd, Shepshed, UK),.

8.2.2 Soil Sampling

Soil samples were collected in January 2007 and 2008 during root core sampling using an 8 cm auger corer. Soil cores where collected from a position equidistant from the three nearest trees at two random locations within each single species subplot and the three species subplot. Three cores were taken at 10 cm increments each location to a depth of 30 cm (192 cores). Soil cores were placed directly into polythene bags and stored at 5 °C in the field. Once at the laboratory soil samples were coarse sieved (8 mm) to remove roots and homogenised before drying at 70 °C overnight. Dried soils were then ground to a powder and passed through a 500 μm sieve before being placed in sealed polythene bags and stored ready for analysis. The two samples from each location were then sub-sampled, bulked and homogenised to limit the number of samples for analysis to 96 each year.

8.2.3 Litter P inputs.

Following observations of leaf senescence, fallen leaf litter was collected on a weekly basis from litter baskets 0.11 m^2 until all leaves had abscised (October to December). A litter basket was located in each of the three single species subplots and the three species mixture subplot (4 in each experimental plot). Litter was returned to the laboratory on the day of collection, washed and sorted into individual species, and then dried at 80 °C for 24 hours. The dry weight of each species was determined and recorded for each species subplot within each ambient and elevated CO_2 plot. Nutrient content of leaf litter was determined by induction coupled plasma (ICP) atomic emission spectroscopy from collected fallen leaf litter. 0.2 g of finely ground leaf material was digested in HNO_3 for 130 °C for 18 hours, and then filtered before analysis.

8.2.4 Soil P Fractionation

Phosphate was determined using a modification of the Hedley sequential P fractionation method (Hedley *et al.* 1982a; Hedley *et al.* 1982b; Khan *et al.* 2008). 0.5 g of soil was placed into a 50 ml plastic centrifuge tube. Water soluble P was extracted by the addition of 10 ml of distilled water and followed by shaking for 16 hours at 200 strokes min^{-1}. Immediately after shaking samples were centrifuged at 1500 g for 10 min, the supernatant was then filtered using Whatman #41 paper and transferred to a new tube. The second plant available fraction was extracted by addition of 30 ml of 0.5 M $NaHCO_3$ pH 8.5 (Olsen *et al.* 1954) to the remaining pellet, the solution was then shaken for 16 hours then centrifuged and filtered as above. The $NaHCO_3$ supernatant was collected and the residual pellet further extracted in 30 ml of 0.1 M NaOH, shaken, centrifuged and filtered as above. The NaOH supernatant was again collected and the pellet extracted in 30 ml of 1 M HCl, shaken, centrifuged and filtered as above. At this point the pellet was dried at 70 °C overnight then ground into a fine powder. A 0.2 g sub-sample was then weighed into glass digestion tube, 1.6 ml concentrated HNO_3 and 0.4 ml $HClO_4$ was added and the samples left overnight at room temperature to remove volatile oxidants. Sampled were then heated to 75 °C for 1 h, 150 °C for 5 h and finally 200 °C for 2 h to complete digestion. Samples were re-suspended in 15 ml HNO_3 prior to filtering with Whatman #541 paper into a new tube. The concentration of molybdate reactive phosphate was determined in each extract colourimetrically (Murphy and Riley, 1962). 200 µl of colour developing solution (1.25 M H_2SO_4, 4.5 mM $(NH_4)_6Mo_7O_{24}$, 250 mM $C_8H_4K_2O_{12}Sb_2$, 30 mM ascorbic acid) was added to 40 µl of sample in 96 well microtitre plates and colour allowed to develop for 30 minutes. Absorbance was measured at 882 nm using a VERSA max plate reader (Molecular Devices). Standards were prepared for each microtitre plate in the same matrix as the extractant. Three measurements were made of each sample and data was expressed as mg P kg^{-1} soil dry weight calculated from the mean.

8.2.5 Fungal Biomass

Saprophytic and mycorrhizal fungal biomass was determined using an in-growth mesh bag technique (Wallander *et al.* 2001). 160 mesh bags (10 cm × 4 cm) were constructed from 39 µm nylon mesh which allows passage of fungal mycelium but excludes plant root in-growth. Each bag was filled with 80 g acid washed sand and

sealed using a thermoplastic adhesive. During June 2007 four bags were vertically inserted into the top 10 cm of soil in each of the single species and three species mixture subplots using a 4 cm corer. To estimate the contribution of saprophytic mycelium to fungal biomass on the perimeter of each treatment plot a 20 cm diameter plastic pipe was inserted into the ground; creating a trench which excludes the input from mycorrhizal mycelium.

Four bags were then inserted into the trenched area using the technique described above. A single mesh bags was removed from each sub-plot in November 2007 in April, July and November of the following year. During collection bags were placed into individual polythene bag then temporarily stored at 4 °C. Once back at the laboratory mesh bags were opened and the contents homogenised then stored in clean polythene bags at -20 °C until analysis.

The extent of fungal colonisation was determined by ergosterol extraction and analysis by high performance liquid chromatography (HPLC) based on the method of (Klamer and Bååth, 2004). 5g of homogenised sand was extracted by the addition of 4 ml 10% KOH in methanol and 1 ml cyclohexane. Samples were shaken for 45 min, and heated to 70 °C for 90 mins. After cooling to room temperature, 1 ml of distilled water and 2 ml cyclohexane was added prior to vigorous mixing and centrifugation (1500 g; 10 min). The upper phase of the supernatant was then carefully transferred to a clean tube; to improve the efficiency of extraction 2 ml cyclohexane was added a second time and supernatant removed as above. Samples were then evaporated under N_2 at 40 °C and resuspended in 100 µl of methanol. 40 µl of the extract was analysed by HPLC (Varian Pro Star) equipped with a C_{18} reverse phase column (5 µm ; 5 cm × 4.6 mm column). Extracts were eluted with 100% methanol at a flow rate of 1 ml min^{-1} and monitored at 282 nm. The standard curve for quantification was obtained using dilutions of ergosterol (Sigma E6510) suspended in methanol. All samples were filtered with a 0.45 µm syringe filter (Whatman) prior to loading onto the column. Ergosterol obtained from trenched samples was assumed to be the product of saprophytic fungi and deducted from non-trenched samples to obtain the contribution of mycorrhizal extramatrical mycelium in each sub-plot.

8.2.6 Statistical Analysis

Data were subjected to repeated measures factorial ANOVA, and Multivariate General Linear Model (SPSS 17.0, SPSS Inc., Chicago, IL) with $P<0.10$ used to determine statistical significance. Treatment, species and depth were used as factors and data were assessed for normality using Shapiro-Wilk's test. Homogeneity of variance was determined using Levene's test. Correlations and graphs were produced using SigmaPlot v 11.0 (Systat Software Inc, Chicago, IL).

8.3 Results

8.3.1 Molybdate reactive Phosphate fractions

Water was used as the initial extraction medium to remove P from a specific pool most representative of where plants access their supply and based sorption models (Sorn-srivichai *et al.* 1988). Elevated CO_2 did not significantly alter the H_2O available P fraction but there was a consistent 20% reduction in both sampling years (Figure 8.1) this water extractable P constituted 1.2 ± 0.1% of the total extracted P.

The second sequentially extracted fraction used $NaHCO_3$; bicarbonate extracts the most plant available inorganic P fraction as the introduced chemical changes are representative of those induced by root respiration. When data was pooled for treatment, $NaHCO_3$ extractable P was slightly reduced by elevated CO_2 in 2007 (31% ; $P=0.070$) and reduced by 46% ($P=0.141$) during the second sampling period in 2008, but was not statistically significant. There was a significant impact on $NaHCO_3$ extractable P with soil depth during 2007, P was reduced by 23% at 10 cm ($P<0.05$), 42% at 20 cm ($P<0.1$) and 28% at 30 cm (Figure 8.1). In the following year (2008) P was more strongly reduced by 38%, 43% and 61% at respective depths of 10, 20 and 30 cm. Overall, the $NaHCO_3$ extractable fraction was reduced in both the ambient and elevated CO_2 plots, but with stronger reduction in the CO_2 treatment plots that increased with depth to a maximum 61% reduction at the deepest sampling point (20-30 cm) (Figure 8.2).

A large reduction of 38 and 56% was observed within the *A. glutinosa* subplots during 2007 and 2008 respectively, however, no significant species specific tree response were apparent. During 2007 and 2008, we observed decreasing amounts of $NaHCO_3$ extractable P between years in ambient and elevated CO_2 conditions for

all species, however, this was only significant in the three species mixture plot ($P<0.1$) during 2007.

The third fraction extracted utilised NaOH; hydroxide extractable P is a relatively stable pool with reduced availability to plants, and thought to consist of amorphous and some crystalline Al and Fe phosphates associated with sesquioxides and humic compounds. The hydroxide extractable P fraction in both years comprised 17% and 22% of total extracted P in FACE and ambient plots respectively. Between 2007 and 2008, there was a substantial increase in hydroxide extractable P of 47% and 37% in ambient and FACE plots respectively. There were no significant differences factored by depth or species. Repeated measures ANOVA showed that the largest amount of variance was explained by sampling year and an interaction between year × treatment ($P<0.05$), whilst also alluding to a significant year × treatment × depth interaction ($P<0.10$).

Dilute hydrochloric acid was used as the fourth sequential extractant to determine Ca associated P. The P in this fraction showed a significant 16% treatment induced decline during 2007 ($P<0.05$). A significant reduction of the fraction was observed in all species 15.9 ± 0.7% and at all depths 15.9 ± 0.6% studied ($P<0.05$). The reduction in HCl extracted fraction became slightly larger (17%) with increasing soil depth. In the second season a greater reduction of 18.9 ± 1.3% was observed, the degree of reduction also increased with depth, reaching a value of 22.5% at 30 cm, however this was not statistically significant ($P=0.80$). Sampling year explained a significant amount of variance ($P<0.05$), but there were no significant interactions when analysed by repeated measures (Table 8.1).

Finally the residue was digested in concentrated nitric and perchloric acid to extract remaining P. This fraction, the largest contained 50% more P in 2008 than the preceding year. Resulting in sampling year explaining a significant amount of variance ($P<0.05$). P in this fraction was lowest in the three species mixture plot and highest in birch plot. However, treatment, species composition or sampling depth had no significant effect on the P content of this fraction.

Table 8.1. F-values of repeated measures ANOVA for each extractable P fraction of the modified Hedley P fractionation scheme and analyzed as Molbdate reactive P (Murphy & Riley, 1962). Soils were collected during January of 2007 and 2008 at three depths (10, 20 and 30 cm) from single species sub-plots and a three species mixture of the ambient and elevated atmospheric CO_2 plots at BangorFACE. Significance is denoted by $^{**}(P<0.05)$ and $^{*}(P<0.1)$.

Source of Variation	H_2O Extracted P	0.5 M $NaHCO_3$ Extracted P	1 M NaOH Extracted P	1 M HCl Extracted P	HNO_3 & $HClO_4$ Extracted P	Total Extracted P
year	0.361	**93.188****	**193.515****	**26.322****	**818.451****	**70.220****
year × treatment	0.000	0.892	**11.797****	2.007	0.097	2.288
year × species	1.727	0.090	1.253	1.388	**5.430****	1.753
year × depth	1.105	**2.906***	**2.737***	1.434	0.737	0.541
year × treatment × species	**3.366****	0.142	0.379	0.405	0.088	0.441
year × treatment × depth	0.035	1.842	**2.738***	0.765	2.153	0.240
year × species × depth	0.568	0.844	0.938	0.800	1.208	0.604
year × treatment × species × depth	1.002	0.273	0.311	0.393	0.998	0.782

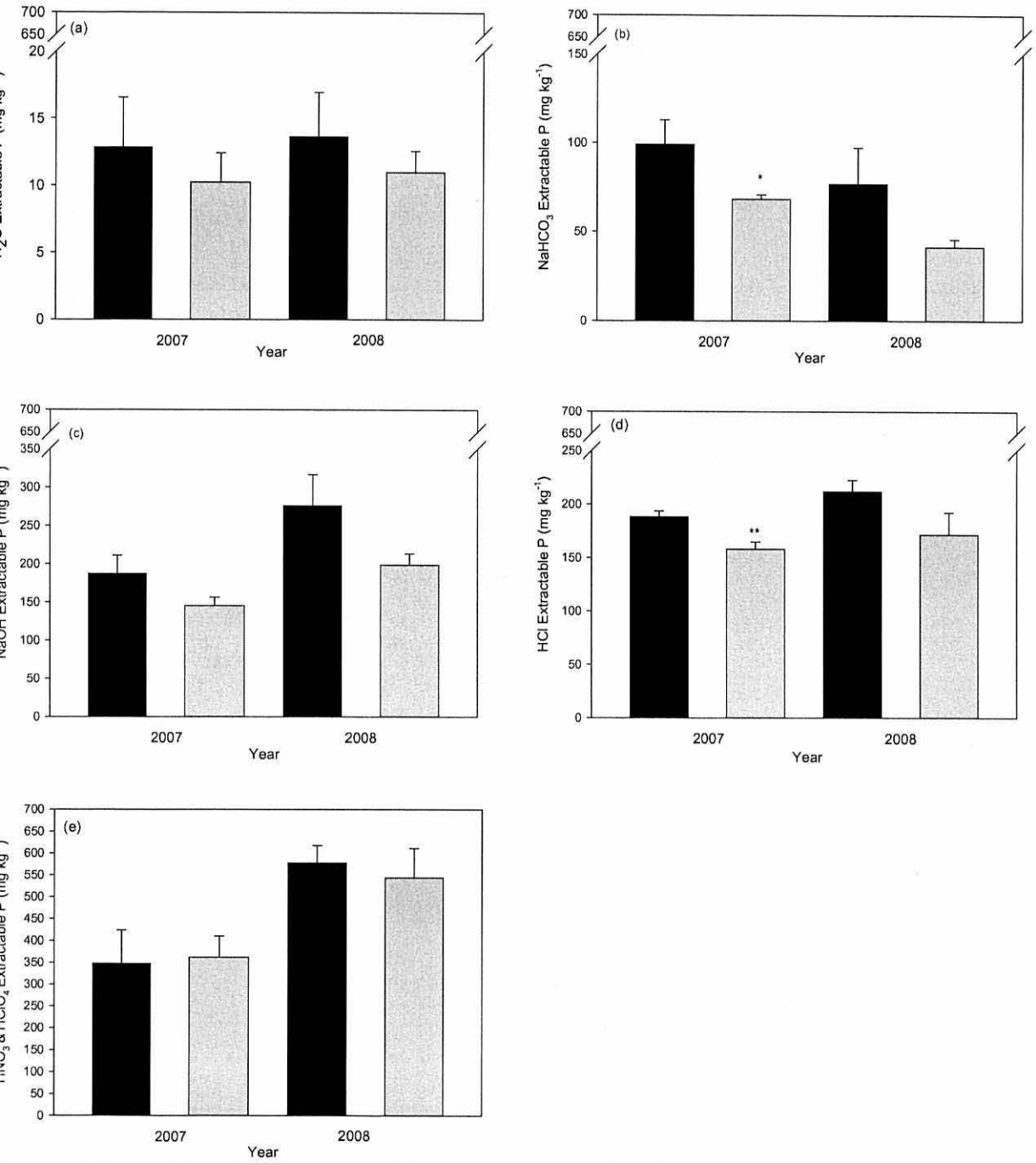


Figure 8.1 Extractable P fractions sequentially extracted from soils collected during root coring in 2007 and 2008. Black bars soil collected from under ambient CO_2 grown trees and gray bars are soil collected from under elevated CO_2 grown trees. Values are pooled means for all species (n=4) whiskers are 1 SE. ■ ambient CO_2, □ elevated CO_2. Significant differences are denoted by **(P<0.05) and *(P<0.1).

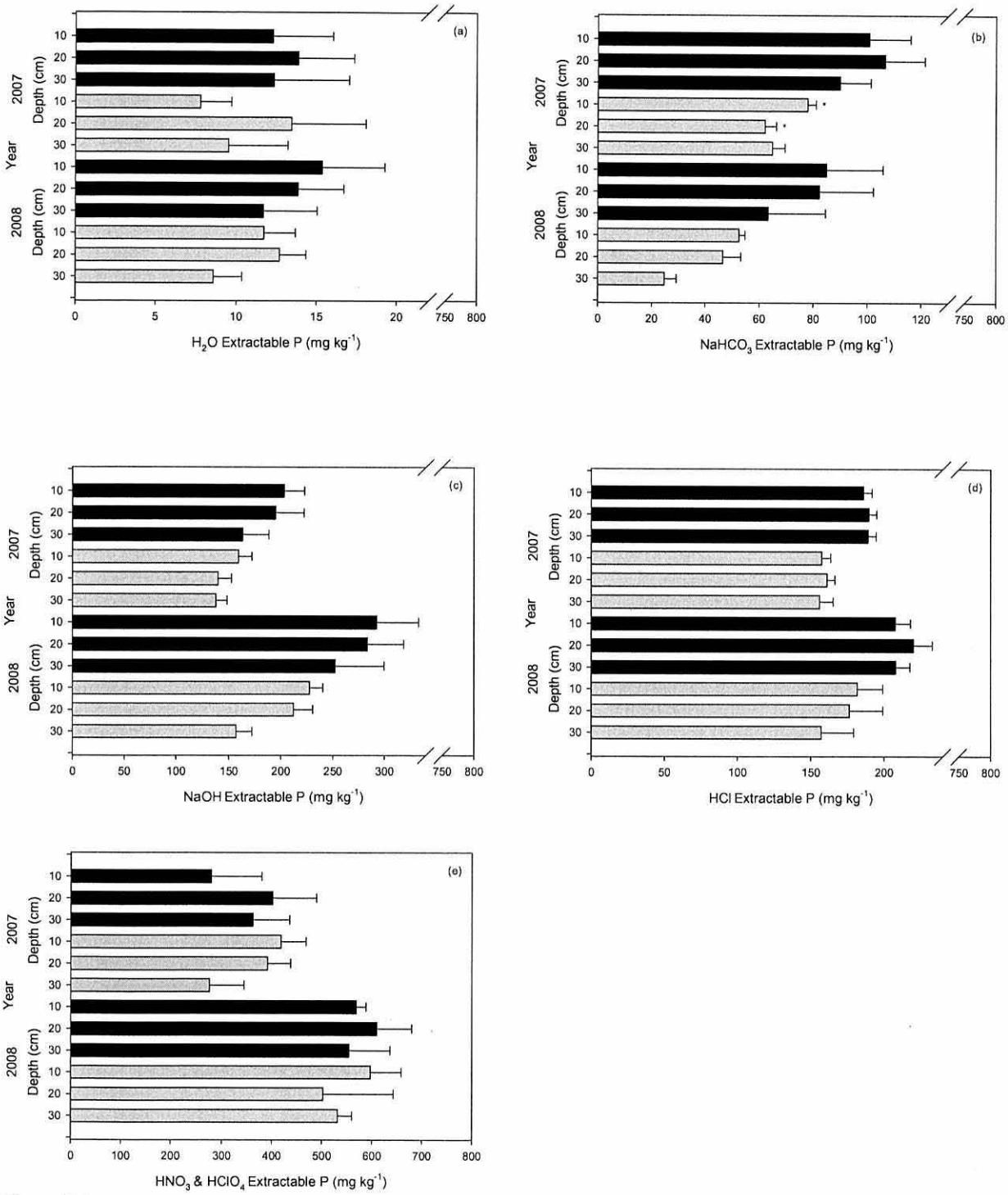
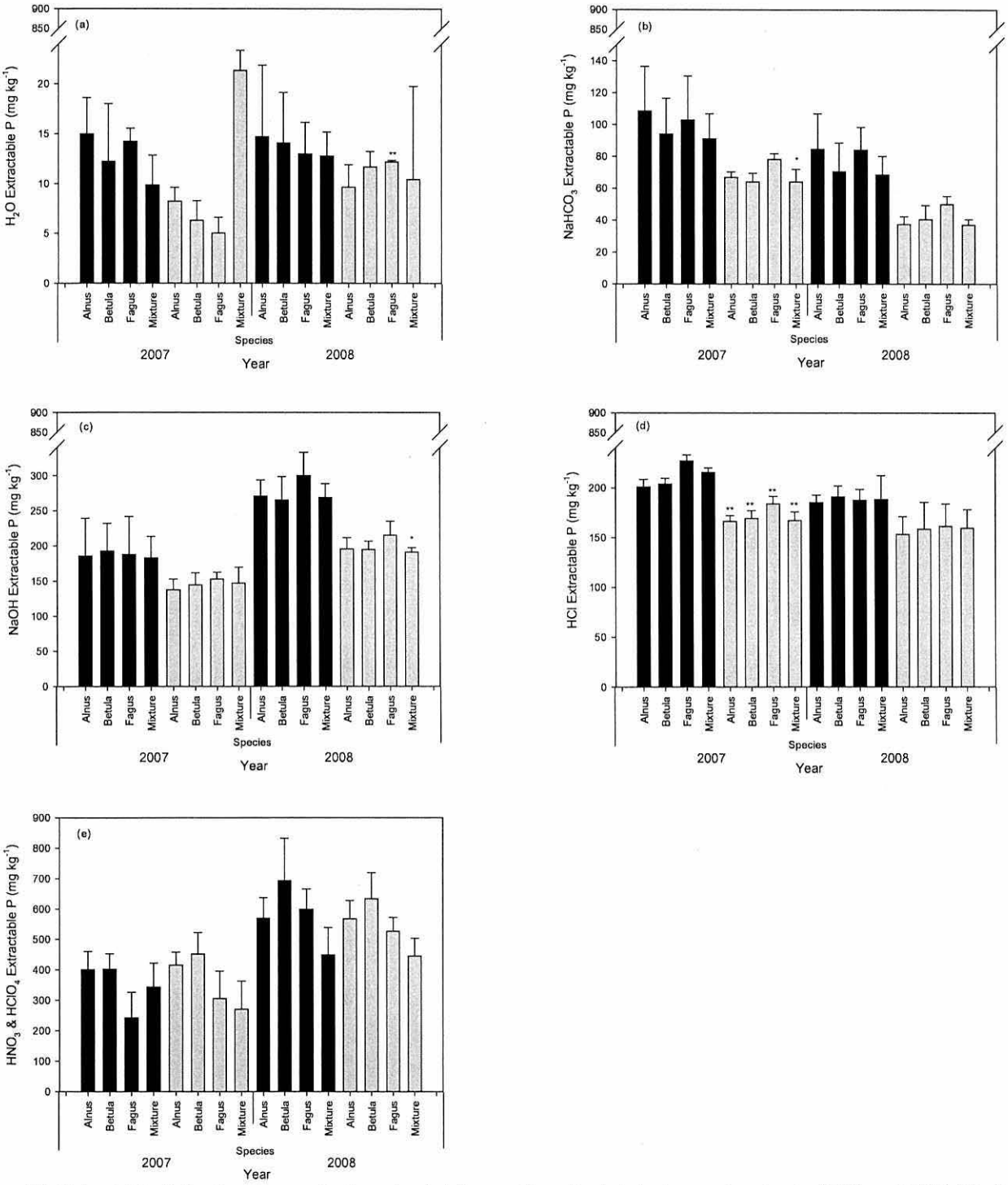


Figure 8.2 Extractable P fractions sequentially extracted from soils collected during root coring in 2007 and 2008. Black bars soil collected from under ambient CO_2 grown trees and gray bars are soil collected from under elevated CO_2 grown trees. Values are pooled means for all species by depth (n=4) whiskers are 1 SE. ■ ambient CO_2, □ elevated CO_2. Significant differences are denoted by **(P<0.05) and *(P<0.1).



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8.3.2 *Fungal biomass*

Elevated CO_2 induced a significant ($P<0.05$) 10-fold increase of ergosterol content within the *F. sylvatica* sub-plots. Although, no other significant increases were apparent, soil ergosterol content doubled in the three species mixture. Ergosterol concentration was greatest within the *B. pendula* sub-plots, being 4-fold greater than in the *A. glutinosa* plots and 2-fold greater than *F. sylvatica* or the three species mixture (Table 8.2).

Table 8.2 Mean±SE ergosterol concentration determined by HPLC from sand within 39 μm mesh bag buried within the single and three species mixture subplots of species grown in ambient and elevated CO_2 ($n=4$). Significance is denoted by **($P<0.05$) and *($P<0.1$)

Species	Ergosterol (μg g^{-1})	
	Ambient	FACE
Alnus glutinosa	0.061 ± 0.050	0.082 ± 0.033
Betula pendula	0.329 ± 0.082	0.384 ± 0.087
Fagus sylvatica	0.013 ± 0.013	**0.198 ± 0.065***
Species Mixture	0.079 ± 0.042	0.147 ± 0.054

8.3.3 *P biomass input from litter.*

The quantity of fallen litter was not altered by elevated CO_2 in either 2007 or 2008 (Figure 8.4). However, in 2008 fallen litter biomass was almost 4-fold greater than 2007 in the *A. glutinosa* plots ($P<0.001$) and more than 2-fold greater in mixed species plots ($P<0.05$) in both the elevated and ambient CO_2 plots. Leaf P content was similar between *B. pendula* and *A. glutinosa.* However, the P levels in *F. sylvatica* were almost half of those the other species. Elevated CO_2 did not impact upon leaf P content (Table 8.3).

Table 8.3 Mean±SE P concentration of fallen leaf litter determined by ICP of singles species grown in ambient and elevated CO_2 ($n=4$). Significance is denoted by **($P<0.05$) and *($P<0.1$)

Species	Phosphorus (mg g^{-1})	
	Ambient	FACE
Alnus glutinosa	2.88 ± 0.89	2.73 ± 1.02
Betula pendula	2.91 ± 0.86	2.82 ± 0.93
Fagus sylvatica	1.43 ± 0.87	1.46 ± 0.56

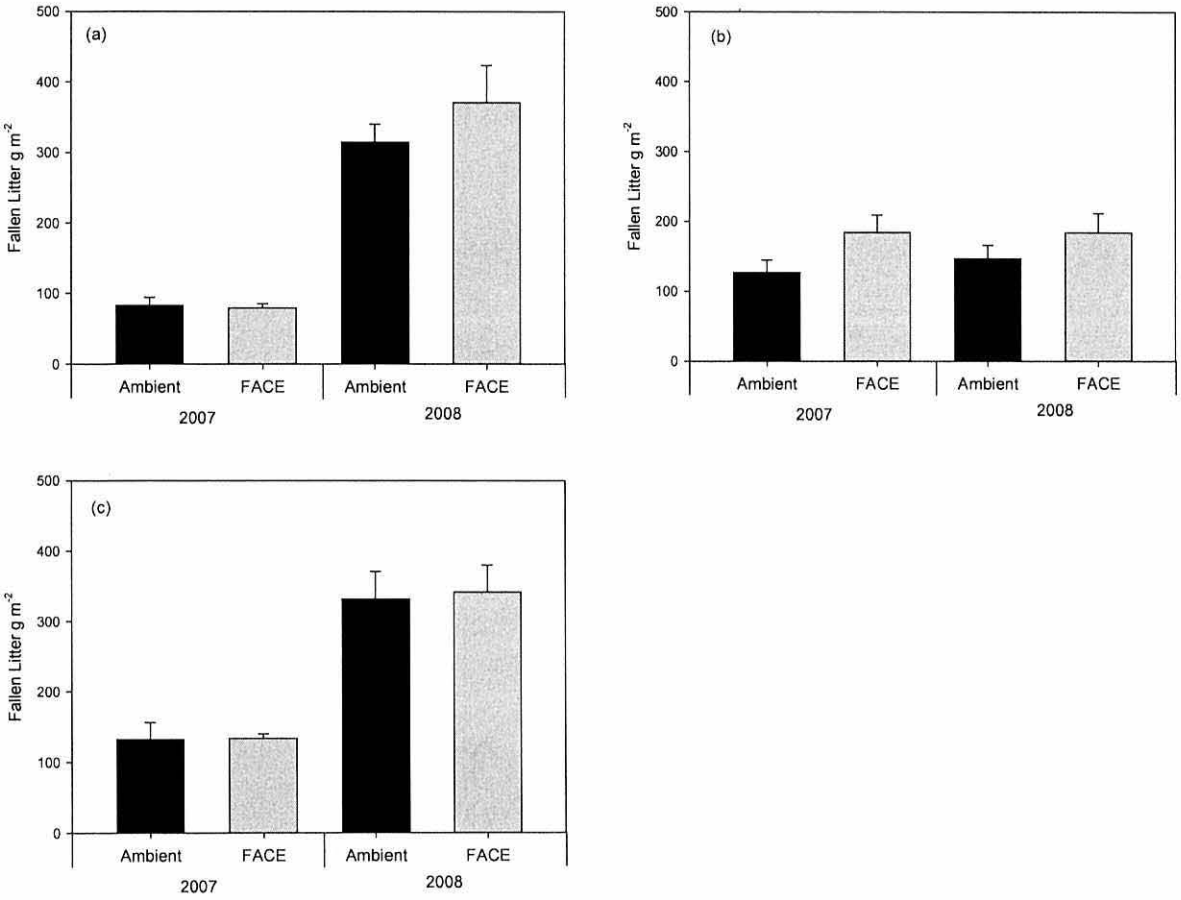

Figure 8.4 Total fallen litter collected from litter baskets within the ambient and elevated CO_2 plots and within the *B. pendula* and *A. glutinosa* single species subplots, panels (a) and (b) respectively. Panel (c) shows the of *A. glutinosa* and *B. pendula* to fallen litter within the mixed species plots. Litter retention prevented inclusion of *F. sylvatica* in the analysis. Values shown are mean ± 1 SE (n=4). Significant differences are denoted by **(P<0.05) and *(P<0.1).

8.3.4 Correlation with extractable P fractions.

Correlations between labile P fractions and microbial biomass revealed a significant positive relationship (r^2=0.45) within the ambient CO_2 plots for H_2O extractable P; whereas within the elevated CO_2 plots a weak negative relationship existed (r^2=0.13; Figure 8.5a). A similar pattern was observed in the correlation between the $NaHCO_3$ extractable P fraction and microbial biomass, where the elevated CO_2 relationship was stronger (r^2=0.35; Figure 8.5b) and the ambient relationship was weaker (r^2=0.03; Figure 8.5b) suggesting that microbially driven processes are maintaining P concentrations within the soil solution. Annual biomass increment was significantly correlated with $NaHCO_3$ extractable P in both ambient and elevated CO_2 plots (r^2=0.52) whereas no

relationship existed for H_2O extractable P (Figure 8.7). The NaOH extractable P which is derived from organic residues was strongly correlated with SOM in the elevated CO_2 plots (r^2 = 0.64) but no relationship existed within the ambient CO_2 plots (Figure 8.7). Whilst the HCl extractable P correlated well with presence of mycorrhizal biomass (r^2=0.56), indicated by the concentration of ergosterol found within mesh bags in the elevated CO_2 plots. However, no correlation was observed between the HCl fraction and ergosterol within the ambient plots (Figure 8.8).

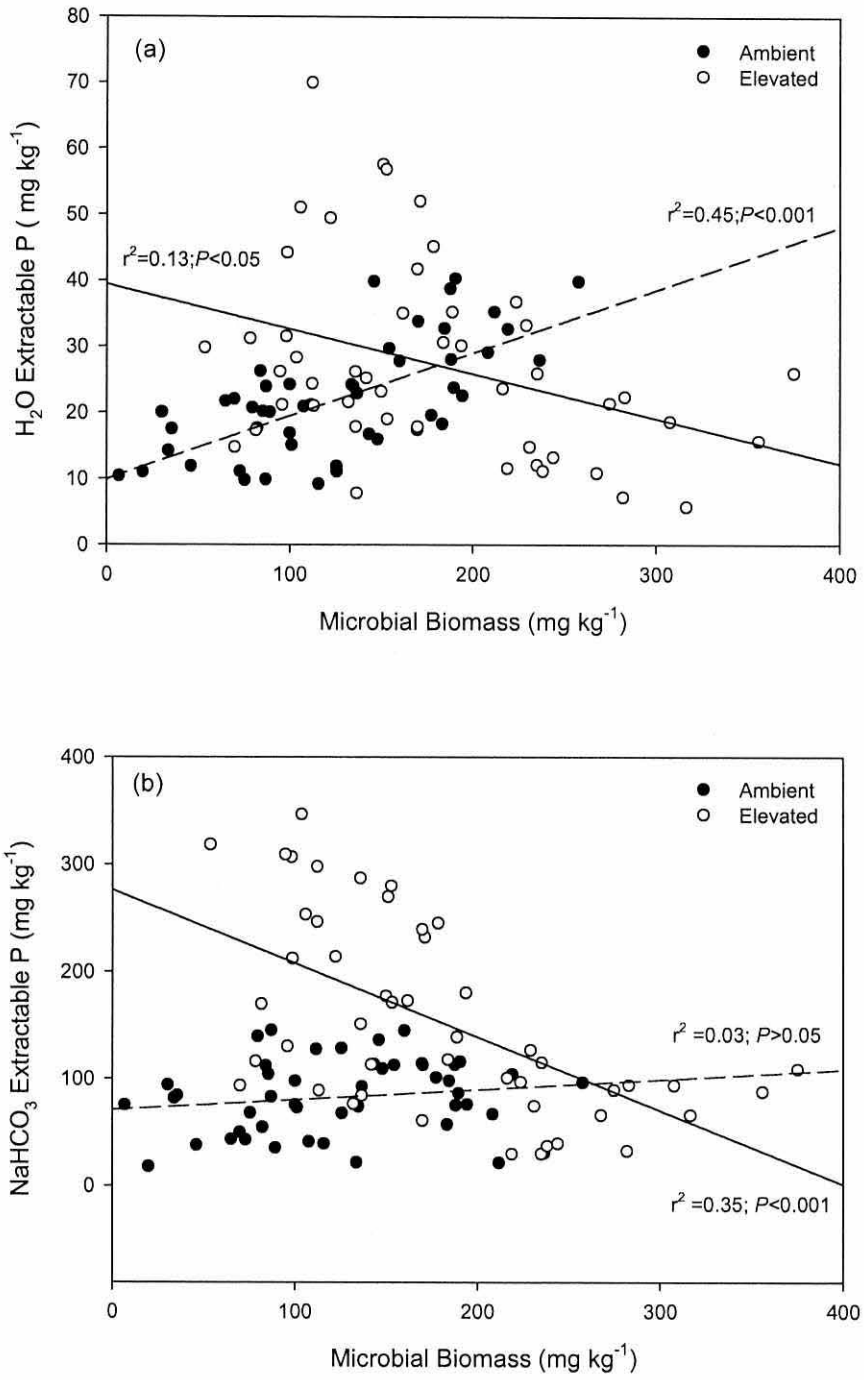

Figure 8.5 The relationship between microbial biomass and labile inorganic P pools extracted in H_2O (panel a) and $NaHCO_3$ (panel b) for *A. glutinosa, B. pendula* and *F.* sylvatica in monoculture and a three species mixture during 2008. Elevated CO_2 plots are shown by hollow O symbols and ambient CO_2 plots by filled ● symbols.

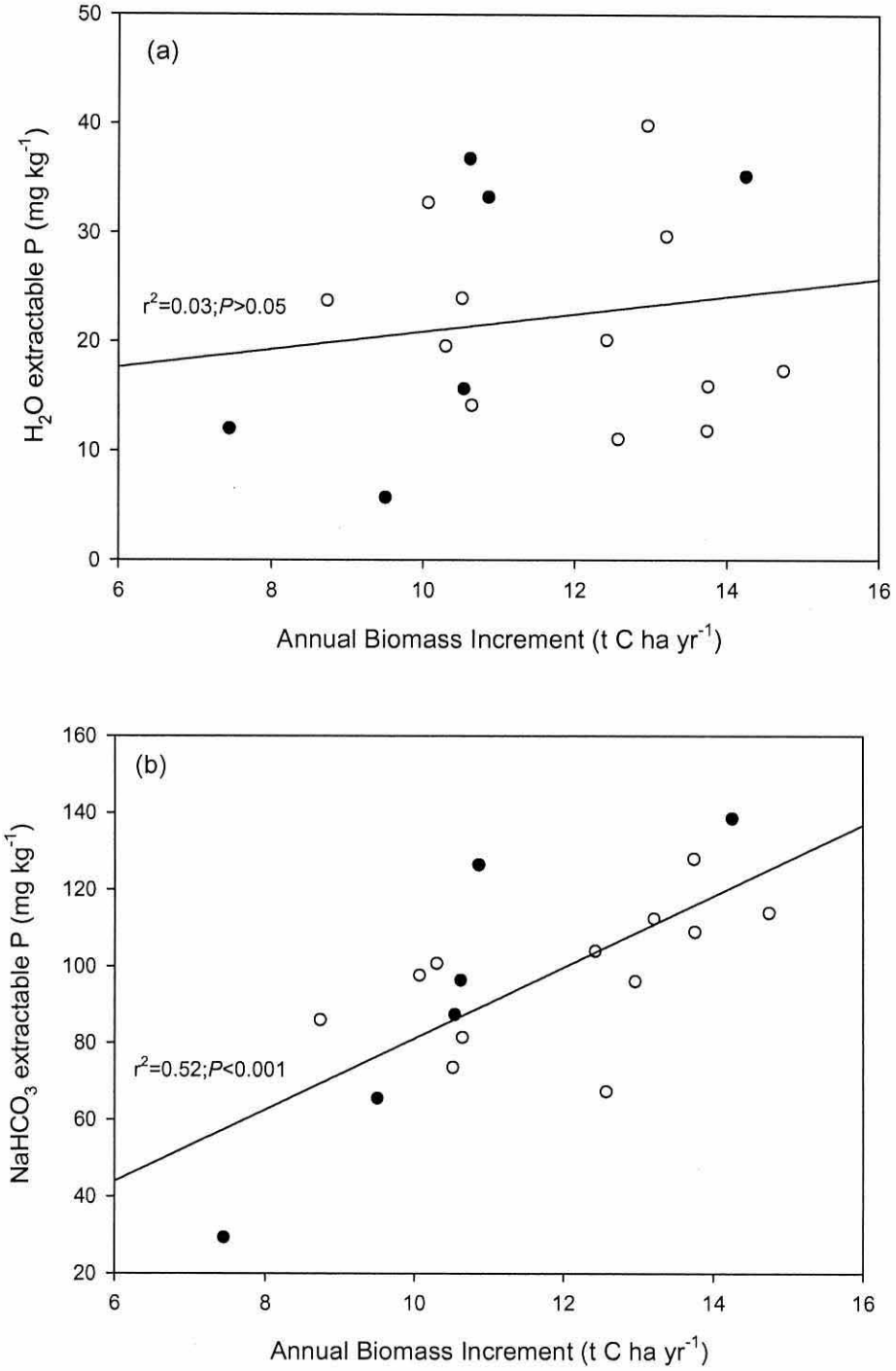

Figure 8.6 The relationship between annual biomass increment and labile inorganic P pools extracted in H_2O (panel a) and $NaHCO_3$ (panel b) in the top soil for *A. glutinosa, B. pendula* and *F. sylvatica* in monoculture and a three species mixture during 2008. Elevated CO_2 plots are shown by hollow O symbols and ambient CO_2 plots by filled ● symbols.

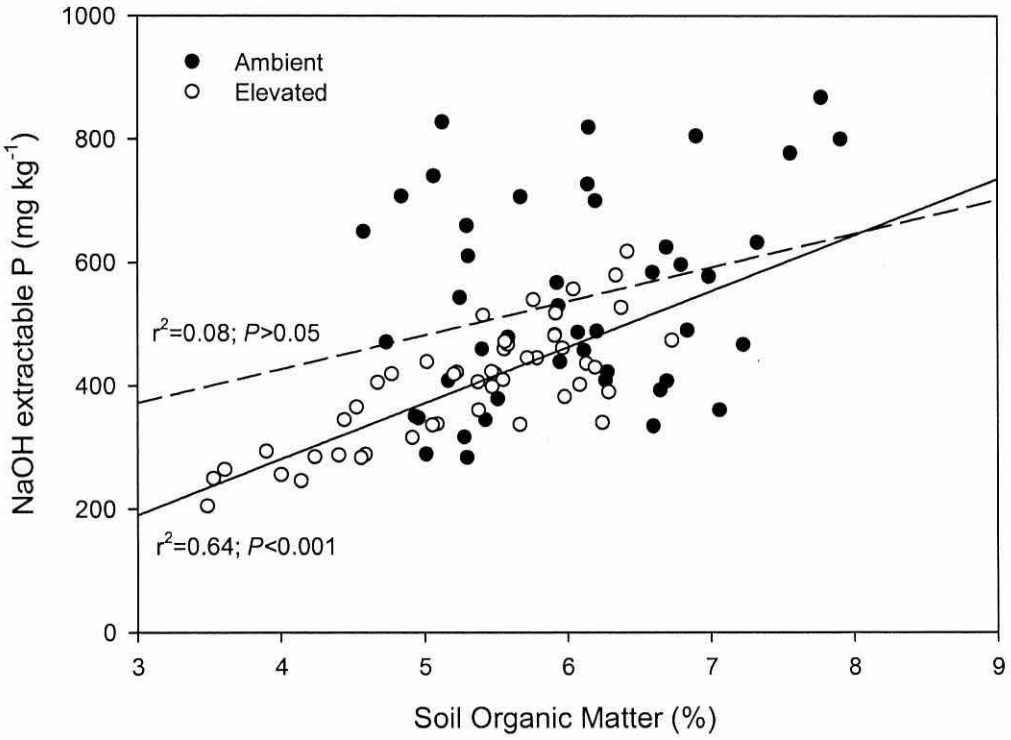

Figure 8.7 The relationship between NaOH extractable P and soil organic matter at three depth (10, 20 and 30 cm) of *A. glutinosa, B. pendula, F. sylvatica* and a three species mixture during 2008. Elevated CO_2 plots are shown by hollow circles and ambient CO_2 plots by filled circles. The solid line represents a linear regression for elevated CO_2 and the dashed line a linear regression for ambient CO_2.

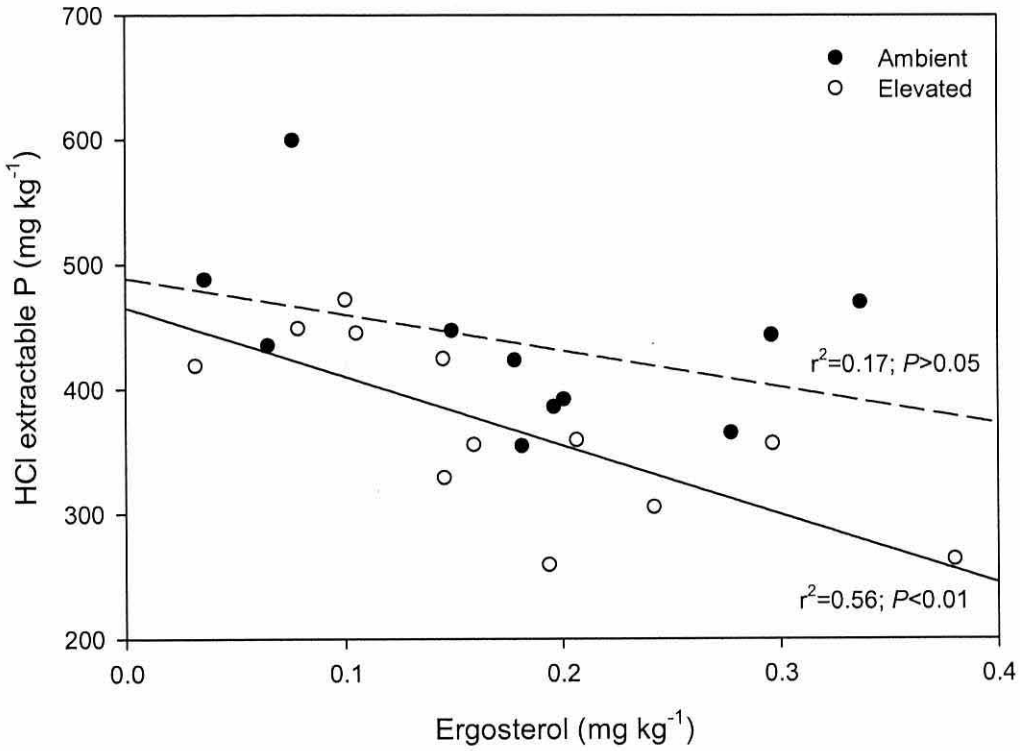

Figure 8.8 The relationship between HCl extractable P and ergosterol extracted from mesh bags within the top soil of *B. pendula, F.* sylvatica and a three species mixture during 2008. Elevated CO_2 plots are shown by hollow circles and ambient CO_2 plots by filled circles. The solid line represents a linear regression for elevated CO_2 and the dashed line a linear regression for ambient CO_2.

8.4 Discussion

Availability of P for plant growth is not only dependant on the concentration of P in soil solution but also physiochemical complexes, and the rate of desorption or mineralisation of numerous inorganic and organic soil constituents. P extraction procedures utilise solutions of mild acidity or alkalinity, for example alkaline sodium bicarbonate (Olsen *et al.* 1954) or acid ammonium fluoride extraction (Bray and Kurtz, 1945). These methods attempt to quantify P pools that are relevant to plant uptake from the soil mineral phase without solubilising phosphatic minerals. Characterisation of P pools that contribute to pedogenesis and P cycling is best achieved by sequential extraction (Tiessen and Moir, 2008). The Hedley P sequential fractionation procedure (Hedley and Stewart, 1982) has become a popular method amongst many biogeochemists studying P cycling in forest ecosystems. Yet despite continued refinement of P extracting procedures, quantitative determination of soil P availability to plants is poorly understood. Plant P availability depends not only on pool sizes of differing forms of P, but also on rates of P absorption and desorption from a variety of inorganic and organic soil constituents, linked to plant P demand, and microbial interactions. A lack of information linking ecosystem operationally defined P pools to chemically extracted P hinders the interpretation of the Hedley P fractionation procedure in forest soils. Albeit, the procedure provides a useful index of biological and geochemical forms of P during soil development in natural ecosystems by estimating P pools of differing solubility (Cross and Schlesinger, 1995). Therefore investigators have continued to measure sequentially extractable P to improve understanding of P cycling in forest ecosystems (Johnson *et al.* 2003a).

Typical values of total P for inceptisol soils in temperate broadleaved forests are 496 ± 258 mg P kg^{-1}, while inceptisols of the Eastern Pyrenees have been reported to contain 700 to 1100 mg P kg^{-1} soil (Johnson *et al.* 2003a; Cross and Schlesinger, 1995). The soil in our study yielded molybdate reactive total P of 774 ± 34 mg P kg^{-1} in 2007 and 1062 ± 43 mg P kg^{-1} in 2008. These values are within the range reported by the aforementioned authors reflecting the young and un-weathered status of the dystrochrept inceptisol at our experimental site.

8.4.1 Labile P fractions

Water extractable P was unaltered by treatment or sampling year ranging between 1.1 and 1.6% of total molybdate reactive P (MRP). The marginal proportion of P in this pool available for plant uptake is representative of the reactivity of P ions in solution which act as important inorganic ligands that form complexes with several metal cations (Lindsay, 1979). We found a mean P concentration of 6.7 ± 0.3 μM within this pool which is consistent with a typical soil solution range between 0.1 and 10 μM (Hinsinger, 2001). Water extractable P has been shown to be highly correlated with plant uptake independent of P buffering capacity and soil type (Sorn-srivichai *et al.* 1988). Furthermore, we found that the annual biomass increment was not correlated to soil solution P suggesting that previously reported relationships that exist for crops (Sorn-srivichai *et al.* 1988) may not apply to forest ecosystems.

0.5M $NaHCO_3$ extracts loosely bound labile P pools by improving solubility of P. Increased solubility is achieved as CO_3^{-2} removes soluble Ca^{2+} forming $CaCO_3$, additionally OH^- ions remove soluble Al^{3+} and Fe^{3+} by formation of Al and Fe oxyhydroxides. An increase in pH also enhances desorption of available P into solution (Marschner, 1995). The $NaHCO_3$ extractable pool was strongly affected by FACE and forest growth, during the first sampling $NaHCO_3$ P was 12.3% of total MRP in ambient conditions but was reduced to 9.2% by FACE. In the subsequent year this pool was further reduced by approximately half in both ambient and FACE plots. A strong positive relationship (r^2=0.52) between annual biomass increment and $NaHCO_3$ extractable P, coupled with a temporal reduction of this pool suggests that (a) forest growth is depleting labile P, and that (b) availability of $NaHCO_3$ extractable P is mediated by tree biomass production. This observation is supported by Johnson *et al.* (2003b) who in a study of fire adapted scrub oak forest exposed to a doubling of atmospheric CO_2 quantified two labile P pools using root simulator probes and bicarbonate extraction. Although Ortho-P extracted from the root simulator probes showed a non-significant decrease, $NaHCO_3$ extractable P significantly decreased following five years of CO_2 enrichment. This suggests that replenishment and distribution of the labile P pool by biological cycling of organic P are unable to replenish this pool. The strong significant positive relationship

between $NaHCO_3$ extractable P and annual biomass increment observed at BangorFACE also alludes to the fact that forests ecosystems may, preferentially access these pools. Forests are known to possess strong mycorrhizal associations that may promote inorganic P liberation through chelation of Ca, Al and Fe by organic acids (Graustein *et al.* 1977). Moreover, microbial consumption of oxalate salts produces CO_2 leading to carbonate dissolution of P and weathering of mineral P (Delucia *et al.* 1997).

8.4.2 Recalcitrant P fractions

0.1M NaOH extractable P is a relative stable pool with reduced availability to plants, but also contains organic P (Chang and Jackson, 1957). At BangorFACE this fraction was decreased by elevated CO_2. We believe that this pool was likely utilised as a source of P for microbial uptake mediated through increased microbial activity which rapidly cycled organic P to meet demand (Chauhan *et al.* 1981). However, we were unable to observe a correlation between microbial biomass and P content to confirm this hypothesis. Following initiation of the experiment, the NaOH P pool size increased temporally, which we attribute to increased biomass inputs as litter (Chapter 10), root production (Chapter 4) and potentially through old SOM priming (Hoosbeek *et al.* 2004). Our result contrasts with the observations of Khan *et al.* (2008) who reported a significant 62% increase in NaOH at the EuroFACE *Populus* plantation in response to CO_2 enrichment. The result of Khan *et al.* (2008) may be explained through increased organic matter inputs to soil, as root turnover was 35-85% higher than ambient at EuroFACE (Lukac *et al.* 2003). However, it is also possible that soil organic matter priming may have contributed to P in this pool (Moscatelli *et al.* 2005).

Cross and Schlesinger (1995) showed that the mineral P pool declines with soil age, as weathering transfers P from highly recalcitrant forms to pools available for plant and microbial uptake. In support of this mechanism, we observed a decline of the HCl extractable pool in the FACE plots, which was correlated with increases in mycorrhizal biomass. The ability of ectomycorrhizae to dissolve mineral particles has been demonstrated in culture (Paris *et al.* 1996), and in natural forests by utilising Sr isotopes to determine the apatite source of Ca^{2+} ions (Blum *et al.* 2002). Furthermore, it is well

known that roots and mycorrhizae exude organic acids that contribute to mineral dissolution (Courty *et al.* 2010). This data strongly suggests that in our experiment, elevated CO_2 mediated a mycorrhizal enhanced dissolution of HCl extractable P. However, conversely the HCl extractable P fraction did not change in a sweetgum (*Liquidambar styraciflua*) plantation enriched by elevated CO_2 (Johnson *et al.* 2004). This may indicate that the functional physiology of specific tree and mycorrhizal species affects there ability to mine for P from primary minerals. Furthermore, early successional species such as *Betula* and their mycorrhizal symbionts might be expected to directly influence primary mineral weathering to access P and aid in soil development. Whereas, later successional species growing on more developed soils, rich in organic residues containing P, would have a reduced requirement to scavenge for P through primary mineral weathering.

8.4.3 Biomass inputs

P content of litter and litter inputs were not significantly affected by elevated CO_2. However, this is contrary to previously published data indicating that an increase in leaf area index of is a relatively consistent response of forests to elevated CO_2 (Ainsworth and Long, 2005). The quantity of litter collected in litter traps at BangorFACE may have been affected by the high winds experienced at the experimental site which resulted in the leaves being removed from the plots. Our observed increase in NaOH extractble P would indicate that organic residues are being cycled within the ecosystem, and we attribute the increases to faster root and mycorrhizal production and turnover (discussed in Chapter 4).

8.5 Conclusion

Our first hypothesis stated that faster plant growth under elevated CO_2 would deplete the labile plant available P pool due to enhanced transfer of soil P to tree biomass. A consistent reduction in the labile P fractions with an increasing temporal depletion of the $NaHCO_3$ pool supported this assertion. The second hypothesis stated that NaOH extractable P would increase as biomass input to the soil was greater under elevated CO_2 as cycling of organic P is the primary pathway for replenishing plant available P. As

expected we observed a temporal increase in the NaOH fraction. However, unexpectedly the NaOH P fraction decreased under elevated CO_2 which we attributed to initial SOM priming at the site. We expect this pool to replenish as biomass inputs replenish SOM fractions as the ecosystem develops. The final hypothesis (primary mineral P dissolution will increase to meet P demand) was supported by the decrease in the HCl extractable fraction in the elevated CO_2 plots and a negative correlation between mycorrhizal extramatrical biomass and the HCl P fraction.

Our data show that in temperate forest ecosystems are able to improve acquisition of P through enhanced primary mineral weathering through NPP related feedback mechanisms. However, despite increased input from mineral sources and biomass cycling the plant available $NaHCO_3$ P fraction appears to continue to decline possibly leading to P limitation in the later years of forest growth.

8.6 Acknowledgements

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9 The Relative Contribution of Mycorrhizal Extramatrical Mycelium and Fine Roots to Soil Carbon in Forest Enriched with CO_2

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Andrew Smith wrote the manuscript, collected data, performed analysis and maintained the FACE facility during 2006-2009. Dr. Nathalie Fenner performed IRMS on supplied soil samples. Dr. Andreas Heinemeyer provided equipment to measure soil respiration and assisted with measurements. Prof. Douglas Godbold is the project principle investigator; he also provided assistance with data interpretation, analysis and manuscript preparation.

9.1 Introduction

Atmospheric CO_2 concentrations are rising at an unprecedented rate, and are expected to double by the end of the century (IPCC, 2007). Forest ecosystems and their soils store almost half of the C of terrestrial ecosystems (Schlesinger and Lichter, 2001). Under elevated CO_2 forest net primary productivity increases due to greater efficiency of the carboxylation enzyme ribulose-1,5-bisphosphate (Long *et al.* 2004; Norby *et al.* 2002; Norby *et al.* 2005). Globally, soil contains 2344 Pg C in the top 3 m and soils are the largest C pool of the terrestrial biosphere (Jobbagy and Jackson, 2000). Putative C inputs to soil organic matter are derived from the turnover or woody biomass, leaf litter, fine root, mycorrhizal mycelium and rhizodeposition (Vogt *et al.* 1986; Malhi *et al.* 1999). Forests are an important component of the global C cycle as their roots contribute up to one third of global terrestrial NPP (Jackson *et al.* 1997). In addition to aboveground biomass inputs to soil, woody root production creates a greater potential to mitigate anthropogenic CO_2 emissions by sequestering C in belowground C pools. Woody roots also contain large amounts of lignin a highly recalcitrant molecule that offers potential for long term storage in soil (Niklaus *et al.* 2006). Transformation of plant detritus to soil organic matter requires perturbation by the soil microbial community. During this process metabolised C compounds are respired back to the atmosphere leaving only the most recalcitrant components to form soil organic matter (Lützow *et al.* 2006).

An often overlooked component of the C cycle is the contribution of mycorrhizal symbionts (Godbold *et al.* 2006). Most of the Earth's forests grow in nutrient poor environment and are highly dependant on their fungal symbionts to increase soil exploration for nutrient acquisition (Smith and Read, 2008). In exchange for nutrients, mycorrhizal symbionts are supplied with carbohydrates that may account for up to 20-30% of plant photosynthate (Jakobsen and Rosendahl, 1990; Nehls *et al.* 2001). Following a girdling experiment Högberg *et al.* (2001) were able to demonstrate that 54% of respired CO_2 in boreal forests was associated with the respiration of ectomycorrhizal fungi and their associated fine roots. The contribution of extramatrical hyphae was determined to be 34% of microbial biomass and 80% of fungal biomass in boreal forest soils (Högberg and Högberg, 2002).

Differentiating between saprophytic and mycorrhizal extramatrical mycelium has been determined using ^{13}C labelling, and an in-growth bag approach (Wallander

et al. 2001.) Using this technique in a spruce and mixed oak-spruce stand, an annual extramatrical biomass increment was estimated at 590 ± 70 kg ha^{-1} yr^{-1} and 420 ± 160 kg ha^{-1} yr^{-1} respectively. Furthermore, a total biomass estimate of between 4.8-5.8×10^3 kg ha^{-1} to a depth of 70 cm alludes to an extensive biomass pool extending deep within the soil profile (Wallander *et al.* 2004). Ectomycorrhizal extramatrical mycelium exhibits considerable morphological variation that alters length, tissue density and aggregation into rhizomorphs (Agerer, 2001). These differences in mycelial structures are likely to be related to foraging strategies and may also affect the cost benefit balance of the C and nutrient fluxes between plant and fungal symbiont (Parrent and Vilgalys, 2007). Determination of mycelial turnover is complex and currently there are no published data for ectomycorrhizal fungi. However, using minirhizotron data and a Laplan-Maier survival analysis Vargas and Allen (2008) determined a median life span of 400 days for rhizomorph structures in a mixed species temperate forest. Whereas Staddon *et al* (2003) reported turnover of arbuscular mycorrhizal hyphae within 5-6 days. With such variation in structure and function it seems apparent that coarse mycelial structures may persist, whilst other finer structures involved in foraging, may turnover extremely rapidly, and therefore represent a significant component of the soil C cycle (Staddon *et al.* 2003)

Elevated CO_2 research has extensively demonstrated increased NPP in forests with larger root systems that explore deeper with faster turnover rates (Rogers *et al.* 1994; Norby *et al.* 2005). Indeed three genotypes of *Populus* increased root biomass by 35-84% while root turnover was stimulated by 27-55% during the EuroFACE experiment (Lukac *et al.* 2003), and in a review of coniferous tree species Tingey *et al.* (2000) showed a median root response of 23% in trees grown in field conditions. A greater allocation of C belowground in elevated CO_2 has also shown to influence mycorrhizal diversity to species that produce greater amount of extramatrical mycelium (Godbold *et al.* 1997). A finding supported by a meta-analysis of ectomycorrhizal (EcM) and arbuscular mycorrhizal (AM) fungi under elevated CO_2 that revealed EcM fungi responded with a 45% increase in extramatrical mycelium dry weight and a 57% increase in hyphal length (Albertson *et al.* 2005).

The large quantity of C allocated belowground, in addition to the recalcitrant quality of constituent C compounds such as lignin and chitin should increase the sequestration potential of forest ecosystem (Iverson *et al.* 2008). Changes in forest

soils are difficult to determine as the soil is a large and heterogeneous environment with comparatively small organic C inputs. These issues are exacerbated by the relatively short duration of most elevated atmospheric CO_2 experiments (Hungate *et al.* 1996). The CO_2 used in FACE systems often is derived from fossil fuel and thus is depleted in ^{13}C relative to ambient CO_2 ($\delta^{13}C$ -8‰) (Schlesinger and Lichter, 2001). Subsequently the abundance of the ^{13}C isotope has been used to identify changes in soil organic matter. Indeed following a 200 μmol mol^{-1} step increase in atmospheric CO_2 for three years Schlesinger and Lichter, (2001) showed that a *Pinus taeda* forest produced 183 g C m^{-2} new soil C. Whereas an experiment utilising a beech – spruce model ecosystem produced 670 g C m^{-2} of new soil C after four years CO_2 enrichment (Hagedorn *et al.* 2003). Soil organic matter derived from plants with the C_4 photosynthetic pathway, and that is not as depleted in the ^{13}C stable isotope ($\delta^{13}C$ -20‰) against the Pee Dee Belemnite (PDB) international standard in comparison to C_3 derived detritus ($\delta^{13}C$ -27‰) is often used to determine new soil organic matter inputs (Hagedorn *et al.* 2003; Godbold *et al.* 2006.). Using C_4 soil and a strongly ^{13}C depleted CO_2 gas ($\delta^{13}C$ -39‰) increases the $\delta^{13}C$ difference of newly fixed C and soil improving accuracy and precision of new soil C calculations. By partitioning mycorrhizal and root input using in-growth cores and soil organic matter derived from C_4 plants data from the EuroFACE experiment showed that up to 62% of new soil C was the product of mycorrhizal mycelium (Godbold *et al.* 2006). In this experiment we tested the hypothesis that mycorrhizal extramatrical mycelium would have a greater input to soil C under elevated CO_2.

9.2 Material and Methods

9.2.1 Site Description and Experimental Design

The BangorFACE experimental plantation was established over an area of 2.36 ha at Abergwyngregyn (53°14'N, 4°01'W) 10 km east of Bangor in March 2004. Two fields, a former agricultural field, and former agricultural pasture which had been used for small scale forestry experiments for the previous eight years were chosen to host the experiment. The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Soils are fine loamy brown earth over gravel (Rheidol series)

and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002), and as a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011). Soil texture is 63% sand, 28% silt and 9% clay determined by laser diffraction (Coulter LS particle size analyzer). Climate at the site is classified as Hyperoceanic. Mean annual temperature collected at hourly intervals throughout 2005-2008 was 11.5 °C with an annual rainfall of 1034 mm. Soil soil characteristics determined during 2008 in the elevated and ambient plots are shown in Table 9.1.

Table 9.1 Average ± SE soil physical and chemical properties for Bangor during 2008 used in the experiment, two ambient and two elevated plots are located in each field.

	P mg kg^{-1}	K mg kg^{-1}	Ca mg kg^{-1}	Na mg kg^{-1}	pH	Moisture (%)	OM (%)
Ambient	$153.2^{\pm 13.0}$	$68.5^{\pm 4.4}$	$741.0^{\pm 50.9}$	$10.9^{\pm 0.4}$	$4.9^{\pm 0.1}$	$22.0^{\pm 0.4}$	$6.0^{\pm 0.1}$
Elevated	$82.6^{\pm 5.0}$	$64.3^{\pm 3.9}$	$695.9^{\pm 23.2}$	$14.1^{\pm 0.7}$	$4.7^{\pm 0.0}$	$21.4^{\pm 0.3}$	$5.3^{\pm 0.1}$

The experimental plots were comprised of eight octagonal plots 8 m in diameter, four ambient and four CO_2 enriched, in a 2 × 4 factorial block design. We used three tree species (*Alnus glutinosa* [L.] Gaertner, *Betula pendula* Roth. and *Fagus sylvatica* L.) that were selected due to their contrasting shade tolerance, successional chronology and to represent a range of taxonomic, physiological and ecological types. Each plot was divided into seven planting compartments and planted in a pattern creating areas of one, two and three species. However, for simplicity this experiment utilised three single species subplots of *B. pendula, A. glutinosa* and *F. sylvatica*, in addition to a fourth subplot which contained a mixture of all three species. Each experimental plot was surrounded by a 10 m buffer of the same species planted at the same density. The remainder of the field was planted with a mixture of species at 10000 stems ha^{-1}. The four FACE plots were enriched to 580 ppm using pure CO_2 depleted in ^{13}C (-39‰ PDB). CO_2 was supplied during the photosynthetically active part of the day through laser drilled holes in horizontal pipes suspended from eight masts and positioned mid canopy (Miglietta *et al.* 2001). Elevated [CO_2] was measured at one minute intervals and was within 30% of the target concentration for 75-79% of the time during 2005-2008.

9.2.2 C_4 soil in-growth cores

Soil in-growth cores were constructed using uPVC pipe 160 mm diameter and 250 mm length. To allow for root and fungal in-growth two windows 150 mm × 110 cm in the opposite sides of each tube were created. These windows were covered with a 2 mm nylon mesh to allow in-growth of fine roots and mycorrhizal extramatrical mycelium, or a 39 μm mesh which excludes roots but is sufficiently large to allow penetration of fungal hyphae (Agerer, 2001). Control cores were also constructed using identical tubing without windows and sealed using a 1 μm mesh on the bottom of the core to completely exclude roots and mycorrhizal hyphae. Field incubation of C_4 soil control cores enabled us to identify the effect of SOM decomposition of the $\delta^{13}C$ signature during the study.

During May 2007 the in-growth cores were installed in both ambient and elevated CO_2 plots at BangorFACE. Two cores, coarse mesh and fine mesh were installed into each of the three single species subplots and the three species mixture subplot. A collar of approximately 2 cm was left protruding above the soil surface to allow the attachment of respiration measuring equipment and to limit contamination of the core by surrounding C_3 soil. The soil used to fill the in-growth cores had been produced under *Zea Mays* continuous cultivation for ten years and was carefully selected to resemble soil chemicophysical characteristics of the native soils originating from BangorFACE (Table 9.2).The $\delta^{13}C$ signal of the selected C_4 soil was -22.5 ‰ relative to the Pee Dee Belemnite (PDB) international standard.

Table 9.2 Comparison of Henfaes soil characteristics with soil produced under maize (C_4 soil)

Soil	C: N	%C	%N	$\delta^{13}C$	pH
C_4 Soil	9.7	3.1	2.6	-22.5	5.36
C_3 Native	10.5	2.9	2.6	-28.0	4.70

Each core was filled with 5500 g of C_4 soil and packed down to a similar bulk density to the native soil. To exclude the contribution of leaf litter a plastic coated wire mesh was placed over each core. Removal of new moss and plant growth was performed regularly to ensure only root and mycorrhizal input was determined.

9.2.3 Sample collection soil, plant and fungal material

Soil samples were taken from in front of the in-growth core mesh windows during February 2009 using a 2 cm auger corer. Following transport back to the laboratory, samples were dried at 80 °C homogenised and a subsample milled to a fine powder.

Root sampling was undertaken during January of 2007 using randomly selected positions that were equidistant to neighbouring trees. Eight cores were taken from each plot, two cores from each of the three single species subplots and two from the three species mixture subplot using an 8 cm auger corer. Extracted cores were immediately placed into labelled polythene bags, transported back to the laboratory and stored at 4 °C. The following day roots were washed free of soil, and separated into distinct species and two size classes, fine (≤2 mm), coarse (>2 mm) before drying at 80 °C for 72 hours. Field soil samples were removed from each of the root cores during processing, dried at 80 °C for 72 hours and finely ground. Leaf litter was collected on a weekly basis from four litter baskets located in each of the three single species subplots and the three species mixture. Litter was returned to the laboratory, washed and sorted into individual species prior to drying at 80 °C for 24 hours.

Fungal hyphae were collected using the mesh bag technique (Wallander *et al.* 2001). Briefly, a 39 μm mesh that allows the in-growth of fungal hyphae was used to create 10 × 2 cm bags which were filled with acid washed quartz sand. Five bags were installed vertically in the single and mixed species subplots during April 2007 and collected at tri-monthly intervals. Fungal hyphae were extracted from the mesh bags using the water floating technique (Miller and Webster, 2005). Unfortunately, not enough hyphal material could be extracted from in-growth bags to allow determination of $\delta^{13}C$ from hyphae alone, therefore sporocarps of the mycorrhizal species *Paxillus involutus* were collected from the experiment plots and dried at 80 °C for 72 hours. Subsamples of the sporocarps and fine roots of each species were ground to a fine powder using a ball mill (Retsch Ltd., UK).and analysed for $\delta^{13}C$ to provide input signals.

9.2.4 Carbon analysis of soil, plant and fungal material

Field soil samples were weighed into tin (Sn) foil cups and analysed for C and N content using a TruSpec CN analyser (LECO Corp., Michigan, USA). Finely ground C_4 soil samples were weighed into pressed silver (Ag) capsules (Elemental Analysis, Kentucky, USA) and placed into microtitre plates. Soil carbonates were removed by moistening the soil contained within each capsule with 20 µl water and exposing to concentrated HCl acid vapour overnight (Harris *et al.* 2001). Ground plant and fungal material were weighed into tin (Sn) capsules. Total C and $\delta^{13}C$ content of the samples were determined by a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Carbon isotope ratio results are expressed as $\delta^{13}C$ per mil (‰) referenced to the Pee Dee Belemnite (PDB) international standard:

$$\partial^{13}C = 1000 \times \left(\frac{R_{sample}}{R_{s\tan dard}} - 1 \right) \qquad \text{(Eqn.1)}$$

Where the isotope ratio R = $\frac{^{13}C}{^{12}C}$

9.2.5 Respiration Measurements

Soil respiration was measured on the 28th May of 2008 using an LI-COR 8100 Automated Soil Respirometer (LI-COR, Lincoln, NE, USA). To ensure accurate measurement of root and mycorrhizal respiration an adapter plate was manufactured to provide a seal between the 160 mm soil core and 200 mm diameter respiration chamber. Measurements were made over a 2 minute integration time and a linear and exponential regression performed to determine the best fit to evolved CO_2. The contribution of saprophytic fungi to soil respiration was removed by deducting the CO_2 flux from the field incubated control cores from the coarse and fine mesh in-growth cores. Finally, to obtain root only respiration the CO_2 flux of fine mesh (fungal) in-growth cores was deducted from the coarse meshed (root and fungal) cores.

9.2.6 *Soil Carbon Calculations*

The fraction of soil derived from labelled plant material (f) was determined using the simple mixing model described by Balesdent *et al.* (1987) Equation 2.

$$f = \left(\frac{\partial^{13}C_{incubated}\ C_4 soil - \partial^{13}C_{initial}\ C_4\ soil}{\partial^{13}C_{plant\ biomass} - \partial^{13}C_{initial}\ C_4\ soil} \right) \qquad \text{(Eqn. 2)}$$

The contribution of fungal biomass to new soil C was first determined from the change $\delta^{13}C$ induced in the fungal only C_4 in-growth cores and the isotopic signal of the fungal material. Root only derived new soil C was determined using new C from the combined root and mycorrhizal $\delta^{13}C$ input minus only mycorrhizal derived C.

9.2.7 *Statistical Analysis*

Statistical procedures were undertaken with SPSS 17.0 (SPSS Inc., Chicago, IL) with $P<0.05$ used as the limit for statistical significance. A General Linear Model was used to calculate univariate analysis of variance. Data were tested for normality using Shapiro-Wilk's test. Homogeneity of variance was determined using Levene's test.

9.3 Results

9.3.1 *Plant and Fungal $\delta^{13}C$ input*

The $\delta^{13}C$ signature of fine roots significantly decreased (Table 9.3) following atmospheric enrichment with ^{13}C depleted CO_2 ($P<0.001$). In the ambient plots the $\delta^{13}C$ signature of fine roots was significantly different between *A. glutinosa* and *F. sylvatica* ($P=0.006$) and *B. pendula* and *F. sylvatica* ($P=0.007$). However, this difference was not present in roots collected from elevated CO_2 plots demonstrating that metabolic fractionation of ^{13}C in *F. sylvatica* results in root tissue more depleted in ^{13}C relative to other species. *Paxillus involutus* was chosen as a common mycorrhizal species with strong symbiotic associations with the Betulaceae and Fagaceae family to represent mycorrhizal input. The $\delta^{13}C$ of sporocarp collected within the elevated plots was -35.4 ± 0.4 ‰ a significant depletion relative to the ambient plots ($P<0.001$; Table 9.3).

9.3.2 *C_4 soil $\delta^{13}C$ signature*

Soil samples taken before the experiment yielded initial $\delta^{13}C$ signatures of -22.5 ± 0.4 ‰. Following 20 months of ^{13}C depleted C input through in-growth of hyphae and roots the $\delta^{13}C$ changed to between -22.7 and -23.3 ‰ in the fungal only cores and between -23.2 and -24.1 ‰ in the root and fungi in-growth cores (Table 9.4). There were no significant differences between the $\delta^{13}C$ of soils incubated in elevated or ambient plots. However, with the elevated plots a significant difference in $\delta^{13}C$ existed between *A. glutinosa* and *B. pendula* (P=0.023). Input from fungi and roots were significantly different for *F. sylvatica* (P=0.048) in ambient plots. Whereas in elevated CO_2 plots there was a significant difference in input between fungi and roots in *A. glutinosa* (P=0.048), *B. pendula* (P=0.050), and the mixture (P=0.023).

9.3.3 *Carbon efflux from C_4 soil cores.*

Root derived soil respiration ranged between 0.49 and 1.20 μmol CO_2 m^{-2} s^{-1} in ambient plots and between 0.98 and 1.77 μmol CO_2 m^{-2} s^{-1} in FACE. Evolved CO_2 in FACE was more than double ambient levels in the three species mixture plots. There also existed a marginal CO_2 efflux increase by FACE in all species; however these were not statistically significant. There were also no differences in root derived respiration between the species studied. Soil respiration resulting from mycorrhiza was between 0.26 and 1.22 μmol CO_2 m^{-2} s^{-1} in ambient plots and between 0.24 and 1.10 μmol CO_2 m^{-2} s^{-1} in FACE. CO_2 evolved from the *A. glutinosa* and the three species mixture subplots was approximately three fold greater than *B. pendula* and *F. sylvatica.* However, there were no significant differences between species or CO_2 treatment (Figure 9.1).

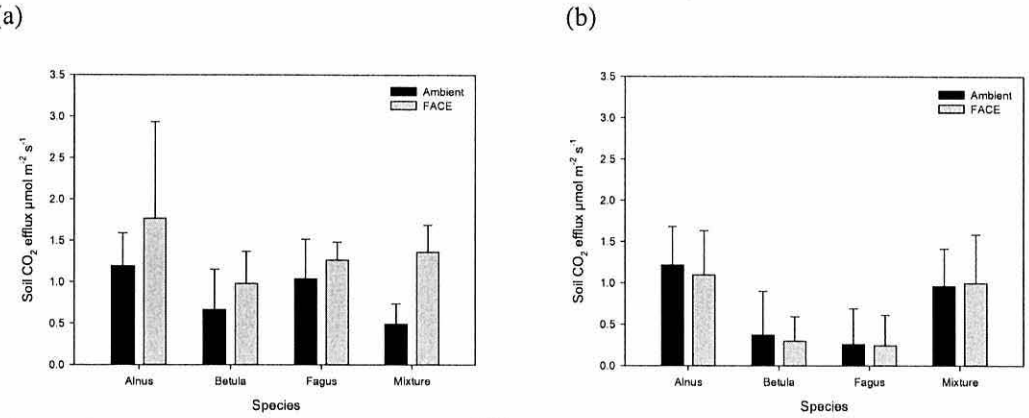

Figure 9.1. Mean C_4 soil in-growth core CO_2 efflux (mean ± SE; n=4) following 1 year of field incubation at ambient and elevated atmospheric CO_2 (a) root derived respiration (b) mycorrhizal mycelium derived respiration.

Dissolved organic C extracted from the cores at the final sampling was between 18.9 ± 0.7 and 20.5 ± 1.0 mg L^{-1} but was not significantly altered by treatment or in-growth core type (Table 9.5).

9.3.4 New Soil Carbon

Soil C derived from root and hyphal inputs are shown in Table 9.6. The largest contribution to calculated soil C was derived from fungi was evident within the ambient plots and was between 375 ± 179 g C m^{-2} and 485 ± 152 g C m^{-2}, with species contributions followed the order *A. glutinosa* > *B. pendula* > *F. sylvatica* > *Mixture*. Whereas in the elevated CO_2 plots hyphal C input accrued between 47 ± 20 g C m^{-2} and 133 ± 48 g C m^{-2} with species contributions following the order *F. sylvatica* > *B. pendula* > *Mixture* > *A. glutinosa*. The contribution of species differed by *A. glutinosa* in elevated CO_2 which was significantly smaller than *B. pendula* (P<0.05) no other species differences where observed. The C resulting from hyphal input was 10-fold greater in the ambient plots for *A. glutinosa* (P<0.05), and 3-fold greater in *B. pendula* (P<0.05), and *F. sylvatica* (P<0.05), and the three species mixture (P>0.05). Roots had a greater contribution to soil C than hyphal derived C under *A. glutinosa* and the three species mixture, yet conversely the contribution of roots was smaller for *B. pendula* and *F. sylvatica*. New C derived from roots ranged between -43 ± 268 g C m^{-2} and 70 ± 146 g C m^{-2} in ambient CO_2 plots. In the elevated CO_2 plots root derived C was greater with values between 90 ± 67 g C m^{-2} and 243 ± 84 g C m^{-2}. In ambient CO_2 conditions the species contributed to soil C in the order, *A. glutinosa* > *B.*

pendula > *F. sylvatica* > three species mixture this switched to the order, three species mixture > *A. glutinosa* > *B. pendula* > *F. sylvatica* in elevated CO_2 conditions. There were no significant differences in root derived C for treatment or between species.

Table 9.3 Mean ± SE $\delta^{13}C$ input signals from root and mycorrhizae used to calculate new soil carbon. Values for roots were obtained from each individual species, the mixture $\delta^{13}C$ input signal is calculated as a mean assuming equal contribution from all three species.

Input Component	Species	$\delta^{13}C$ Ambient	$\delta^{13}C$ Elevated
Root	*Alnus glutinosa*	-28.0 ± 0.4	-36.3 ± 1.2
	Betula pendula	-28.1 ± 0.3	-37.7 ± 0.6
	Fagus sylvatica	-29.7 ± 0.3	-36.9 ± 2.0
	Mixture	-28.6 ± 0.1	-36.1 ± 0.9
Mycorrhiza	*Paxillus involutus*	-26.6 ± 0.9	-35.4 ± 0.4

Table 9.4 Mean $\delta^{13}C$ values (‰ ± SE; *n=4*) for C_4 soil samples collected from root and mycorrhizal in-growth cores following 20 months of field incubation.

	Alnus glutinosa		*Betula pendula*		*Fagus sylvatica*		*Mixture*	
mesh size	Ambient	FACE	Ambient	FACE	Ambient	FACE	Ambient	FACE
39 µm	-23.2 ± 0.2	-22.7 ± 0.1	-23.2 ± 0.1	-23.3 ± 0.2	-23.2 ± 0.1	-23.1 ± 0.2	-23.1 ± 0.3	-23.0 ± 0.1
2 mm	-23.6 ± 0.2	-23.4 ± 0.2	-23.4 ± 0.1	-24.1 ± 0.3	-23.7 ± 0.2	-23.6 ± 0.1	-23.2 ± 0.2	-24.1 ± 0.4

Initial $\delta^{13}C$ C_4 soil was -22.5‰ PDB

Table 9.5 K_2SO_4 extractable dissolved organic carbon (DOC) determined from soil collected from the C_4 soil cores at the experiment conclusion. Data are mean ± SE; *n*=4.

CO_2 Treatment	In-growth core	DOC mg L^{-1}
Ambient	Mycorrhizal	20.5 ± 1.0
	Root	20.2 ± 2.5
Elevated	Mycorrhizal	18.9 ± 0.7
	Root	19.7 ±0 .6

Table 9.6 Mean new C values (g C m^{-2} ± SE; *n=4*) for C_4 soil samples collected from root and mycorrhizal in-growth cores following 20 months of field incubation.

	Alnus glutinosa		*Betula pendula*		*Fagus sylvatica*		*Mixture*	
	Ambient	FACE	Ambient	FACE	Ambient	FACE	Ambient	FACE
Root	70 ± 146	182 ± 56	-6± 127	123 ± 41	-24 ± 103	90 ± 67	-43 ± 268	243 ± 84
Fungal	485 ± 152	47 ± 20	461 ± 102	178 ± 35	436 ± 66	133 ± 48	375 ± 179	98 ± 29

9.3.5 Field Soil Carbon

Bulk soil C content at the experiment initiation was 2823 ± 57 g C m^{-2} and 2748 ± 99 g C m^{-2} in the elevated and ambient CO_2 plots respectively. Throughout the four years of plantation establishment soil C content steadily increased to 3516 ± 68 g C m^{-2} in ambient plots and 3536 ± 54 g C m^{-2} in elevated CO_2 plots (Figure 9.2a). Over the course of the experiment soil C in the top 0-10 cm increased by 24% in ambient and 30% in CO_2 enriched plots. Repeated measures analysis revealed a significant ($P<0.05$) year × treatment interaction and significant ($P<0.05$) effect of sampling year on soil C. However, the rate at which C accumulation occurred varied dramatically, both temporally and with depth. Initially, a greater rate of accumulation was observed in ambient plots, which in later years was superseded by the CO_2 enriched plots. Deeper within the soil profile (10-20 cm) soil C appeared to decline until 2008 (Figure 9.2b) whereas at 20-30 cm a decline in both ambient and elevated CO_2 plots was observed until the end of the experiment (Figure 9.2c). The mean rate of C accumulation was 173 g C m^{-2} yr^{-1} and 197 g C m^{-2} yr^{-1} for ambient and elevated CO_2 plots respectively.

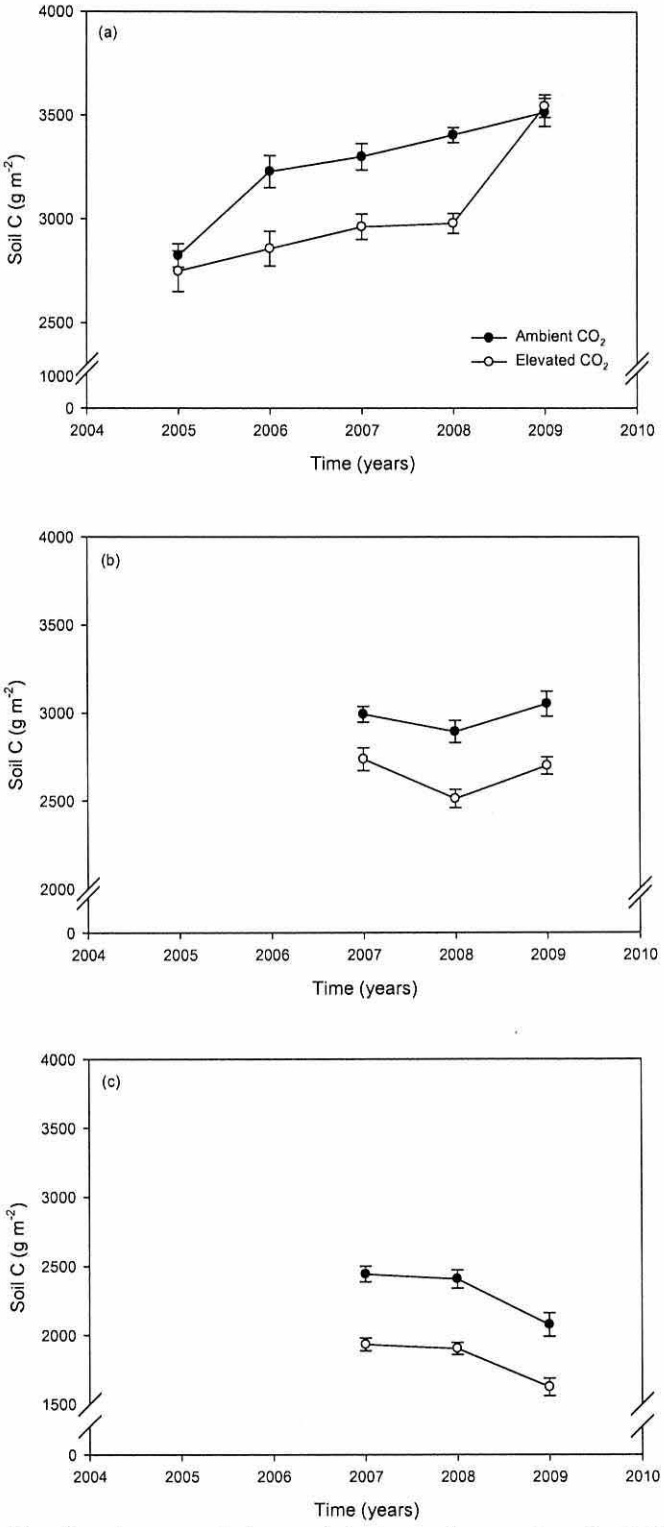

Figure 9.2 Mean field soil carbon sampled annual during each year since the initiation of experiment at (a) 0-10 cm and in the last three years at (b) 10-20 and (c) 20-30 cm. Filled circles indicate ambient CO_2 plots and hollow circles indicate elevated CO_2 plots. Error bars indicate ±1SE around the mean .

9.4 Discussion

9.4.1 Field Soil Carbon

During the four years following initiation of the experiment, soil C content increased at a rate of 173 g C m^{-2} yr^{-1} in ambient plots, and 197 g C m^{-2} yr^{-1} in FACE plots, an additional 24 g C m^{-2} yr^{-1} as a result of CO_2 enrichment. We attribute the increased C accumulation in ambient conditions to afforestation of agricultural land. Our results are broadly consistent with the results from the field afforested AspenFACE project, where an accrual of 298 g C m^{-2} was observed to a depth of 20 cm after four years, an accrual rate of 74.5 g C m^{-2} y^{-1} (Loya *et al.* 2003). At EuroFACE total soil C increased at a mean rate of 161 g C m^{-2} y^{-1}, and 36 g C m^{-2} yr^{-1} over three years for ambient and FACE respectively. In the current study, the rate of C accumulation was faster in ambient plots, and as the experiment matured the accumulation rate became faster in the elevated CO_2 plots. This observation is in agreement with that of Hoosbeek *et al.* (2004) who reported that soil C content increased more under ambient CO_2 treatments than under FACE at the EuroFACE plantation using two *Populus* species, and a *Populus* hybrid during the first two years following afforestation. Temporal variation in C accrual rates coupled with the heterogeneous forest environment, and the short duration of the experiment may not reflect the long term effect of elevated atmospheric CO_2 on soil C accumulation (Hungate *et al.* 1996). Subsequent analysis of soils collected at EuroFACE revealed that soil C was accumulating faster in the elevated CO_2 plots during the second rotation (Hoosbeek *et al.* 2006). Again this observation is consistent with the soil C accumulation at BangorFACE as during the latter half of the experiment C accumulation increased under FACE. Faster C accumulation in ambient CO_2 plots may be explained by the priming effect hypothesis (Kuzyakov *et al.* 2000), where following CO_2 enrichment changes in C inputs and environmental perturbations that favour mineralisation of old soil organic matter. Atmospheric CO_2 enrichment of plants increases the flux of labile C compounds to the soil through increased root production and turnover (Lukac *et al.* 2003), exudation (Phillips *et al.* 2006; Phillips *et al.* 2009) and decomposition of detritus. This increased flux of labile C, is thought to raise the rate of soil organic matter mineralisation and loss of C through co-metabolism of old and new C by microbial growth or activity (Fontaine *et al.* 2002; Fontaine *et al.* 2004). Indeed in a C

limited ecosystem, large labile C inputs may reduce limitations on microbial nutrient acquisition from organic matter creating a positive feedback on soil priming processes. In congruence with this hypothesis, Hoosbeek *et al.* (2004) postulated the occurrence of organic matter priming at EuroFACE to explain an initial reduction in soil C.

At the ORNL FACE facility at Oak Ridge, Lichter *et al.* (2005) observed an accrual of 52 g C m^{-2} y^{-1} in the organic forest floor C after six years, although FACE had no affect on the C content of mineral soil. Soil organic matter priming was again hypothesised to explain the lack of C accrual in mineral soils (Lichter *et al.* 2005). Whereas at the Duke FACE facility, Jastrow *et al.* (2005) reported a linear increase of soil C content under sweetgum (*Liquidambar styraciflua*) accruing 44 g C m^{-2} yr^{-1} in elevated CO_2 plots and no change in ambient plots. The lack of a priming effect was attributed to organic matter physical protection from mineralisation through the formation of microaggregates. Soil aggregation may also result in conditions that favour formation of organomineral complexes during detrital breakdown (Lützow *et al.* 2006). At the conclusion of the experiment at BangorFACE the soil C content at 20-30 cm depth was continuing to decline. We believe that this reduction is a result of deep soil priming resulting from supply of oxygen and labile C products deeper in the soil profile as a result of deeper rooting and enhanced production under FACE, which is supported by the observations of Fontaine *et al.* (2007). However, as with the upper soil horizons we expect that new C input and sequestration at this depth will eventually exceed C loss.

9.4.2 New soil carbon

New soil C calculated using the C_4 soil technique and averaged across all species produced 451 g C m^{-2} yr^{-1} and 274 g C m^{-2} yr^{-1} in ambient and elevated CO_2 respectively. In comparison to field C accrual measurements the rate of C accumulation is over three fold greater in ambient and two fold greater in elevated CO_2. Consistent with greater root production in elevated CO_2 root derived C was greater in the elevated CO_2 plots being 11 g C m^{-2} yr^{-1} in ambient and 159 g C m^{-2} yr^{-1} in elevated CO_2 plots. In the only other study to use the C_4 soil technique to measure root and fungal derived soil organic C, conducted at EuroFACE, Italy, Godbold *et al.*

(2006) reported annual increments of 347 g m^{-2} yr^{-1} ambient plots and 299 g m^{-2} yr^{-1} in FACE for root derived C, whereas mycorrhizal mycelial C yielded 552 g m^{-2} yr^{-1} in ambient plots and 488 g m^{-2} yr^{-1} FACE.

9.4.3 Root Detrital Input

Root drived C at EuroFACE was thirty fold greater in ambient and two fold greater in elevated CO_2 that in the present study. We attribute the large difference in root C input to greater root production and faster turnover of the species used (*Populus*). Lukac *et al* (2003) reported a mean annual root production of 542 g C m^{-2} yr^{-1} in ambient and 894 g C m^{-2} yr^{-1} in elevated, considerably higher than the 28 g C m^{-2} $yr^{-1}$and 34 g C m^{-2} yr^{-1} in ambient and elevated CO_2 plots in the present study. Furthermore, mean root turnover at the site was 1.75 yr^{-1} in comparison to only 0.45 yr^{-1} at BangorFACE. The magnitude of root detrital input may suggest a larger difference between the two sites than we have observed, although proportional increases in microbial mineralisation may balance our observation though losses of C during respiration and leaching.

If using annual root production and turnover measurements to predict root detrital C input we would expect a range between 13-15 g C m^{-2} yr^{-1} which is approximately half the input calculated using $\delta^{13}C$. It is likely that isotopic fractionation during plant metabolism and decomposition would account for these observed differences, as enzymatic and diffusional metabolic processes determine differences isotope composition of plant organs or biochemical molecules (Figure 6.3). In a recent study Badeck *et al.* (2005) determined that cellulose was 1.29‰ ^{13}C enriched compared with lipids and lignins that were 4.67 and 3.17‰ depleted respectively. Plant residues are composed of complex mixture of organic molecules, 50-60% non structural carbohydrates, 15-20% lignin, and 10-20% proteins, lipids, and waxes. Structural composition alters their bioavailability to mineralisation and soil residence time (Lützow *et al.* 2006). The abundance of molecules of variable recalcitrance differs between plant species, Godbold *et al.* (2006) calculated that if lignin was used as the major contributor of C to soil and was 5‰ ^{13}C depleted it would result in 30% more C.

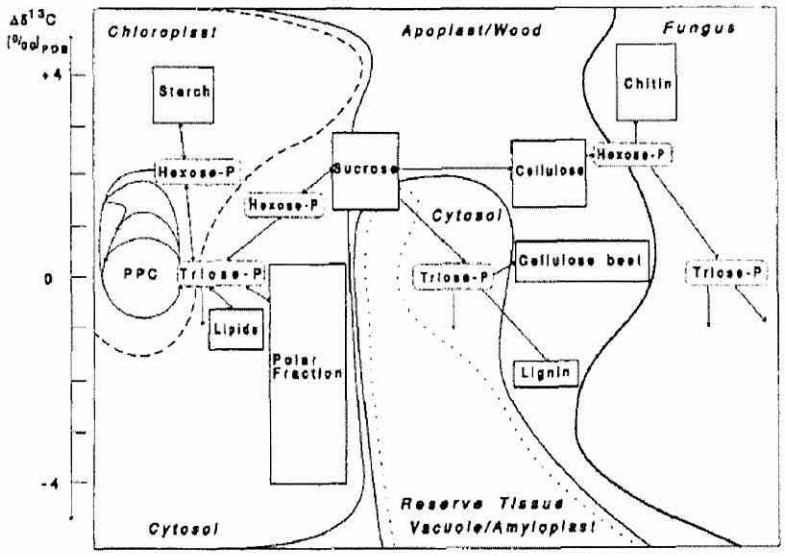

Figure 9.3 $\delta^{13}C$ (‰ PDB) of primary and secondary plant compounds in different cell compartments and in decomposing Basidomycete. The vertical scale indicates the relative amount of ^{13}C enrichment or depletion (Gleixner *et al.* 1993).

9.4.4 Mycorrhizal extramatrical mycelium input

C derived from mycorrhizal hyphal input in the ambient plots was of similar quantity to that reported by Godbold *et al.* (2006). Annual new C input averaged across species was 439 g C m^{-2} yr^{-1} in ambient and 114 g C m^{-2} yr^{-1} in elevated CO_2. Although we did not quantify the relative contribution of saprophytic or mycorrhizal fungal biomass within the cores, the large difference between the ambient and elevated CO_2 plots was surprising as measurements of the fungal biomarker ergosterol suggested no significant difference in mycorrhizal biomass due to CO_2 treatment (Smith *et al. in prep*).

Mycorrhizal fungi affect the formation of soil structure via a variety of biochemical and biophysical mechanisms. Mycorrhizal fungi contain chitin, a relatively recalcitrant compound thought to significantly contribute to the formation of soil organic matter (Rillig and Mummey, 2006). Information on chitinous residues in soil are sparse, although recent estimates of residence time are 49 ± 19 years for chitin derived pyrolysis products (Gleixner *et al.* 2002). To confirm our observed C production we calculated hyphal turnover from measured mycorrhizal biomass at BangorFACE using hyphal in-growth mesh bags. When averaged across all species subplots mycorrhizal biomass ranged between 17 g C m^{-2} and 107 g C m^{-2}. Based on

mycorrhizal biomass and annual hyphal C production in C_4 soil cores we quantified in this study a mycelial turnover rate between 9-19 days. Although interspecies variation is likely to confer differential life expectancy, our data is broadly consistent with those of Godbold *et al.* (2006) using a similar approach and those estimated using ^{14}C of 5-6 days (Staddon *et al.* 2003). Given that new C is based on $\delta^{13}C$ signature there are several possible hypotheses that may alter $\delta^{13}C$ of hyphal input, these include (i) mycorrhizal species composition, (ii) isotopic fractionation, (iii) mycelial anastomosis, and (iv) soil aggregate protection from microbial mineralisation.

In the present study we were unable to gather enough hyphal material from mesh in-growth bags in order to perform IRMS and subsequently had to rely on $\delta^{13}C$ signature of *Paxillus involutus* sporocarps which were observed in abundance at the site. In recent work, Bakker *et al.* (2009) showed that hyphae and sporocarps have have a comparable $\delta^{13}C$ signatures; however, using a single species input signature may not have accurately represented the signature of the species present and their relative contribution to soil C. At the Swiss Web-FACE facility the $\delta^{13}C$ signature of eleven mycorrhizal sporocarps within the CO_2 enriched forest was assessed and revealed a 5.8‰ variation between species (Keel *et al.* 2006). As mycorrhizal species form large anastomotic networks, it is possible that ambient CO_2 may enrich the $\delta^{13}C$ signature of fungi within the FACE plots resulting in the variation observed. Furthermore, elevated CO_2 has been shown to influence mycorrhizal species abundance and community composition (Godbold *et al.* 1997) which may result in different ispotopic signature input between treatments.

Fungi have demonstrated different isotopic fractionation to other phylum, basidiomycetes preferentially metabolises the lighter C isotope resulting in enriched cellular compounds, with chitin being enriched by 2‰ (Gleixner *et al.* 1993). If these compounds have a greater contribution to soil organic matter than other cellular constituents we would expect a reduction in C derived from extramatrical mycelium than observed.

9.4.5 Control C_4 soil core incubation and impact on C values

During the analysis of the data reported in this manuscript we found that calculated soil C values were not consistent with root and fungal biomass measurements previously obtained and discussed in Chapter 4. Contrary to our expectations C efflux

from the C_4 soil cores as respiration or dissolved organic C leachate was not detectably different between ambient and elevated CO_2 plots. Without a differential C efflux between treatments we were unable to satisfactorily explain the differences in soil C accrual between treatments. We believe that a C accrual anomaly exists between elevated and ambient CO_2 plots, which may be explained by differential mineralisation of the C_4 soil in the field. The initial $\delta^{13}C$ signature of the C_4 soil used for the in-growth cores was -22.5‰. To account for changes in the $\delta^{13}C$ signature of the soil during the experiment, eight field incubated exclusion cores were installed. The exclusion of hyphae and roots was achieved using a 1 μm mesh which reduced the core drainage, and increased soil moisture, occasionally resulting in the core flooding. We believe that during the 20 month experiment the $\delta^{13}C$ signature of the field incubated core became more depleted (-23.2‰) as a result of increased moisture, whereas the same C_4 soil kept in a dry storage vessel became ^{13}C enriched (-21.4‰). It appears likely that different mesh pores sizes differentially altered the soil moisture content, and therefore mineralisation of C_4 soil within each experimental core. If ^{13}C was mineralised in ambient cores the new C calculated from inputs with a small $\delta^{13}C$ signature would be large. Further confounding our results was the productivity of the BangorFACE forest ecosystem which was not high enough to produce a comparatively strongly depleted $\delta^{13}C$ within the elevated CO_2.

9.5 Conclusion

We found no evidence to support our hypothesis that mycorrhizal extramatrical mycelium C inputs would result in greater C inputs to soil under elevated CO_2. Indeed, contrary to our expectations C input to via the mycorrhizal pathway were greater in ambient CO_2 conditions. Firstly, we suspect that mycorrhizal community dynamics may introduce experimental artefacts that influence $\delta^{13}C$ isotopic signal resulting in an underestimate of C input in elevated CO_2 plots. Secondly, we believe that preferential mineralisation of ^{13}C occurred within the reference C_4 soil cores incubated in field conditions due to differing soil moisture artefacts within each core negating the results. What remains clear from our data is the importance of mycorrhizal extramatrical mycelium to soil C sequestration. Mycorrhizal mycelium is pervasive throughout terrestrial ecosystems and rapidly turned over. Therefore, it is

likely mycorrhizal mycelium has a fundamental role in the translocation of C to soil organic matter, and the global C cycle.

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10 Elevated atmospheric CO_2 enrichment delays autumnal senescence of *Betula pendula* in an aggrading mixed species temperate forest.

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Contributions:

Andrew Smith wrote the manuscript, collected the data, performed the analysis and maintained the FACE facility during 2006-2009. Dr. Martin Lukac designed, built and maintained the experiment during 2004-2006. Iftekhar Ahmed provided specific leaf area measurements collected during his MSc in 2006. Prof. Douglas Godbold is the project principle investigator who designed and constructed the experiment. He also provided assistance with data interpretation, analysis and manuscript preparation.

10.1 Introduction

Studies investigating the impact of global environmental change on terrestrial ecosystems have identified a consistent pattern of phenological change in the northern hemisphere (IPCC, 2007). Analysis of normalised difference vegetation index (NDVI) remote sensing data gathered during 1985-1999, has revealed an 18 day extension of the growing season in Eurasia (Zhou *et al.* 2001). Multiple drivers have been attributed to differentially influencing plant phenophases. Specifically, atmospheric warming has been correlated with earlier bud burst (Menzel *et al.* 2006), and delayed senescence to interactions with temperature and elevated atmospheric CO_2 concentrations (Taylor *et al.* 2008).

Leaf area and leaf phenology area are important attributes of the forest canopy because they determine the duration and rate of C fixation. Leaf area index (LAI), the projected leaf area per unit of ground area, is related to the amount of intercepted light available for canopy photosynthesis, and therefore ecosystem productivity (Herrick and Thomas, 2003). LAI is also an essential indicator of ecosystem functioning that may be affected by global climate change and atmospheric chemistry. Leaf phenology may be affected by the process of senescence, which is basically governed by developmental age, but also influenced by various integrated endogenous and environmental signals (Lim *et al.* 2007). Environmental factors influencing leaf senescence can be grouped into: (i) abiotic factors that include, drought, nutrient limitation, extreme temperatures, ozone induced oxidative stress, and (ii) biotic factors including, pathogen infection or shading by other plants (Li *et al.* 2000). Endogenous factors influencing senescence include, C source-sink relationships, phytohormones, particularly jasmonic (JA), abscisic acid (ABA), ethylene and salicylic acid (SA). The aforementioned phytohormones initiate senescence through cellular signalling pathways in response to various abiotic and biotic stresses that promote the expression of senescence inducing genes (Morris *et al.* 2000).

Anthropogenic activities since the industrial revolution have accelerated atmospheric CO_2 concentrations (IPCC, 2007). Elevated atmospheric CO_2 is known to increase forest net primary productivity by *ca.* 23% due to greater efficiency of the carboxylation enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) during photosynthesis (Long *et al.* 2004; Norby *et al.* 2005). These changes are of global significance as forests occupy one third of the land surface of the Earth, and

account for almost half of C stored in the terrestrial biosphere (Schlesinger and Lichter, 2001). Subsequently changes in tree leaf senescence may influence nutrient availability by reducing or increasing nutrient input to soil through fallen litter (Norby *et al.* 2003). Furthermore, LAI changes and phenological changes may alter light availability, and impact upon terrestrial ecosystems by altering plant community dynamics. Ecosystem productivity and plant community dynamics are intrinsically linked, consequently there is potential for direct and indirect feedback mechanisms to alter the global C balance (McConnaughay *et al.* 1996). For example, Goulden *et al.* (1996) showed that annual productivity in temperate deciduous forests was 500 kg C ha^{-1} greater when senescence was delayed by 5-10 days. Studies of the effects of elevated atmospheric CO_2 on autumnal phenophase of trees have produced conflicting results. Elevated CO_2 advanced senescence in two varieties of *Pinus ponderosa* (Houpis *et al.* 1988) and also in *Populus trichocarpa* (Sigurdsson, 2001), yet conversely delayed senescence of *Quercus myrtifolia* (Li *et al.* 2000), and *Populus* species grown in freely rooted field conditions during the AspenFACE and POPFACE studies (Taylor *et al.* 2008). However, at the DukeFACE experiment no effect on leaf phenology was observed in *Liquidambar styraciflua* (Herrick and Thomas, 2003).

Central to our understanding of the impact of environmental change on leaf phenology is elucidating how leaf senescence is initiated. Several studies utilising molecular genetic approaches have indicated that high concentrations of leaf sugars reduces photosynthetic activity, presumably through a negative feedback system, which induces leaf senescence (Swartzberg *et al.* 2010 ; Tallis *et al.* 2010 ; Solfanelli *et al.* 2006). For example, it has been demonstrated that hexokinases that catalyses phosphorylation of hexose, glucose and fructose are associated with accelerated senescence (Pourtau *et al.* 2006). Moreover, the mRNA expression of senescence associated genes (SAGs) have been analysed in response to senescence inducing treatments that included phytohormones (Quirino *et al.* 2000). For example, ethylene was found to induce chlorophyll loss and expression SAGs, while the application of abscisic acid lead to expression of only half of the age related SAGs. Conversley, genetic studies have also revealed that expression of the genes in tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*) leading to delayed senescence, are similar to gene expression associated with increased cytokinin levels (Quirino *et al.*

2000; Swartzberg *et al.* 2010). Furthermore, recent transcriptome analysis of *Populus* trees grown at the POPFACE elevated CO_2 experiment revealed up-regulation of genes determining anthocyanin production (Tallis *et al.* 2010)

The aims of this manuscript were to identify and quantify the effect of atmospheric CO_2 enrichment on delayed autumnal senescence in two broadleaved temperate forest tree species grown under elevated CO_2 using FACE technology for four years.

10.2 Methods

10.2.1 Site Description

BangorFACE experimental facility is located at Henfaes Research Station, Abergwyngregyn, North Wales, UK (53°14'N, 4°01'W). The site has a north westerly aspect, is 13-18 m above sea level with a shallow gradient of 1-2 ° on an alluvial fan. The water table is approximately 1-6 m below the soil surface. Soils are fine loamy brown earth over gravel (Rheidol series) and classified as Dystric Cambisol in the FAO system (Teklehaimanot *et al.* 2002), and as a Dystic Fluventic Eutrudept in the USDA system (Hoosbeek *et al.* 2011). Soil texture is 63% sand, 28% silt and 9% clay. Climate at the site is classified as Hyperoceanic with a mean annual temperature throughout 2005- 2008 of 11.5 °C and an annual rainfall of 1034 mm.

The experiment plots were located on two fields, a former agricultural field, and former agricultural pasture which had been used for small scale forestry experiments for the previous eight years. The experiment comprised of eight octagonal plots 8 m in diameter, four ambient and four CO_2 enriched, in a 2 × 4 factorial block design. We used three tree species (*Alnus glutinosa* [L.] Gaertner, *Betula pendula* Roth. and *Fagus sylvatica* L.) that were selected due to their contrasting shade tolerance, successional chronology and to represent a range of taxonomic, physiological and ecological types. Each plot was divided into seven planting compartments and planted in a pattern creating areas of one, two and three species. However, for simplicity this experiment utilised three single species subplots of *B. pendula, A. glutinosa* and *F. sylvatica*, in addition to a fourth subplot which contained a mixture of all three species. The experimental plots were surrounded by a 10 m buffer of the same species planted at the same density. The remaining field was planted with a mixture of species at 10000 stems ha^{-1}. The four FACE plots were

enriched to 580 μmol mol^{-1} using pure CO_2 depleted in ^{13}C (-39 ‰ PDB). CO_2 was supplied during the photosynthetically active part of the day through laser drilled holes in horizontal pipes. Two layers of pipes were suspended from eight masts and positioned mid canopy (Miglietta *et al.* 2001). Elevated [CO_2] was measured at one minute intervals and was within 30% of the target concentration for 75-79% of the time during 2005-2008.

10.2.2 Leaf measurements

Following observations of leaf senescence, fallen leaf litter was collected on a weekly basis from litter baskets 0.11 m^2 until all leaves had abscised (October to December). A litter basket was located in each of the three single species subplots and the three species mixture subplot (4 in each experimental plot). Litter was returned to the laboratory on the day of collection, washed and sorted into individual species, and then dried at 80 °C for 24 hours. The dry weight of each species was determined and recorded for each species subplot within each ambient and elevated CO_2 plot. Measurements of leaf area were made with a LI 3000A portable area meter (LI-COR, Lincoln, NE, USA) from fresh leaves collected during 2006. Immediately following area measurement leaves were dried at 80 °C for 24 hours, and weighed to determine specific leaf area according to Equation 1. Leaf area index was then calculated according to Equation 2 (McCarthy *et al.* 2007). Juvenile *Fagus sylvatica* was excluded from the calculations as a consequence of senesced litter retention until bud burst the following season. Leaf retention was deduced by subtracting fallen litter at each sampling collection from the total fallen litter after all the canopy leaves had abscised.

$$specific\,leaf\ area = \frac{leaf\ area}{leaf\ weight} \qquad \text{(Eqn. 1)}$$

$$leaf\ area\ index = fallen\ litter\ mass \times specific\ leaf\ area \qquad \text{(Eqn. 2)}$$

10.2.3 Statistical Analysis

Data were subjected to repeated measures factorial ANOVA, and Multivariate General Linear Model (SPSS 17.0, SPSS Inc., Chicago, IL) with $P<0.05$ used to determine statistical significance. Treatment, species were used as factors and data were assessed for normality using Shapiro-Wilk's test. Homogeneity of variance was determined using Levene's test. Graphs were produced using SigmaPlot v 11.0 (Systat Software Inc, Chicago, IL).

10.3 Results

10.3.1 Leaf Area Index

When species were grown in monoculture, elevated CO_2 caused a mean increase in LAI of 16% in *B. pendula,* and mean decrease of 6% in *A. glutinosa*. During the four years of CO_2 enrichment LAI of *B. pendula* was between 0.8-2.7 m^2 m^{-2} in elevated CO_2 and 1.1-2.3 m^2 m^{-2} in ambient CO_2 plots, whereas LAI of *A. glutinosa* was between 1.4-5.6 m^2 m^{-2} and 1.4-5.1 m^2 m^{-2} in elevated CO_2 and ambient plots respectively (Figure 10.1). Elevated CO_2 initially increased LAI of *B. pendula* by 37%, however this effect gradually declined to a decrease of -24% by conclusion of the experiment. The LAI of *A. glutinosa* was marginally increased (3%) at the experiment initiation but the FACE effect varied considerably for the duration of the CO_2 enrichment resulting in an mean reduction in LAI of -6% (Table 10.1). When species were grown in polyculture the LAI of *A. glutinosa* was decreased by 26% under elevated CO_2, conversely, LAI of *B. pendula* was increased by 16% (Table 10.1). Repeated measures ANOVA showed a significant year × species, and year × treatment interaction for species grown in monoculture ($P<0.05$; Table 10.2), however, analysis of tree species grown in polyculture revealed only a year × species interaction (Table 10.2).

Table 10.1 The effect of elevated CO_2 on LAI for *A. glutinosa* and *B. pendula* grown in monoculture and polyculture. Values are the percentage effect of elevated CO_2 relative to the control plots.

	Monoculture		Polyculture	
Year	*A. glutinosa*	*B. pendula*	*A. glutinosa*	*B. pendula*
2005	3	37	-36	-8
2006	-12	34	-21	1
2007	9	15	-27	66
2008	-22	-24	-21	3
Overall	-6	16	-26	16

Table 10.2 Analysis of the LAI of trees grown in monoculture and a three species mixture under ambient and elevated atmospheric CO_2 between 2005-2008 using repeated measures ANOVA.

	Monoculture		Polyculture	
Source of Variation	F-Value	Probability	F-Value	Probability
year	123.480	**<0.001****	44.828	**<0.001****
year × treatment	3.572	**0.044***	0.140	0.936
year × species	59.358	**<0.001****	18.143	**<0.001****
year × treatment × species	1.590	0.225	2.002	0.131

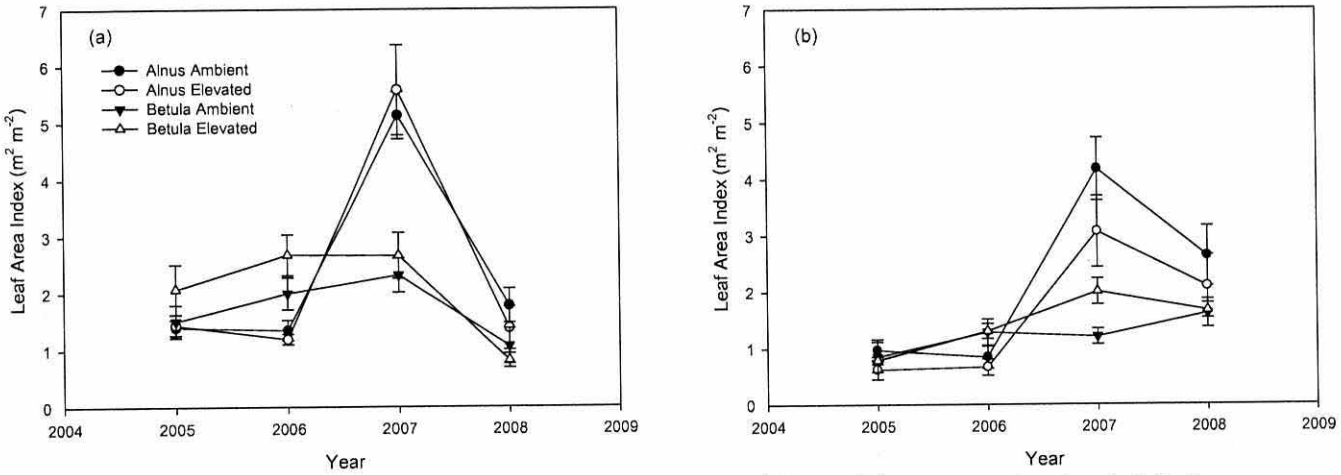

Figure 10.1 Measured leaf area index for *A. glutinosa* and *B. pendula* grown under elevated (hollow symbols) and ambient (filled symbols) atmospheric CO_2 in (a) monoculture and (b) polyculture. Values are mean ± 1 SE.

10.3.2 Environmental drivers

During the four year experiment annual minimum temperature ranged between -5.5 °C and -3.3 °C, whilst maximum temperature was between 25.4 °C and 34.3 °C. Growing degree days (GDD), and accumulative ozone over the threshold of 40 ppb were highest during 2006 when leaf retention was greatest, however, there no correlation between leaf retention and GDD was observed when other years were included. (Table 10.3)

Table 10.3 Environmental variables and there relation to leaf crown retention at BangorFACE throughout the four years of CO_2 enrichment.

Year	T_{min} (°C)	T_{max} (°C)	*GDD (base 10°C)	Rain (mm)	Ozone (AOT40)	Ambient CO_2 leaf retention (days)	Elevated CO_2 leaf retention (days)
2005	-3.5	27.0	1910	726	9058	201	201 (+0)
2006	-5.5	34.3	2065	1111	12931	176	190 (+14)
2007	-3.3	24.3	1672	705	3783	172	172 (+0)
2008	-4.5	25.4	1788	1077	7561	165	177 (+12)

*growing degree days (GDD) = $\left(\frac{T_{min}+T_{max}}{2}\right)$ - 10

10.3.3 Differential tree species leaf retention

Leaf retention was observed in *B. pendula* during two of the four growing seasons, (2006 and 2008) and only within atmospheric CO_2 enrichment plots (Figure 10.2). In 2006 litter collection was initiated on the 20th September, and by 11th October 61% of the forest canopy was retained in the crown of the ambient CO_2 plots, whereas 80% of the forest canopy was present in the crown of elevated CO_2 plots. The canopy of the *B. pendula* growing in the elevated CO_2 plots approached 61% of the total canopy 14 days later on the 25th October. In 2008 litter collection started on the 29th September, and by the 24th October only 4% of the total canopy litter had not abscised in the ambient plots, whereas 11% remained in the canopy of the elevated CO_2 plots, leaf abscission to levels observed in the ambient CO_2 plots required a further 12 days. No differences were observed within the *A. glutinosa* plots during the experiment or in *B. pendula* during 2005 or 2007. Whilst the litter retention appears evident in Figure 10.2, repeated measures ANOVA revealed no significant treatment or species × treatment interactions.

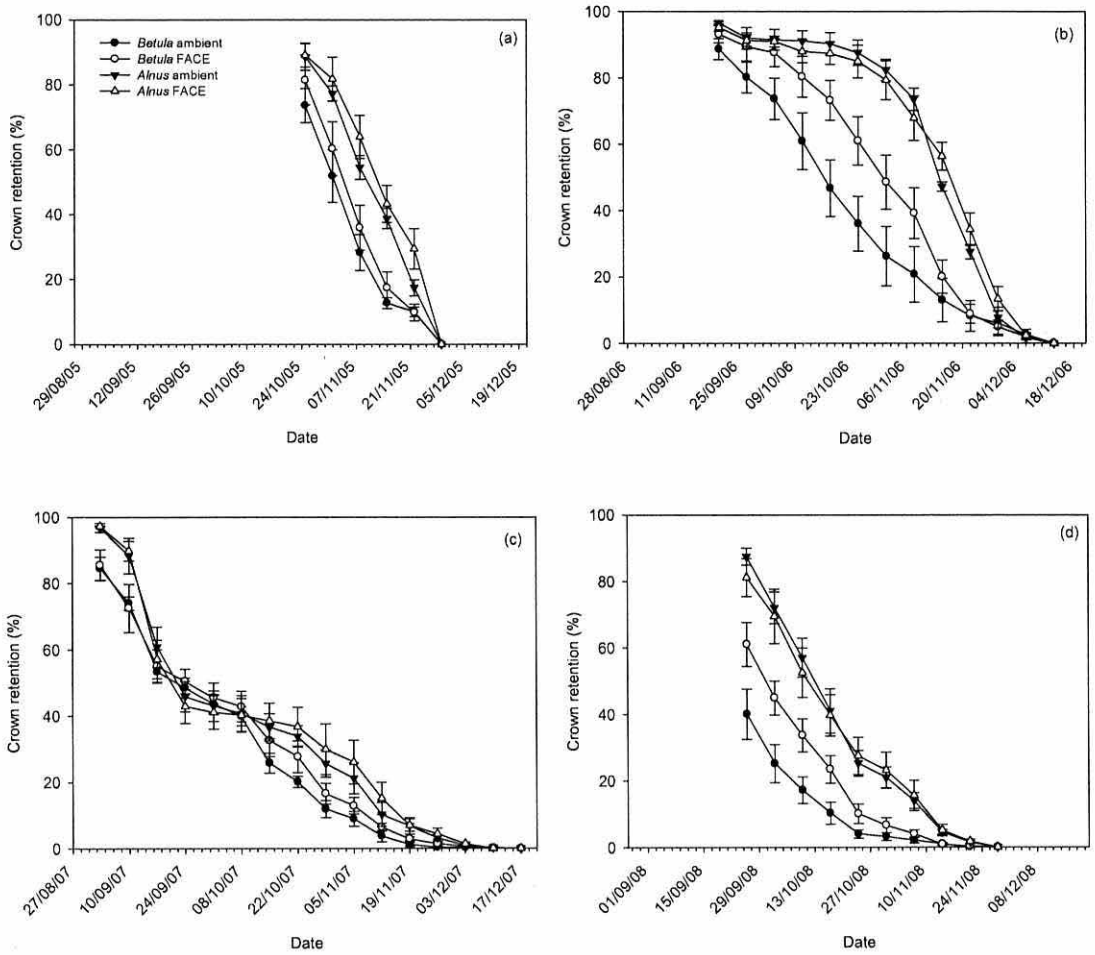

Figure 10.2 Mean ± SE tree crown retention for *A. glutinosa* (triangular symbols) and *B. pendula* (circular symbols) in ambient and elevated atmospheric CO_2 plots. Panels (a-d) denote growing seasons from 2005-2008.

10.4 Discussion

Plant senescence is a complex process influenced predominantly by environmental factors such as, temperature, light, N availability, soil moisture, in addition to plant physiological interactions that include phytohormones, leaf sugar content and the plant source-sink status (Winger *et al.* 2006; Taylor *et al.* 2008). The data presented here demonstrates that not only are senescent processes differentially altered by tree species, but that inter-annual variation in senescence indicates that external environmental drivers may have a significant interaction with elevated CO_2.

Many of the laboratory studies investigating senescence have utilised model organisms, such as *Arabidopsis,* which provide an excellent opportunity to increase

our mechanistic understanding of the biochemical processes involved in senescence. Annual plant species may not realistically represent autumnal senescence of woody plants in their natural environments, however, molecular genetic analysis has enabled the determination of specific gene expression patterns correlated to senescence environmental drivers that are likely relevant to explaining our results. Indeed, many of the environmental drivers of senescence processes such as N availability, soil moisture, phytohormones and intercellular sugar content influenced by source sink relationships have been previously shown to be altered by elevated CO_2 and are likely to be involved biochemical signalling pathways elucidated by laboratory studies.

10.4.1 Influence of sugars and plant source-sink relationships

Plant growth in an elevated CO_2 atmosphere is often associated with an accumulation of leaf starch and sugars, whilst leaf N and Rubisco contents are reduced (Ainsworth and Long, 2005). Reductions in leaf N content observed in elevated CO_2 experiments occur in conjunction with slowly depleting soil N reservoirs, and as a result of N being sequestered in biomass and soil organic matter. A progressive reduction in N availability suggests that foliar N resorption will become increasingly important, as foliar N resorption is an important mechanism that plants utilise to enhance their N use efficiency. Perturbations to the leaf sugar and N balance may potentially alter leaf N resorption rates or occurrence, substantially altering sugar and N feedbacks processes mediating senescence. Studies of *Arabidopsis* have demonstrated that leaf senescence can be induced by low N availability, and that N deficiency can result in leaf sugar accumulation (Pourtau *et al.* 2004). Therefore, the interaction between N content reduction and sugar accumulation creates a positive feedback accelerating senescence, and indicating that N supply has an important role in sugar metabolism. Indeed, Winger *et al.* (2006) speculated that N deficiency may lead to the accumulation of sugars as a result of decreasing demand for C rich skeletal structures during amino acid and protein synthesis. The aforementioned mechanisms allude to an advancement of senescence under elevated CO_2, as found at the Duke FACE experiment with sweetgum (*Liquidambar styraciflua*) trees (Herrick and Thomas, 2003). Conversely, Li *et al.* (2000) found that a close correlation between leaf N and leaf chlorophyll concentration or Rubisco activity and suggested that N metabolism was probably a major factor controlling the delayed senescence of *Quercus myrtifolia*

in a scrub-oak community. Generally, sugar content increases during the processes of leaf senescence (Quirino *et al.* 2001). It would therefore appear that leaves are not starved of C during senescence initiation, suggesting that a positive photosynthetic C balance may not be a senescence signal precursor (Herrick and Thomas, 2003). Molecular genetic work on *Arabidopsis* has identified several sugar sensing pathways, for example, leaf chlorosis and expression of *SAG12* can be repressed by the application of glucose, sucrose or fructose to detached leaves, whereas hexose accumulation promoted senescence signalling and activated pathways of N remobilisation (Pourtau *et al.* 2006). Furthermore, involvement of glucose and hexose signalling was revealed during delayed senescence in the *Arabidopsis* mutant *gin2-1* lacking hexokinase catalytic activity (Moore *et al.* 2003). Pourtau *et al.* (2006) showed that hexose content was 50-fold greater in *Arabidopsis* leaves during senescence, and concluded that a lack of hexose accumulation in senescing *gin2-1* plants suggested that delayed senescence is likely the result of altered sugar metabolism (Pourtau *et al.* 2006). In support of the complex interactions of sugar metabolism, a girdling experiment of sugar maple (*Acer saccharum*) showed that leaf sugar accumulation, initiated the formation of anthocyanin which was associated with delayed senescence (Murakami *et al.* 2008). Furthermore, in recent work using *Populus* specific cDNA microarrays up-regulated gene expression of leucoanthocyanidn dioxygenase (LDOX) and dihydroflavonol reductase (DRF), two enzymes involved in the biosythesis of anthocyanin was observed, in addition to increased autumnal leaf sugar accumulation (Tallis *et al.* 2010). These data allude to complex biochemical interactions of leaf senescence and plant sugar source-sink relationships and N metabolism that may be differentially altered in a high CO_2 atmosphere.

10.4.2 Influence of cytokinin on leaf senescence

Cytokinins are known to delay leaf senescence (Yong *et al.* 2000), and usually an excellent negative correlation between leaf cytokinin content and autumnal phenophase exists during senescence (Buchanan-Wollaston, 1997). However, the physiology and biochemistry relating to the production of cytokinins and their interactions with senescence processes are poorly understood. Many researchers consider cytokinins to be predominantly root-sourced plant hormones, which are

translocated from the roots through the xylem (Dong *et al.* 2008). The supposition that cytokinin synthesis occurs primarily in roots was supported by the discovery of IPT-genes that control cytokinin synthesis in plants (Chang *et al.* 2003); consequently, as elevated CO_2 has been shown to increase C allocation to roots and mycorrhizal symbionts (Iverson *et al.* 2010; Chapter 4), elevated CO_2 may also raise cytokinin production and subsequently increase leaf concentrations of cytokinins. In support of this hypothesis, a study of drought-stressed alfalfa (*Medicago sativa*) revealed that leaf concentrations of cytokinins were greatest in plants with mycorrhizal associations (Goicoechea *et al.* 1995). Alluding to additional ecophysiological interactions, Yong *et al.* (2000) showed that cotton (*Gossypium hirsutum* L.) grown under elevated CO_2 significantly increased cytokinin delivery to leaves under low N conditions, whereas cytokinin concentrations were found to be at similar levels in plants grown in high N at both ambient and elevated CO_2. The finding of Yong *et al.* (2000) is particularly prevalent in the light of progressive N limitation (PNL) (Reich *et al.* 2006). When an ecosystem is enriched with elevated atmospheric CO_2 photosynthesis stimulation is through the enhanced carboxylation of Rubisco (Long *et al.* 2004). Elevated CO_2 enhanced C assimilation ultimately increases C stored in recalcitrant biomass pools such as long-lived woody structures and soil organic matter, whilst also sequestering N. Subsequently, without alternative N inputs, mineral N pools slowly become depleted in a high CO_2 atmosphere. At the POPFACE experiment, Calfapietra *et al.* (2007) reported a reduction in N availability, and an increase in the N use efficiency (NUE; net primary productivity per unit annual N uptake) of *Populus* was increased following three years of CO_2 enrichment. Although at BangorFACE there was evidence of changes in N cycling in the ecosystem, which lead to alternative sources of N uptake, trees were able to maintain their N requirements (Millet *et al. submitted*; Chapter 11). Therefore there was no evidence of N limitation or greater N use efficiency within the time frame of the study, leading us to postulate that enhanced production of cytokinin would not have occurred through N limitation.

10.4.3 Influence of ozone on leaf senescence.

Plant growth in an elevated CO_2 atmosphere improves water use efficiency through a reduction in stomatal conductance (Medlyn *et al.* 2001; Long *et al.* 2004). In periods of high tropospheric O_3 concentration low stomatal conductance could reduce plant uptake of O_3, thereby protecting plants from ozone induced senescence, and

increasing the crown leaf retention of elevated CO_2 grown species. On the other hand, as high O_3 tropospheric O_3 concentrations occur during periods of high temperatures improved water use efficiency may result in higher stomatal conductance at high temperatures in elevated CO_2 leading to a larger flux of O_3 into leaves inducing a O_3 mediated senescence.

10.5 Conclusion

The data presented here contributes to the growing body of evidence suggesting that autumnal senescence in forest ecosystems is differentially delayed as anthropogenically driven environmental change increases the global atmospheric CO_2 concentration. In two of the four years of CO_2 enrichment *B. pendula* extended its growing by retaining leaves for approximately two weeks longer than when grown in ambient CO_2 conditions. Conversely, *A. glutinosa* growing within the same enrichment plots, showed no change in leaf retention throughout the experiment. However, our results show an inter-annual variability in delayed autumnal senescence suggesting biochemical and physiological changes induced by elevated CO_2 not only differentially alter tree species leaf longevity, but also interact with currently unknown environmental variables to delay autumnal senescence.

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11 N-fixation and N dynamics in *A. glutinosa*, *B. pendula* and *F. sylvatica* exposed to free air CO_2 enrichment (BangorFACE).

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Dr. Jonatham Millett wrote the manuscript, collected ^{15}N leaf data, and performed analysis. Prof. Douglas Godbold is the project principle investigator who designed and constructed the experiment. Andrew Smith, collected ^{15}N soil data, performed analysis, and maintained the FACE facility during 2006-2009. Dr. Helen Grant conducted ^{15}N analysis.

11.1 Introduction

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

The growth of C_3 plants is limited by present atmospheric CO_2 concentrations (Long *et al.* 2004). Therefore, forest growth is predicted to be stimulated by elevated atmospheric CO_2 (Norby *et al.* 2005). However, tree growth in natural systems is also in general limited by nitrogen (N) availability (Korner 2003; Millard *et al.* 2007). Furthermore, trees may become increasingly N limited as atmospheric CO_2 concentrations rise. This is because increased growth is accompanied by increased N requirement which may not be met by increased root N uptake (Luo *et al.* 2004). As a result the 'fertilisation' effect of elevated CO_2 may be reduced (Oren *et al.* 2001; Ainsworth and Long 2005; Reich *et al.* 2006). However, elevated CO_2 might also stimulate increased N uptake (Finzi *et al.* 2007), through increased plant investment in N capture to support increased growth. N and C use are clearly strongly interdependent. Therefore, understanding the interactions between elevated atmospheric CO_2 and N use and cycling in forests is essential for the accurate prediction of future global C dynamics (Reich *et al.* 2006). In particular the role of atmospheric N-fixation in plant and ecosystem responses to elevated CO_2 has been relatively little studied in forest ecosystems. As a result there are some key gaps in our knowledge of this important process.

By directly accessing N fixed from the atmosphere by symbiotic bacteria N-fixing plants are able to reduce their reliance on root derived N to some extent (Postgate, 1998; Vessey *et al.* 2005). The growth of N-fixing plants may therefore show a larger response to elevated CO_2 than non N fixing plants, at least when N availability is limiting (Bucher *et al.* 1998; Poorter and Navas, 2003). Furthermore, N-fixation may also be stimulated by elevated CO_2 (Hungate *et al.* 1999; Temperton *et*

al. 2003a; Feng *et al.* 2004), though this effect may disappear in the long term (Hungate *et al.* 2004). Additionally, N limitation in the entire plant community might be reduced when N-fixing plants are present (Roggy *et al.* 2004; Daudin and Sierra 2008), which might influence the response of the community to elevated CO_2. N 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). As a result N-fixation may play an important role in determining tree and forest responses to elevated atmospheric CO_2. However, no FACE studies in forest systems have to-date included N-fixing tree species. Therefore, our current knowledge of the response of N-fixing trees to elevated CO_2 is potentially limited by the experimental methods used to-date. These shortcomings can be overcome through the use of FACE systems (Long *et al.* 2004).

Plant-plant interactions structure plant communities and can determine ecosystem responses to environmental change (Grace and Tilman 1990; Grime 2001; Bruno *et al.* 2003). Plants rarely grow in isolation and their response to elevated CO_2 can be affected by the extent and type of plant-plant interactions they experience (Poorter and Navas, 2003). However, while changes in negative, competitive interactions between plants in response to elevated CO_2 have been considered in some detail, no studies that we are aware of have considered the impact of elevated atmospheric CO_2 on positive, facilitative interactions. This is despite their obvious importance in plant community structure and function (Bruno *et al.* 2003). When growing with N-fixing plants, non N-fixing plants may be able to access some fixed-N through direct transfer by release from nodulated roots, or 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 N-fixing plants. For example, 5-15% of the N in *Pinus contorta* growing with *A. glutinosa* was N that had been fixed from the atmosphere by *A. glutinosa* and transferred below ground to *P. contorta* (Arnebrant *et al.* 1993) and 31% of the N contained in the grass *Dichanthium aristatum* was from N fixed by co-occurring *Gliricidia sepium* (Daudin and Sierra, 2008). Nonetheless, as far as we are aware no study to-date has considered the impact of elevated atmospheric CO_2 on the transfer of fixed-N between N-fixing and non N-fixing plants.

In this study we used the ^{15}N natural abundance method to measure the proportion of N that was derived from atmospheric fixation (NDFA) for the N fixing tree *Alnus glutinosa* (L.) Gaertn. growing in monoculture or mixture in a free-air CO_2 enrichment experiment (FACE) study (BangorFACE). It was not possible to add labelled N to the site because of the potential for disturbing the N cycle and because the site is used for ongoing long-term studies. Therefore, the ^{15}N natural abundance method was used. Specifically we aimed to address the hypotheses that symbiotically fixed N contributes a significant source of the N required for the growth of the N fixing tree species *A. glutinosa*, fixed N is transferred to the non N-fixing tree species *Betula pendula* (L.) and *Fagus sylvatica* (L.) when growing in mixture with *A. glutinosa*, enhanced growth of *A. glutinosa* under elevated concentrations of atmospheric CO_2 is supported by an increase in N-fixation and enhanced growth of non N-fixing tree species under elevated concentrations of atmospheric CO_2 is supported by increased transfer of fixed-N.

11.2 Methods

11.2.1 Site description and sampling methods

The BangorFACE site is located at the Henfaes research station of the University of Wales, Bangor (UK Grid ref: SH655730; Lat. 53.23, Long. -4.02), covers a total area of 2.36 ha and was planted in March 2004. The climate is Hyperoceanic, with annual rainfall of about 1000 mm. Soils are fine loamy brown earth over gravel (Rheidol series), classified as a Distric Cambisol in the FAO system (Teklehaimanot and Sinclair 1993). The soil texture is 63% sand, 28% silt and 9% clay. The topography consists of a shallow slope of approximately 1-2° on a deltaic fan. The aspect is north-westerly, at an altitude of 13-18 m above sea level. Water table depth ranges between 1-6 m.

Trees were planted into two fields a former agricultural field which was used as pasture and to grow linseed, and a former pasture which has been used for small scale forestry experiments for the previous 8 years. The experimental plots are 8 m in diameter, and planted at 80 cm spacing in a hexagonal design (c. 18000 stems ha^{-1}) with *B. pendula*, *A. glutinosa* and *F. sylvatica*. The tree species were planted within the plots in a pattern that created areas with mixtures containing one, two and three

species. For the purpose of this study trees in four of these areas were measured – three single species sub-plots and the sub-plot containing a mixture of all three species. The plots are surrounded by a 10 m buffer strip of *B. pendula*, *A. glutinosa* and *F. sylvatica* planted at the same density. The rest of the plantation is planted with a mixture of tree species at a slightly lower density. Four FACE and four ambient plots were randomly located in the plantation in a complete replicated block design. CO_2 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 2 minute intervals and were within 30% deviation from the pre-set target concentration of 580 ppm CO_2 for 75-79% of the time during the photosynthetically active part of 2005-2008. Measurements were made for 3 trees from each species growing in monoculture and in mixture (n=18 trees per ring), in each of 4 ambient and elevated FACE rings (total n=144 trees).

The trees to be sampled were chosen from those in the centre of each stand (i.e. monoculture or mixture), from where they were selected at random. Trees were considered to be growing in monoculture when all 6 nearest neighbours (accounting for the hexagonal planting design) were the same species. Trees were considered to be growing in mixture when the 6 nearest neighbours contained at least one individual from each of the three species. For each, tree collar diameter of the main stem (stem diameter at 22.5 cm height) and height were measured. Additionally a leaf sample (n=5 per tree) was taken. $\delta^{15}N$ of tree leaves may be dependent on their position in the canopy (Domenach *et al.* 1989). Therefore, to ensure that the leaf sample was representative, a stratified random sample of leaves was taken from the canopy of each tree. 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 between the 16th and 20th August 2009. Soil samples were obtained from each of the four stands in each ring during root coring in January 2008. An 8 cm auger corer was used to collect samples at three depths 0-10, 10-20 and 20-30 cm.

11.2.2 Data analysis

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 (NAREA) and mass (NMASS) 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 and sieved <2 mm before being ground to a fine powder, $\delta^{13}C$ of the soil samples was also being measured. Therefore, they were HCl acid fumigated to remove carbonates according to Harris *et al.* (2001). Approximately 20 mg was weighed into Ag pressed foil cups, fumigated overnight and re-wrapped in Sn foil cups before analysis by IRMS with a Finnigan MAT Delta Plus XL continuous flow mass spectrometer at Duke University, Stable Isotope Laboratory. Results are given using the δ notation expressed in units of per mil (‰) where $\delta = (R_{sample}/R_{reference}) - 1 \times 1000$, and $R = {}^{15}N:{}^{14}N$. Data are reported with respect to N in air. The mean $\delta^{15}N$ of each core (i.e. mean of 3 depths) was used in subsequent data analysis. $\delta^{15}N$ of atmospheric N is by definition, while that of N taken up from the soil by plant roots tends to be relatively enriched in the heavier isotope resulting in higher values of $\delta^{15}N$ (Boddey *et al.* 2000). Therefore, the $\delta^{15}N$ of N-fixing plants is determined by the contribution of soil and atmosphere derived N to the plants N budget, integrated over the life of the plant and subject to alteration through any loses of N from the plant. By comparing $\delta^{15}N$ of the N-fixing plant, non N-fixing species growing in the same area as the N fixing species and N-fixing species grown with no root N addition, the contribution of N derived from the atmosphere (Ndfa) to the N budget of the N-fixing plant, over the life of the plant, can be estimated (Boddey *et al.* 2000). $\delta^{15}N$ of *B. pendula* and *F. sylvatica* growing in monoculture were used as the reference plants. N-fixation was calculated on a per leaf basis as follows (after Shearer and Kohl, 1986):

$$\%Ndfa = (\delta^{15}NTREE - \delta^{15}NFIXED)/(\delta^{15}NREF - \delta^{15}NFIXED) \times 100 \qquad \text{(Eqn. 1)}$$

Where %Ndfa is the percentage of leaf N fixed from the atmosphere, δ^{15}NFIXED is the δ^{15}N of trees for which the only source of N is derived from atmospheric fixation, δ^{15}NREF is the δ^{15}N of trees for which the only source of N is through soil uptake and δ^{15}NTREE is the δ^{15}N of the tree of interest. δ^{15}NREF of -1.9‰ was used. This is based on nodulated *A. glutinosa* plants growing in an N-free medium (Domenach *et al.* 1989). δ^{15}NREF was calculated from the mean δ^{15}N of leaves growing in the same position on *F. sylvatica* and *B. pendula* growing in monoculture in the same ring. As such, reference plants growing in ambient CO_2 concentrations were used to calculate %Ndfa and Ndfa for *A. glutinosa* growing in ambient CO_2 concentrations and reference plants growing in elevated CO_2 concentrations were used to calculate %Ndfa and Ndfa of *A. glutinosa* growing in elevated CO_2. %Ndfa and Ndfa were calculated separately using *F. sylvatica* or *B. pendula* as reference plants and using the mean value for the two species. Thus data are presented in the text as a range and a mean value. Soil-leaf N enrichment factor (EF) was calculated as follows for each tree (after Garten *et al.* 2007):

$$EF = \delta^{15}NLEAF - \delta^{15}NSOIL \qquad \text{(Eqn. 2)}$$

The potential for contamination between stands within the FACE and ambient rings (for example by *A. glutinosa* litter/below-ground N transfer), due to the relatively close proximity and small size of the stands, was tested. A sample of *B. pendula* leaves was taken from a larger *B. pendula* stand within the wider planting scheme at the site, where there was minimal possibility of contamination from other stands. This stand was c. 15 m × 15 m and c. 20 m from the comparative experimental ring. A sample of approximately 100 leaves was taken from 10 trees in the centre of this stand. These leaves were pooled and the δ^{15}N measured as above. Collar diameter of the main stem of each tree was used to estimate tree and total leaf mass using allometric equations based on trees harvested in 2006 from the buffer zone around the FACE and ambient rings (Chapter 3). These estimates of total leaf mass were combined with measurements of leaf N detailed above to calculate the total amount of leaf N (NTOTAL), the total amount of leaf N derived from the atmosphere (Ndfa) and the soil (Ndfs) on a per tree basis. The five separate leaf measurements were averaged over the whole tree to give one 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 (CO_2) treatments. Mixture/monoculture (MixMon) was treated as a subplot within ring and species was nested within mixture/monoculture. The model used was:

CO_2+ Ring(CO_2) + MixMon + Species + MixMon × Ring(CO_2) + Species × Ring(CO_2) + CO_2 × Species + CO_2 × MixMon + Species × MixMon + Species × MixMon × Ring(CO_2) + CO_2 × Species × MixMon.

Ndfa and %Ndfa 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 the same model but with terms containing 'MixMon' omitted and a 'Stand' term replacing 'Species'. Fisher's protected LSD was used for post-hoc multiple comparisons. *B. 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. *B. pendula* and *F. sylvatica* in monoculture were included as a 'zero *A. glutinosa* neighbours' group. Where appropriate data were Log10 transformed to conform to the assumptions of normality and heteroscedacity. α of 0.1 was used because the small number of replicates for CO_2 treatment increases the risk of a type II error. While this increases the risk of a type I error this was considered an acceptable trade-off.

11.3 Results

There were large between species differences in tree biomass with *A. glutinosa* = *B. pendula* >> *F. sylvatica* (Figure 11.1a, Table 11.1). This difference was largest for trees growing in mixture rather than monoculture (Spp. X Mix/Mon interaction, Table 11.1) due to increased biomass of *A. glutinosa*, and *B. pendula* and decreased biomass of *F. sylvatica*. Overall tree growth increased by 7.6% under elevated CO_2 but this increase was not statistically significant. The three tree species differed in their growth response with *A. glutinosa* and *F. sylvatica* both exhibiting higher growth rates under elevated CO_2 (11.1% and 10.6% increase respectively) and *B. pendula*

showing no change (1.0% increase) There were significant differences in soil $\delta^{15}N$ between stands, with $\delta^{15}N$ of soil in the mixed stand significantly lower than that of the monoculture stands (Figure 11.2). There was no statistically significant impact of elevated CO_2 on soil $\delta^{15}N$. However, soil $\delta^{15}N$ was consistently higher under elevated CO_2 in all four stands, by an average of 0.42‰ (GLM results: CO_2 - F1, 6=1.46, P=0.27; Stand – F3, 18=2.80, P=0.07; CO_2 X Stand – F3, 18=0.33, P=0.80). There were significant differences in $\delta^{15}N$ between leaves on the three tree species when growing in monoculture with *A. glutinosa* << *B. pendula* < *F. sylvatica* (Table 11.1, Figure 11.3a). Overall there was a difference of 2.45 ‰ and 2.90 ‰ between *A. glutinosa* and *B. pendula* or *F. sylvatica* respectively. $\delta^{15}N$ of tree leaves was reduced under elevated CO_2 for all species by on average 0.38‰, but this effect was only statistically significant for *F. sylvatica* (*A. glutinosa* = 0.33‰, *B. pendula* = 0.05‰, *F. sylvatica* = 0.76‰; Table 11.1, CO_2 effect and CO_2 × Species interaction). Additionally, leaf $\delta^{15}N$ followed the pattern of soil $\delta^{15}N$ and was consistently and significantly lower in mixtures compared with monocultures. $\delta^{15}N$ of the leaf sample taken in the *B. pendula* stand outside of the experimental plot but within the wider planting scheme was almost identical to that of the *B. pendula* monoculture in the corresponding plot (2.17‰ and 2.19‰ respectively).

The $\delta^{15}N$ of leaves of the non N-fixing species was significantly affected by the number of *A. glutinosa* neighbours. Leaf $\delta^{15}N$ decreased within increasing numbers of *A. glutinosa* neighbours (Figure 11.4, Kruskal-Wallis results: d.f. = 4, $\chi 2$= 12.94, P=0.01). The soil-leaf N enrichment factor (EF) followed patterns of leaf $\delta^{15}N$ to a large extent, with the exception of differences between trees growing in mixture and monoculture for which there were no significant differences (Table 11.1, Figure 11.3b). Overall a more negative EF was observed in trees growing in elevated CO_2, though this CO_2 effect was largest and only statistically significant for *F. sylvatica*. The EF was considerably more negative for *A. glutinosa* with no significant difference between *F. sylvatica* and *B. pendula*. Total leaf N was increased in all trees in all treatments under elevated CO_2, by an average of 14% (Table 11.1, Figure 11.1b). However, the CO_2 effect was not statistically significant. Differences between species followed those in tree mass with *A. glutinosa* = *B. pendula* >> *F. sylvatica*. Furthermore, there was a significant difference between total leaf N of trees growing in mixture or monoculture, but this was due to a large, significant difference between

total leaf-N of *A. glutinosa* in mixture (=20.0 g. tree^{-1}) and in monoculture 1 (=12.8 g. tree^{-1}). There was no difference between the other two species growing in mixture and monoculture. When $\delta^{15}N$ was used to estimate %Ndfa, 59.2 – 63.3% (mean=62.0%) of the N in leaves of *A. glutinosa* was obtained from atmospheric fixation (Figure 11.1b). There was no effect of elevated CO_2 on %Ndfa, though there was a trend to increased reliance on Ndfa for trees growing in mixture (%Ndfa in mixture: ambient CO_2 = 57.1-63.5%, mean=61.3%; elevated CO_2 = 66.8-68.6%, mean=68.3%. %Ndfa in monoculture: ambient CO_2 = 55.3-62.2%, mean=59.8%; elevated CO_2 =57.4-58.8%, mean=58.8%) (Table 11.2). *A. glutinosa* gained on average 10.1 – 10.6 g. tree^{-1} (mean=10.4 g. tree^{-1}) of N from fixation. The 'CO_2 effect' on Ndfa was far larger for trees growing in mixture than in monoculture (an increase under elevated CO_2 of 45.5 and 9.9% respectively), though the CO_2 × 'MixMon' interaction was not statistically significant (Figure 11.1b, Table 11.2). 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. Patterns of NAREA and NMASS were broadly similar (Figures 11.1c, 11.1d; Table 11.1). For both measures of leaf N content there were significant differences between species, with *A. glutinosa* = *B. pendula* > *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 CO_2, NAREA and NMASS were affected differently. There was no impact of elevated CO_2 on NMASS. However, there was a significant reduction in NAREA, by an average of 5.3%. This reduction was consistent for all species.

Table 11.1 Results of univariate GLM for characteristics of trees of three species (*A. glutinosa*, *B. pendula* and *F. sylvatica*) growing in monoculture or mixture (Mix/Mon) at ambient or elevated (ambient + 200 ppm) CO_2 growing in the BangorFACE experiment. Presented are the F and P-values from the analyses of $\delta^{15}N$, soil to leaf nitrogen enrichment factor (EF), total leaf N per tree (NTOTAL), leaf N per unit area (NAREA), N per unit mass (NMASS), N derived from soil (Ndfs) and tree mass. Significant ($P<0.1$) effects are emboldened.

		$\delta^{15}N$		EF		N_{total}		N_{area}		N_{Mass}		N_{dfs}		Tree Mass[1]	
Effect	df	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
CO_2	1,6	**6.00**	**0.05**	**4.17**	**0.09**	0.70	0.43	**6.73**	**0.04**	0.003	0.96	0.09	0.928	0.47	0.52
Species	2,12	**302.84**	**<0.001**	**236.21**	**<0.001**	**530.95**	**<0.001**	**179.48**	**<0.001**	**76.23**	**<0.001**	**36.22**	**<0.001**	**216.85**	**<0.001**
Mix/Mon	1,6	**35.89**	**<0.001**	1.64	0.25	2.54	0.16	0.11	0.75	**3.95**	**0.09**	0.731	0.571	0.53	0.49
CO_2 × Species	2,12	**3.95**	**0.05**	**4.31**	**0.04**	0.05	0.95	0.76	0.49	1.61	0.24	0.208	0.815	0.13	0.88
CO_2 × Mix/Mon	1,6	0.49	0.51	0.33	0.59	0.03	0.86	2.69	0.15	1.05	0.34	0.324	0.590	1.38	0.28
Species × Mix/Mon	2,12	1.07	0.39	0.25	0.78	**12.56**	**<0.001**	**11.08**	**0.002**	**7.94**	**0.01**	2.842	0.098	**22.19**	**<0.001**
CO_2 × Species × Mix/Mon	2,12	1.77	0.21	1.86	0.20	0.29	0.76	1.80	0.21	0.87	0.44	0.560	0.585	0.50	0.61

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

Table 11.2 Results of univariate GLM for impacts on N-fixation in *A. glutinosa* growing in monoculture or in mixture with *B. pendula* and *F. sylvatica* (Mix/Mon) at ambient or elevated (ambient + 200 ppm) CO_2 growing in the BangorFACE experiment. Presented are the F and P-values from the analyses of %Ndfa and Ndfa. Significant ($P<0.1$) effects are emboldened. Values are calculated based on the mean obtained from using both *B. pendula* and *F. sylvatica* as reference plants.

		Ndfa		%Ndfa	
Effect	d.f.	F	*P*	F	*P*
CO_2	1,6	1.35	0.29	0.87	0.39
Mix/Mon	1,6	**5.55**	**0.06**	**5.21**	**0.06**
CO_2 × Mix/Mon	1,6	1.71	0.15	2.64	0.16

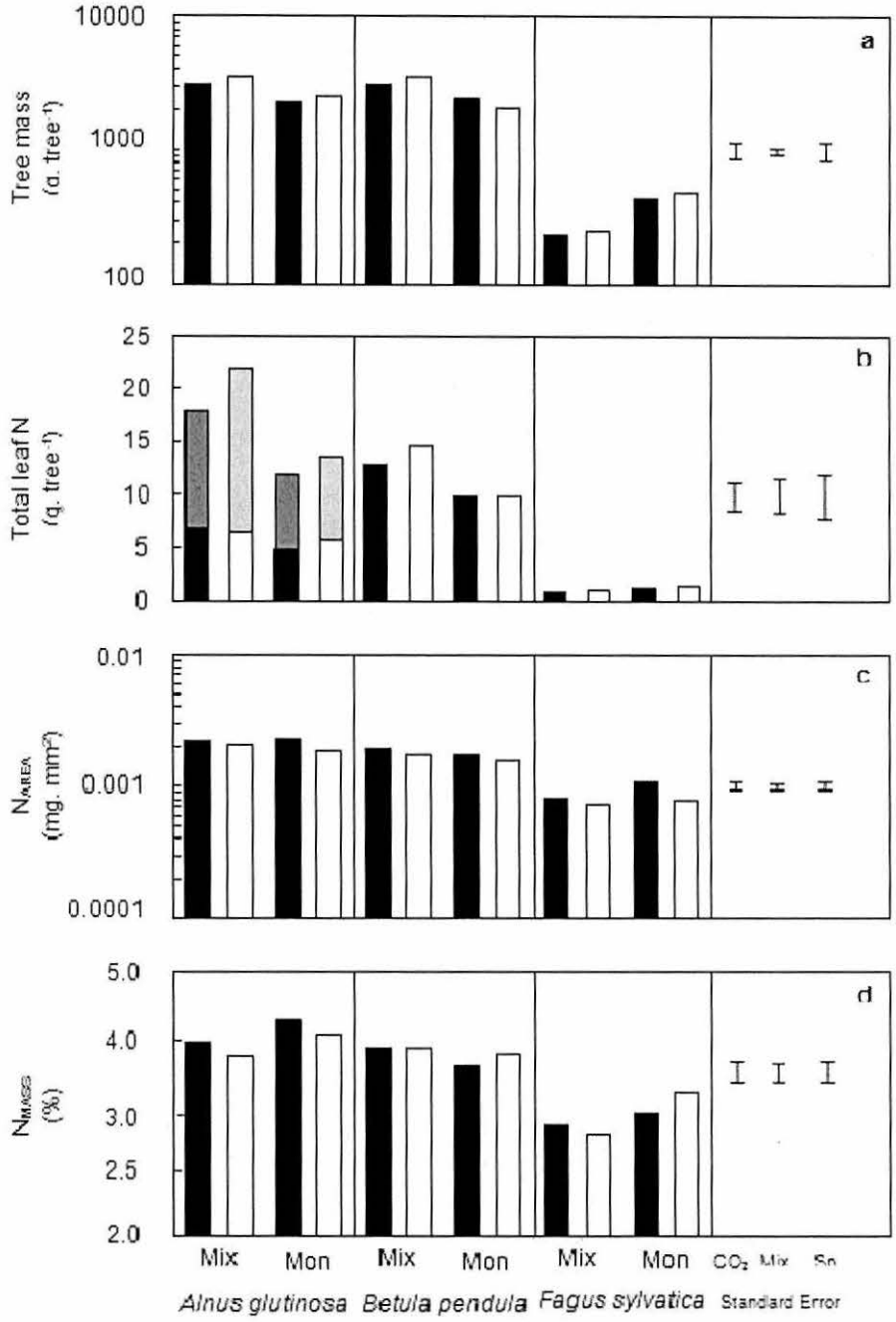

Figure 11.1 Characteristics of three tree species growing in the BangorFACE experiment. Presented are: a: total tree dry mass; b: total leaf N content per tree (upper parts of bars for A. glutinosa indicate Ndfa, all other bars are Ndfs); c: leaf N content on an area basis (NAREA); d: leaf N content on a mass basis (NMASS) trees growing in monoculture (Mon) or in a mixture (Mix) of all three species at ambient (shaded bars) or elevated (ambient + 200 ppm) (open bars) atmospheric CO_2. Data for total leaf N and NMASS (b and d) are means; data for Tree mass and NAREA (a and c) are geometric means and are presented with a log y axis for ease of interpretation of SEM's. SEM for the main effect of CO_2, Mixture/monoculture (Mix.) and species (sp.) are shown on the left of each graph.

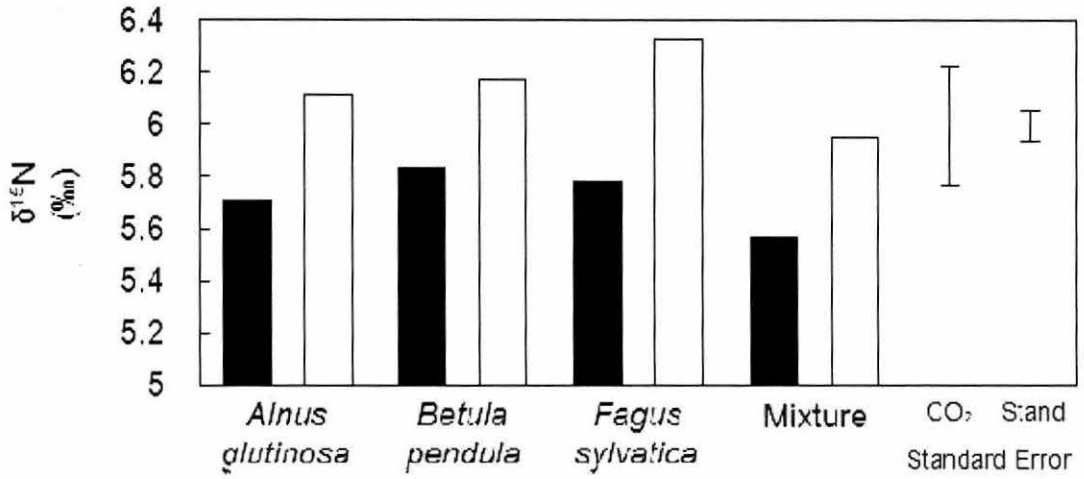

Figure 11.2 $\delta^{15}N$ and of soil in three stand types in the BangorFACE experiment. Presented are mean of trees growing in *A. glutinosa*, *B. pendula* and *F. sylvatica* monoculture or in a mixture of all three species at ambient (shaded bars) or elevated (ambient + 200 ppm) (open bars) atmospheric CO_2. SEM for the main effect of CO_2, Mixture/monoculture (Mix.) and species (sp.) are shown on the left of each graph.

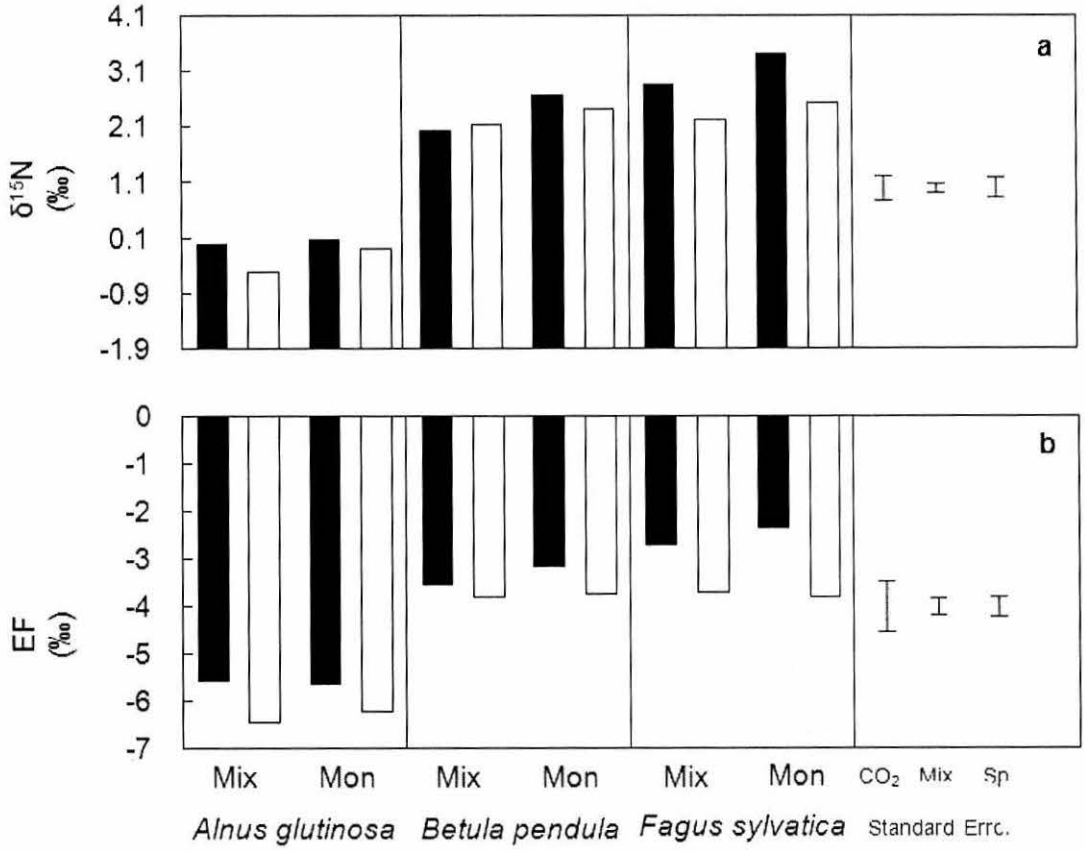

Figure 11.3 $\delta^{15}N$ (a) and soil-leaf N enrichment factor (EF = soil $\delta^{15}N$ – leaf $\delta^{15}N$) (b) of leaves of three tree species growing in the BangorFACE experiment. Presented are mean of trees growing in monoculture (Mon) or in a mixture (Mix) of all three species at ambient (shaded bars) or elevated (ambient + 200 ppm) (open bars) atmospheric CO_2. SEM for the main effect of CO_2, Mixture/monoculture (Mix.) and species (sp.) are shown on the left of each graph. Note that the x-axis minimum is -1.9. This is the expected $\delta^{15}N$ for *A. glutinosa* growing with no root N.

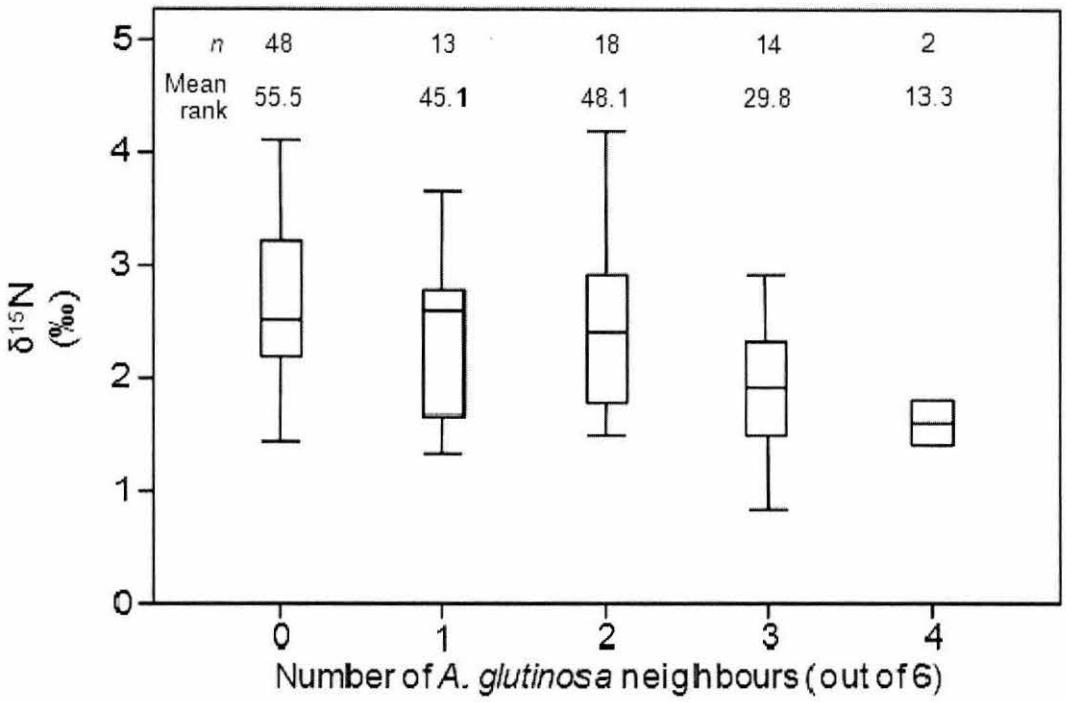

Figure 11.4 $\delta^{15}N$ of leaves of *B. pendula* and *F. sylvatica* trees growing in the BangorFACE experiment. Box-plots show the median and 25th and 75th percentile; whiskers show the minimum and maximum for trees growing with different numbers of *A. glutinosa* neighbours. Note that the values for zero (0) neighbours are from *B. pendula* and *F. sylvatica* growing in monoculture; the remaining data are for *B. pendula* and *F. sylvatica* trees growing in a mixture of all three tree species. Numbers of individual trees are shown for each group.

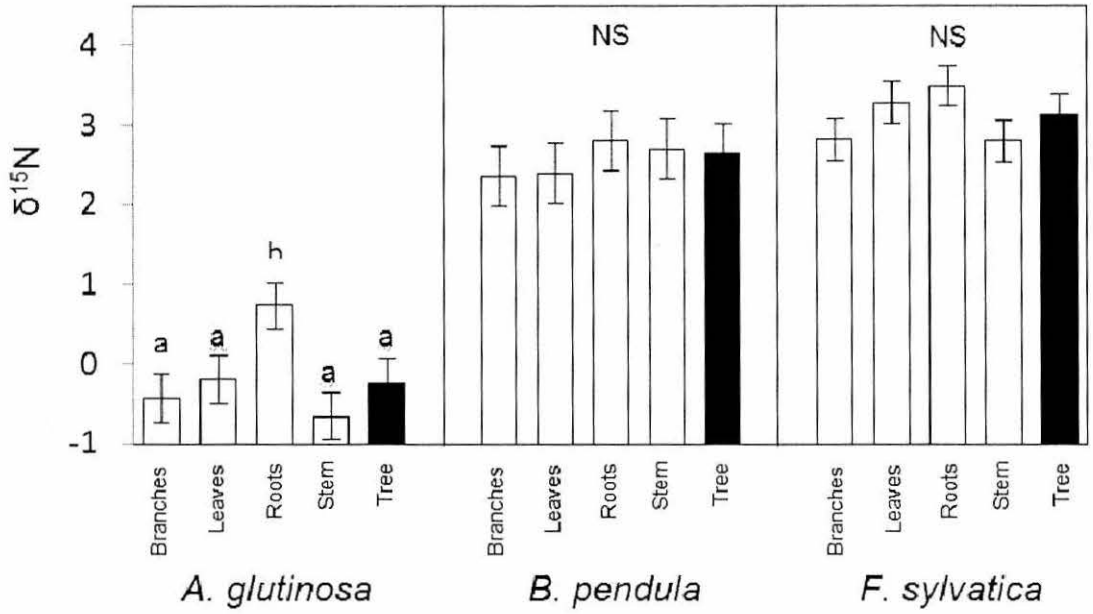

Figure 11.5 $\delta^{15}N$ of different plant tissues for three tree species growing in the BangorFACE experiment. Data presented are mean±SEM $\delta^{15}N$ for each tissue type (open bars) and the mean weighted $\delta^{15}N$ for the whole tree (shown in the filled bar for ease of comparison). Letters above the bars indicate that the mean for those tissue types are significantly different from each other (fisher's LSD). NS indicates that there were no significant differences for that species.

11.4 Discussion

The use of the ^{15}N natural abundance method for quantifying N fixation has been reviewed and evaluated by (Boddey *et al.* 2000 and Unkovich *et al.* 2008). Unkovich *et al.* (2008) consider this method to provide robust quantification of N-fixation when rates of N-fixation are high and when the plants can be demonstrated to be actively fixing N. The relatively large (2.7‰ on average) differences in $\delta^{15}N$ between *A. glutinosa* in monoculture and *F. sylvatica* and *B. pendula* in monoculture indicates that rates of N fixation are high. Additionally, it is clear that the *A. glutinosa* in this study were fixing N. This is demonstrated by the consistently reduced $\delta^{15}N$ and root nodulation which was observed in roots excised from the same plots for other studies. For the non N-fixing species growing in mixture with *A. glutinosa* likely amounts of Ndfa were small (only small decreases in $\delta^{15}N$ of these species in mixture relative to the same species in monoculture). Furthermore, it was not possible to demonstrate definitively that transfer of fixed N was taking place. As a result the quantification of %Ndfa for the two non N fixing species was not carried out as it was not considered to be robust. Boddey *et al.* (2000) and Unkovich *et al.* (2008) highlight the importance of reference plant choice in determining Ndfa. They 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 feel that our experimental design has addressed many of the potential problems associated with reference plant choice. Two reference species were used in this study and all three species were trees and were planted at the same time. Additionally, the rooting depth of the three species used here are similar, though rooting depth of *F. sylvatica* tends to be shallower than that of *A. glutinosa* and *B. pendula* (Atkinson, 1992; Claessens *et al.* 2010; Bakker *et al.* 2008). Furthermore, the calculations of Ndfa and %Ndfa using each reference species were in broad agreement suggesting that any differences between these two species was relatively small.

The use of reference trees from monoculture stands rather than in mixture with *A. glutinosa* means that they had no access to fixed N_2 from *A. glutinosa*. This is supported by the very small difference between $\delta^{15}N$ of *B. pendula* trees growing in the plots and those growing in a larger monoculture stand of *B. pendula* and suggests that the size of the stands in the FACE site is large enough to prevent contamination between stands within each ring. 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-fixing plants. Furthermore, N fixation was calculated using *A. glutinosa* and reference trees from within the same ring. Thus the environmental conditions would have been very similar, and importantly *A. glutinosa* trees growing in ambient CO_2 were compared with reference trees growing in ambient CO_2 and *A. glutinosa* trees growing in elevated CO_2 were compared with reference trees growing in elevated CO_2. Similarity in the $\delta^{15}N$ of the sources of all three species and in fractionation within the trees is assumed. The broad similarities of $\delta^{15}N$ in *F. sylvatica* and *B. pendula* leaves suggests that this assumption holds (the difference in $\delta^{15}N$ between *F. sylvatica* and *B. pendula* was very small (0.45‰) in comparison to the difference between these reference plants and *A. glutinosa* (2.7‰).

11.4.1 Does fixed N comprise a significant source of N for Alnus glutinosa?

There is clear evidence that the *A. glutinosa* trees in this study obtained a significant proportion of their N from biological fixation. $\delta^{15}N$ of the leaves of *A. glutinosa* plants was considerably lower than that of *F. sylvatica* or *B. pendula* in the same plots suggesting that a large proportion (c.62%) of the N contained in the plant was fixed from the atmosphere. Thus we can support our first hypothesis that fixed-N contributed a large proportion of the leaf-N content of *A. glutinosa* trees in this study. 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 contributed between 45% and 90% of total N, with the variation explained by N availability. As a result of the fixed-N *A. glutinosa* in the present study contained more N and had higher leaf-N concentrations than the two non N-fixing species. Additionally, *A. glutinosa* relied on root derived N to a far smaller extent than the non N –fixing species.

11.4.2 Is fixed N transferred to the non N-fixing tree species?

The use of the ^{15}N natural abundance method did not enable the quantification of the amount of fixed-N in the non N-fixing trees, due to the relatively small difference in $\delta^{15}N$ and the lack of direct evidence that transfer was occurring. However, when growing in mixture with *A. glutinosa*, $\delta^{15}N$ of leaves of *F. sylvatica* and *B. pendula* was reduced relative to that of these species growing in monoculture. Furthermore, *F. sylvatica* and *B. pendula* with greater numbers of *A. glutinosa* trees as direct neighbours had significantly lower $\delta^{15}N$ than those with fewer. It seems likely that these changes in $\delta^{15}N$ are explained by the incorporation of fixed N into these tissues. Thus we can support our second hypothesis. This interpretation is consistent with other studies where $\delta^{15}N$ of N-fixing trees is compared with co-occurring non N-fixing species (e.g. Daudin and Sierra 2008) and where the transfer of fixed-N specifically has been measured. For example the contribution of transferred N to total N was 5-15% (Arnebrant *et al.* 1993) and c.9% (Ekblad and Huss-Danell, 1995) between *A. glutinosa* and *P. contorta* and *A. incana* and *P. sylvestris* respectively. These relatively small amounts of fixed N are too small for the robust measurement of %Ndfa using the ^{15}N natural abundance method (Unkovich *et al.* 2008).

11.4.3 Is enhanced growth of A. glutinosa supported by N fixation?

While not statistically significant, the growth of *A. glutinosa* was stimulated under elevated CO_2. Ndfa also increased under elevated CO_2, though this was also not significant. Therefore, we conclude that there is some evidence to support our third hypothesis, though this is insufficient to be conclusive. It seems likely that the increased N demand under elevated CO_2 stimulated N fixation due to the control of N-fixation by N requirements and leaf N status (Baker *et al.* 1997). This supports the findings of (Temperton *et al.* 2003a) who found evidence that N fixation increased for *A. glutinosa* when atmospheric CO_2 was elevated in an OTC study, though again the effect of elevated CO_2 was not statistically significant. It seems likely that N-fixation might support N requirements of enhanced growth for *A. glutinosa* under future CO_2 concentrations. However, we must add the caveat that since the CO_2 effect in both studies was not statistically significant it is impossible to confirm conclusively that this is a real effect. There is evidence that N-fixation in *A. glutinosa* was stimulated by inter-specific interactions. For *A. glutinosa* growing in mixture with *B.*

pendula and *F. sylvatica* the amount of N fixed per tree was nearly double that fixed by trees growing with only intraspecific interactions. This increased the total amount of N contained in those trees. N fixation in *A. glutinosa* is controlled by N demand and the N status of the leaves (Baker *et al.* 1997). The larger size and greater leaf-N content of the trees when growing in mixture would result in an increased demand for N. Furthermore, leaf N content was reduced, which might stimulate N fixation. There is some evidence that the stimulation of N-fixation in *A. glutinosa* by interspecific competition was enhanced under elevated CO_2. This may be because N retention is increased in the *A. glutinosa* trees. Increased retention would reduce the amount of N lost from the tree, and therefore the amount of N available for co-occurring trees. Temperton *et al.* (2003b) found that internal N cycling in *A. glutinosa* was significantly affected by elevated CO_2. Tree growing under elevated concentrations of CO_2 stored more N over winter than those growing under ambient concentrations of CO_2 and remobilised more stored N for new growth in the following spring. These results are in contrast to those found in other FACE studies (Finzi *et al.* 2007), where no increase in N retention was found. This might indicate that N-fixing trees respond differently to elevated CO_2 than non N-fixing trees and suggests that the impacts on internal N cycling identified by (Temperton *et al.* 2003b) have consequences for wider ecosystem N cycling.

11.4.4 Are non N-fixing tree species supported by transfer of fixed-N?

As discussed above the ^{15}N natural abundance methods was not considered to give robust measurements of fixed-N contained in *B. pendula* and *F. sylvatica* growing in mixture with *A. glutinosa*. As a result it is not possible to determine whether our fourth hypothesis is supported. Ecosystem C and N pools are tightly linked (Chen and Coops, 2009). Therefore, forest responses to elevated atmospheric CO_2 are dependent on and impact upon ecosystem N availability and cycling (Oren *et al.* 2001; Ainsworth and Long, 2005; Norby and Iversen, 2006; Reich *et al.* 2006; Zak *et al.* 2006; Finzi *et al.* 2007). Increased growth under elevated CO_2 must be supported by increased N uptake and/or increase N use efficiency (NUE). Evidence from forest FACE studies has shown that both these can occur (Calfapietra *et al.* 2007; Finzi *et al.* 2007) though increased uptake appears to be the dominant process (Finzi *et al.* 2007). The response of N fixing tree species is uncertain because no forest FACE study to-date has included N-fixing species. This is the first forest FACE study to include an

N-fixing tree species (*A. glutinosa*). When atmospheric CO_2 concentration was elevated two of the three tree species increased in size; *B. pendula* did not. However, the total leaf-N content increased in all three tree species. This suggests that N uptake is increased under elevated CO_2. This response is common to other forest FACE studies (Finzi *et al.* 2007). There is some evidence that the trees are altering the sources of N that they use, to support this increased N requirement. Soil N uptake was not increased in *A. glutinosa*, with the increased N requirements met through increased N-fixation. The reduced $\delta^{15}N$ of the leaves of trees growing under elevated CO_2 follows the trend for woody plants identified by (Bassirirad *et al.* 2003). This depletion can be interpreted as being due to changes in the N cycle within the plant-soil system. For *A. glutinosa* some of the decreased $\delta^{15}N$ can be explained by an increased contribution of fixed-N, at least when the trees are growing in mixture. For *B. pendula* and *F. sylvatica* and possibly for *A. glutinosa* this decreased $\delta^{15}N$ might result from increased uptake of mycorrhizal derived N, which tends to reduce ^{15}N of mycorrhizal plants (Hobbie *et al.* 2000). Alternatively, these changes might be explained by differences in the $\delta^{15}N$ of N taken up from the soil. This might be due to exploitation of different soil N pools, or due to changes in the $\delta^{15}N$ of the entire soil N pool. The contrasting response of soil and leaf $\delta^{15}N$ to elevated CO_2 does not rule out this possibility because bulk soil $\delta^{15}N$ might not reflect $\delta^{15}N$ of available and/or exploited soil N. This change in $\delta^{15}N$ might also represent changes in internal processes within the trees, resulting in altered fractionation. Forests are predicted to become progressively more N limited under future atmospheric CO_2 concentrations (Johnson, 2006; Reich *et al.* 2006). There is some evidence of reduced N availability in the forest stands studied here. The soil-leaf N enrichment factor (EF) might provide an indirect measure of site N availability (Garten *et al.* 2007), with a more negative EF indicating reduced N availability. EF was more negative for all trees in all stands under elevated CO_2. This provides some evidence that N availability within the stands is reduced under elevated CO_2. Whether this has resulted in the trees being more N limited is difficult to ascertain. There was no impact of elevated CO_2 on NMASS but a clear, consistent, significant reduction in NAREA. This might not indicate N limitation but an alteration in N use, possibly indicating increased NUE in the trees.

11.5 Conclusion

In conclusion, we found that N-fixation provided a large proportion of the N contained in *A. glutinosa* trees in this study. There was some evidence that the proportional contribution of fixed-N to the N nutrition of these trees was affected by elevated CO_2, but only when the trees were growing in mixture with non N-fixing species. There was evidence that fixed N was transferred from *A. glutinosa* to *F. sylvatica* and *B. pendula* contributing a small proportion of the N contained in these trees. However, we could not quantify this and could not determine how this was altered under elevated CO_2. The response of the N-fixing tree species to elevated CO_2 was not qualitatively different to the non N-fixing species. Main difference was that the N-fixing tree species was able to utilise the additional N source that was mainly unavailable to the other tree species. There was evidence of changes in N cycling in the plant-soil system. Changes in $\delta^{15}N$ suggest changes in N sources for the trees when growing in elevated CO_2. This enabled the trees to increase N uptake. There is little evidence of N limitation but changes in N cycling might be an indication that PNL may have become important in these stands should CO_2 enrichment have continued beyond the 3.5. year duration of this study.

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12 General Conclusion

12.1 Introduction

This thesis has addressed several major aspects of biogeochemical cycling in an aggrading broadleaved forest ecosystem growing within a CO_2 enriched atmosphere for four years. The methodologies and results from experimental work have been presented and discussed individually in nine experimental chapters (3-11). This final chapter serves to summarise and integrate the data presented and relate the findings to the overarching questions raised during the inception of this research.

12.2 Overarching hypotheses

Elevated atmospheric CO_2 growth response is greatest in pioneer tree species.

Analysis of biomass accrual at annual intervals throughout the experiment revealed a differential species response to elevated atmospheric CO_2 (Chapter 3). The growth response was determined using diameter as a predictor of biomass using species specific allometric relationships determined in 2006. In monoculture, *A. glutinosa* produced the largest and most consistent response maintaining a positive growth response until the experiment conclusion. Whilst in contrast, the growth response of *B. pendula* and *F. sylvatica* diminished throughout time. In polyculture, the aboveground growth response to elevated CO_2 was strongly reduced in comparison to species grown in monoculture. Furthermore, a negative CO_2 effect growth response was observed in the final growing season for *F. sylvatica* as the species became rapidly overtopped and suppressed. The data presented here suggests that elevated atmospheric CO_2 enrichment induces a greater growth response of pioneer species for the duration of this experiment. However, competitive interactions reduced inter specific responses of both late and early successional species grown in monoculture. Whereas, in agreement with our hypothesis intra-specific competition between trees grown in polyculture favoured pioneer species.

Elevated atmospheric CO_2 increases tree belowground carbon allocation resulting in larger and deeper root systems.

In agreement with our hypothesis we found that atmospheric CO_2 enrichment increased the C allocation to roots (Chapter 4). It was also apparent from our data that the magnitude of fine root response increased with soil depth and was strongly differentiated by species. When species were grown in monoculture *A. glutinosa* was best able to exploit soil resources deep within the soil profile and displayed the largest magnitude response with depth. Whereas, *B. pendula* demonstrated a greater root proliferation throughout the soil profile, in addition to a greater magnitude response with increased depth. When species were grown in polyculture a greater response to elevated CO_2 was observed at all depths throughout the soil profile, suggesting that species grown in mixtures would be better able to exploit soil resources. The ephemeral nature of fine roots coupled with enhanced root proliferation and greater production at depth may have major implications for the flux of C to long term C sequestration in the soil C pool. Our data challenges our current understanding of biogeochemical cycling in response to elevated CO_2 as root proliferation may deplete soil resources faster and alter C cycles feedbacks. Existing biogeochemical cycling models parameterised with data from species grown in monoculture may be dramatically underestimating the belowground response to global change.

Tree species diversity has a positive effect on above and below ground biomass accumulation when trees are grown under elevated atmospheric CO_2.

The data contained within Chapter 5 of this thesis revealed that when tree species of contrasting ecological and successional characteristics were grown in polyculture biomass accrual was greater than that of species grown in monoculture, indicating a positive interactive effect of species richness on productivity. Surprisingly, when trees were grown under elevated atmospheric CO_2 aboveground overyielding was reduced compared to ambient CO_2. Whilst conversely, belowground fine root, overyielding was positively affected by species richness in both ambient and elevated CO_2 plots. However, the magnitude of the fine root overyielding response was greatest in

response to elevated CO_2 enrichment. In conjunction with ecological theory, experimental data gathered at BangorFACE clearly demonstrates that increases in tree productivity can occur when species are grown in polyculture. However, this thesis reveals for the first time, how interactions between species diversity and elevated atmospheric CO_2 alter C allocation in forest ecosystems. The potential ramifications of these finding on the global C cycle have not previously been considered by the scientific community.

Elevated atmospheric CO_2 enhances rhizodeposition altering the soil microbial community structure, function and soil carbon turnover.

To determine how elevated atmospheric CO_2 affected soil microbial community dynamics and soil C turnover, I examined low molecular weight (LMW) C substrate mineralization kinetics in two experiments involving: (i) a simulated root exudate cocktail, and (ii) sixteen individual low molecular weight substrates. Using individual substrates enabled the determination of a community level physiological profile which allowed detection of changes in community structure and function. Using the data presented in Chapters 6 and 7 we were able to extend our current understanding of LMW C pool turnover and partitioning, develop a three pool exponential decay regression model, and identify environmental drivers of C pool kinetics. Season and tree species had a large effect on LMW mineralization kinetics suggesting that labile C input was removing C limitation from the microbial community. Microbial catabolic activity was reduced in mixed species plots, regression and PCA analysis suggested that external resource limitation was constraining microbial catabolic activity in mixed species plots where a greater resource use efficiency of species diverse communities. Furthermore, the catabolism of LMW compounds increased with depth under elevated CO_2 which was attributed to greater C input through prolific root and hyphae exploration at depth. The data presented here suggests that species-specific biochemical treatment responses and differential LMW compound input to soil, partly to enhance resource acquisition, is responsible for altering the community structure and function. Furthermore, our data showed that microbial function has been altered by elevated CO_2 treatment which we are tightly coupled to stand productivity and nutrient acquisition; subsequently we accept our hypothesis.

Elevated atmospheric CO_2 increases demand for P in forest ecosystems and depletes soil P pools.

The Hedley sequential phosphate fractionation procedure was used to identify and characterize the cycling of P pools at BangorFACE. A reduction in the labile plant available P pool was correlated to a transfer of P to tree biomass which occurred faster in CO_2 enriched plots, supporting our hypothesis that forest growth in elevated CO_2 would increase P demand. Surprisingly, in the elevated CO_2 plots, the NaOH extractable, molybdate reactive phosphate decreased. We attributed this decrease to soil organic matter priming which reduced the source of organic residues to replenish this pool. We speculate that this pool would increase as a result of organic inputs through root turnover and leaf litter inputs as the ecosystem develops. Depletion of the HCl extractable P pool was correlated with an increase in the biomass of mycorrhzial mycelium leading us to suggest that replenishment of P pools was mediated through mineral P acquisition of mycorrhizae. Our data shows that temperate forest ecosystems are able to improve acquisition of P through enhanced primary mineral weathering through both NPP related feedback mechanisms (eg. exudation and organic residue cycling). However, despite increased input from mineral sources and biomass cycling the plant available $NaHCO_3$ P fraction continues to decline under elevated CO_2 likely leading to P limitation in the later years of forest growth.

Elevated atmospheric CO_2 increases the accumulation of soil C through enhanced root and mycorrhizal hyphal production and turnover.

In Chapter 4 and 5 we showed that elevated CO_2 enhanced root biomass, and in Chapter 8 that mycorrhizal biomass was increased using the biomarker ergosterol. The resulting data in these chapters would suggest that elevated CO_2 increased the C flux to soil resulting to greater accumulation of C. However, in Chapter 9 we attempted to determine the input from roots and mycorrhizal extrametrical hyphae using stable isotopes and the changes of C isotope ratio of incurred when trees grown were grown in C_4 soil. We found no evidence to support our hypothesis that

mycorrhizal extramatrical mycelium C inputs would result in greater C inputs to soil under elevated CO_2. Indeed, contrary to our expectations C input to via the mycorrhizal pathway were greater in ambient CO_2 conditions. Although we suspect that mycorrhizal community dynamics may introduce experimental artefacts that influence $\delta^{13}C$ isotopic signal resulting in an underestimate of C input in elevated CO_2 plots. Furthermore, we believe that methodological issues, relating to changes of the field incubated C_4 control soil isotopic signal contributed to this conflicting and unintuitive result. We were therefore unable to determine the contribution of new C in the experimental plots, and consequently are unable to accept our hypothesis that elevated CO_2 increases the accumulation of soil C. However, as mycorrhizal mycelium is pervasive throughout terrestrial ecosystems it likely has a fundamental role in the translocation of C to soil organic matter, and the global C cycle.

12.3 Overall Conclusion

The BangorFACE study and data presented in this thesis has demonstrated that elevated atmospheric CO_2 dramatically impacts upon the biogeochemical cycling of an aggrading forest ecosystem. Atmospheric CO_2 enrichment increased tree growth, and caused trees to allocate more C to roots, producing larger systems with a greater response deeper in the soil profile. Within the experimental time frame nutrient demand was met by improved resource use efficiency. Nevertheless, there was some indication that resource limitation was altering microbial and physiological processes that may impact nutrient and C cycling processes as the ecosystem develops. Significantly, tree species diversity, was not only found to increase forest productivity, but also to interact with elevated atmospheric CO_2 to increase belowground C allocation. These findings are fundamental to improving our understanding of forest ecosystem biogeochemical cycling and its impact on the global C balance.

Appendix A

```
#include <stdio.h>
```

```
#include <windows.h>
#pragma hdrstop
#pragma argsused
/*
        Author: Andy Smith (a.r.smith@bangor.ac.uk)
        Date:   25th October 2006
        Full Description:
        FACE system CO2 level datalogger
        Reads status of FACE control systems from serial port
        sent by telemetry system of the FACE control system
        microprocessors.

        Collects all data sent and appends to a daily file
        which is the uploaded to Bangor (publix.bangor.ac.uk)
        using FTP. A cron job runs on the UNIX server
        publix.bangor.ac.uk that polls the FTP server and
        make regular uploads of the current data.
        A separate PERL script check the data and reports on the
        performance of the system every 3 hours to alert us for
        problems.

        Brief Desription of Operation:
        1. Open a file with the correct time date format
        2. Open a serial port
        3. Read all serial data and write to the file
           cycle the file on a daily basis
*/
/*
** P R O T O T Y P E S
*/
void openSerial( HANDLE *hCom, char *pcCommPort);
void myReportError( char *funcname );
int CreateDataFile( HANDLE *hFILE, char *filename);

/*
** M A I N
*/
int main(void)
{
   SYSTEMTIME st;
   SYSTEMTIME nt;
   HANDLE      hFile ={0};
   HANDLE      hSerial = {0};
   char        *PORT = "COM1";
   char        *FILEDIR = "C:\\FACE\\DATA\\";
   char        filename[256] = {0};
   BOOL        rResult = 0;
   BOOL        wResult = 0;
   char        inBuffer[256] = {0};
   DWORD       nBytesToRead = 0;
   DWORD       nBytesWritten=0;
   DWORD       nBytesRead=0;

   GetSystemTime(&st);
   sprintf(filename, "%s%d%02d%02d.dat",FILEDIR, st.wYear, st.wMonth, st.wDay);
   printf("%s\n",filename);
   CreateDataFile(&hFile, &filename);
   openSerial(&hSerial, PORT);

   while (1)
   {
               /*
               ** check to see if the date has changed
               ** if so then close the old file and reopen a new one
               ** before continuing with listening to the serial port!
               */
          GetSystemTime(&nt);
               if (nt.wDay != st.wDay) {
                       sprintf(filename, "%s%d%02d%02d.dat",FILEDIR, nt.wYear,
nt.wMonth, nt.wDay);
                       st = nt;
                       CloseHandle(hFile);
                       CreateDataFile(&hFile, filename);
                       printf("Date change data flipped to new file %s\n",filename);
               }
               sleep(1);
               do {
                       rResult = ReadFile(hSerial, &inBuffer, sizeof(inBuffer),
&nBytesRead, NULL);
               } while (rResult && 0 == nBytesRead )  ;
               if (rResult) {
                       wResult = WriteFile(hFile, &inBuffer, strlen(inBuffer),
&nBytesWritten, NULL);
                       FlushFileBuffers(hFile);
                       memset(inBuffer, 0, sizeof(inBuffer));
                               if (!wResult) {
```

```
                                        myReportError("Serial write failed");
                                        break;
                                }
                        }
                }
        return 0;
}

/*
** F U N C T I O N S
*/
void myReportError( char *funcname ) {
        CHAR szBuf[80];
        DWORD dw = GetLastError();
        sprintf(szBuf, "Function: %s returned error %u\n",funcname,dw);
        MessageBox(NULL, szBuf, "Error", MB_OK);
        ExitProcess(dw);
}

void  openSerial(HANDLE *hCom, char *pcCommPort)
{
   DCB dcb;
   BOOL fSuccess;
   *hCom = CreateFile( pcCommPort,
                                        GENERIC_READ | GENERIC_WRITE,
                                        0,
                                        NULL,
                                        OPEN_EXISTING,
                                        0,
                                        NULL
                                        );

   if (*hCom == INVALID_HANDLE_VALUE)
   {
           printf ("CreateFile failed to open serial port with error %d.\n",
GetLastError());
           return (1);
   }
   fSuccess = GetCommState(*hCom, &dcb);

   if (!fSuccess)
   {
          printf ("GetCommState failed with error %d.\n", GetLastError());
          return (2);
   }

   // Serial port configuration parameters.
   dcb.BaudRate = CBR_9600;
   dcb.ByteSize = 8;
   dcb.Parity = NOPARITY;
   dcb.StopBits = ONESTOPBIT;

   fSuccess = SetCommState(*hCom, &dcb);

   if (!fSuccess)
   {
          printf ("SetCommState failed with error %d.\n", GetLastError());
          return (3);
   }

   printf ("Serial port %s successfully reconfigured.\n", pcCommPort);
   return (0);
}


int CreateDataFile( HANDLE *hFile, char *filename)
{

                *hFile = CreateFile(filename,
                                                GENERIC_WRITE,
                                                FILE_SHARE_READ,
                                                NULL,
                                                CREATE_NEW,
                                                FILE_ATTRIBUTE_NORMAL,
                                                NULL);
                /*
                ** If the return value is invalid see if a file exists
                ** we can then append to the existing file
                */
                if (*hFile == INVALID_HANDLE_VALUE)
                {
                        DWORD error = {0};
                        error = GetLastError();

                        // Stupid windows has two error codes for the same thing!?!
                       if( ERROR_ALREADY_EXISTS == error || ERROR_FILE_EXISTS == error )
```

```
                        {
                                unsigned long size = 0;
                                printf("The file already exists opening in append
mode\n");
                                *hFile = CreateFile( filename,
                                                        GENERIC_WRITE,
                                                        FILE_SHARE_READ,
                                                        NULL,
                                                        OPEN_EXISTING,
                                                        FILE_ATTRIBUTE_NORMAL,
                                                        NULL);
                        }else{
                                myReportError("File open/append ERROR");
                                return -1;
                        }
                }
                return 0;
}



#!/usr/bin/perl -w
# Author: Andy Smith
# 25th October 2006
# cleans up a FACE raw data file and outputs data to new csv files
# also calculates averages, stddev, time with 5,10,20% of target 580ppm
# for each input file ( .dat suffixed files read from $DIRPATH )
# takes -o as an argument to not output the datafiles
use strict;
use FileHandle;
use Data::Dumper;
my $DIRPATH = "/homedir/afp422/FACE CO2 DATA";
my $CO2TARGET = 580;
my $FILE_OUTPUT = 0;
my $TODAY = 0;
my $date;
if ( defined($ARGV[0]) ){
        if ($ARGV[0] eq "-o" ){
                $FILE_OUTPUT = 1;
                print "File output enabled\n";
        }elsif( $ARGV[0] eq "-today" ){
                $TODAY = 1;
                my ($today,$month,$year) = (localtime(time))[3,4,5];
                $year  +=1900;
                $month +=1;
                $date = sprintf("%d%02d%02d",$year,$month,$today);
        }
}
my $file = &get_file_via_ftp();
print "RING\tCO2\tSTDDEV\t5%\t10%\t20%\tWIND\tVALVE\tRECORDS\tS1  S2  S3  S4  S5  S6
S7  S8\n";
my $fh = new FileHandle;
$fh->open("$DIRPATH/$file") or die("Failed to open $file: $!");
my $parsed = &parsefile($fh);
&calculate_statistics_output($parsed);
$fh->close();

sub parsefile {
        my $fh = shift;
        #
        # First remove the non printable characters!
        #
        my @file;
        my @line;
        my $end=0;
        while(my $read =  $fh->getline()){
                my @a = split(//,$read);
                foreach (@a){
                        $end++;
                        # only keep ascii printable characters
                        if( ord($_) < 126 && ord($_) > 33 ){
                                push(@line,$_);
                        }
                        # keep newlines 0x0A or 0x0D to separate the data
                        # plus a bit of cleaning up of random chars
                        # and blank lines
                        if ( ord($_) == 10 || ord($_) == 13 ){
                                push(@line, "\n");
                                my @snip = splice(@line,0,$end);
                                my @line = split(",",join("",@snip));
                                $line[0] =~ s/\S*(\d+)$/$1/g;
                                push(@file, \@line);
                                delete($file[$#file]) if $end < 2;
                                delete($file[$#file]) if $#line < 7;
                                $end=0;
```

```
                        }
                }
        }
        return \@file;
}

sub calculate_statistics_output {

        my $file = shift;
        #
        # Organise the data into rings keeping the original array order
        #
        my $ring;
        my $averages;
        foreach my $line (@{$file}){
                # Remove data when the rings aren't on
                my @time = split(":",$line->[2]);
                # Currently between 6am - 7pm
                # Currently between 8am - 6pm
                if ( $time[0] >= 9 and $time[0] <= 17 ){
                        # Put daily average data into the structs
                        # line[0] = ringno
                        # line[1] = date
                        # line[2] = time
                        # line[3] = valve open
                        # line[4] = IRGA measurements
                        # line[5] = wind
                        # line[6] = wind direction (in degrees)
                        # pipe section that is active
        #
        # Beware: Indentation altered to improve readability
        #
        $averages->{$line->[0]}->{$line->[1]}->{time}->{$line->[2]} = $line->[2];
        $averages->{$line->[0]}->{$line->[1]}->{irga}->{$line->[2]} = $line->[4];
        $averages->{$line->[0]}->{$line->[1]}->{wind}->{$line->[2]} = $line->[5];
        $averages->{$line->[0]}->{$line->[1]}->{valve}->{$line->[2]} = $line->[3];
        chomp($line->[7]);
        $averages->{$line->[0]}->{$line->[1]}->{sector}->{$line->[2]} = $line->[7];
        $averages->{$line->[0]}->{$line->[1]}->{total} += $line->[4];
        $averages->{$line->[0]}->{$line->[1]}->{number} += 1;
        push(@{$ring->{$line->[0]}},$line);
        #
        # Beware: Indentation normal from here.
        #
                }
        }
        #
        # Write the ring separated data to files in append mode
        #
        foreach my $ringno ( keys(%{$ring}) ) {

                # File Output
                open(RING, ">>RING${ringno}.csv") or
                                die("Failed to open ring output file: $!\n");
                foreach my $aref ( @{%{$ring}->{$ringno}} ){
                        print RING join(",",@{$aref}) if $FILE_OUTPUT;
                }
                # Averages
                foreach my $dates ( keys(%{$averages->{$ringno}}) ) {
                        #print "$dates\t";
                        print "$ringno\t";

                        #mean
                        my $t = $averages->{$ringno}->{$dates}->{total};
                        my $n = $averages->{$ringno}->{$dates}->{number};
                        $averages->{$ringno}->{$dates}->{mean} = $t/$n;

                        printf("%2.1f\t", $averages->{$ringno}->{$dates}->{mean});

                        #
                        #  Calculate the STDEV
                        #  sqrt(1/n * sum (x-mean)2)
                        #
                        my $sum=0;
                        my $numberReadings=0;
                        my $on5target=0;
                        my $on10target=0;
                        my $on20target=0;
                        my $valveOpenNotEnriching=0;
                        my $windTotal=0;
                        my $valveTotal=0;
                        my @sector;
                        my $windMin=0;

        foreach my $times ( values(%{$averages->{$ringno}->{$dates}->{time}}) ) {
                my $value = $averages->{$ringno}->{$dates}->{irga}->{$times};
```

```
                $windTotal += $averages->{$ringno}->{$dates}->{wind}->{$times};
                my $wind = $averages->{$ringno}->{$dates}->{wind}->{$times};
                $windMin = $wind if $windMin > $wind;
                $valveTotal += $averages->{$ringno}->{$dates}->{valve}->{$times};
                $sector[$averages->{$ringno}->{$dates}->{sector}->{$times}]++;
                $numberReadings++;
                if ($value <= ($COTARGET*1.05) and $value >= ($COTARGET*0.95)) {
                        $on5target++;
                }
                if ($value <= ($COTARGET*1.10) and $value >= ($COTARGET*0.90)) {
                        $on10target++;
                }
                if ($value <= ($COTARGET*1.20) and $value >= ($COTARGET*0.80)) {
                        $on20target++;
                }
                $sum += abs($value - $averages->{$ringno}->{$dates}->{mean})^2;

        }
        #print Data::Dumper->Dump([@sector]);
        printf("%2.1f\t", sqrt(((1/($n))*$sum))); #print stdev
        printf("%2.1f\t", ($on5target/$numberReadings)*100);
        printf("%2.1f\t", ($on10target/$numberReadings)*100);
        printf("%2.1f\t", ($on20target/$numberReadings)*100);
        printf("%2.1f\t", ($windTotal/$numberReadings));
        printf("%2.1f\t", ($valveTotal/$numberReadings));
        printf("%2.1f\t", $numberReadings);
        ## sector[0] = 0 (actually sector 8)
        ## sector[1] = 1
        ## so start at 1 instead of 0 until 7 then on 8 extract 0
        for(my $i=1 ; $i<8 ; $i++ ){
                my $x = $sector[$i];
                $x = 0 if !defined($x);
                printf("%03d ",$x);
        }
        printf("%03d ",(defined($sector[0])? $sector[0]: 0));
        print "\n";
        #if outside of 20% and valve open above 20
        # should include a check on windspeed too!
        if( (($valveTotal/$numberReadings) > 20) and
((($on20target/$numberReadings)*100) < 50)){
                print STDERR "WARNING: CO2 valve is above 20 but enrichment inside 20%
of target <50% of time\n";
        }
                #print $windMin, "\n";
        }
        close(RING);
}
}

use strict;
use Net::FTP;
use POSIX;

sub get_file_via_ftp {
        my $date=`/usr/bin/date +%Y%m%d`; # hack hack hack
        chop($date);
        my $file = "${date}.dat";
        my @line;

        my $time = time();
        my $month = {Jan => 0,
                     Feb => 1,
                     Mar => 2,
                     Apr => 3,
                     May => 4,
                     Jun => 5,
                     Jul => 6,
                     Aug => 7,
                     Sep => 8,
                     Oct => 9,
                     Nov => 10,
                     Dec => 11 } ;

        eval {
                my $ftp = Net::FTP->new("147.143.187.22", Debug => 0);
                if (!$ftp) {
                        print $@, "\n";
                        die();
                }

                $ftp->login("bangorface",'XXXXXXXX');
                $ftp->binary();
                $ftp->cwd("/FACE/DATA/");
                my @list = $ftp->dir();
                my %files;
                my %filetime;
```

```
            foreach my $line (@list){
                    my @t = split(/\s+/, $line);
                    my $mon = $t[5];
                    my $mond = $t[6]; # starts at zero
                    my ($hour,$min) = split(/:/,$t[7]);
                    next if ! defined($min);
                    next if ! defined($hour);
                    #-rwxrw-rw- 1 root  root   119292 May 18  0:27
                    my $filetime = \
                    POSIX::mktime(0, $min, $hour, $mond, $month->{$mon}, 07);
                    my $dftime = POSIX::difftime($time, $filetime);
                    $files{$dftime} = $t[8];
                    $filetime{$dftime} = $filetime;

            }

            my @sort = sort(keys(%files));
            $file = $files{$sort[0]};
            my $time = $filetime{$sort[0]};
            print "Retrieving most recent file from Henfaes: $file\n";
                            if ( $file !~ $date) {
                    #print "$file ${date}.dat \n";
                   print "WARNING: downloaded file does not match todays date!!!\n";
            }
            $ftp->get("$file", "/homedir/afp422/My Documents/FACE CO2 data/$file");
            $ftp->quit;
    };
    if ($@ ){
            #print $@, "\n";
            print "\n";
            print "\n";
            print "**********************************\n";
            print " Connectivity problem\n";
            print "**********************************\n\n";
            print "Possibilities:\n";
            print "\tNo network between Bangor and Henfaes\n";
            print "\tFTP server down at Henfaes\n";
            print "\tPower cut at Henfaes\n\n";
            exit(-1);
    }
    return $file;
}
```