Ozone and Nitrogen Controls on Carbon Allocation within Plants and Soil

A thesis submitted for the degree of Doctor of Philosophy

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I, Kirsten Victoria Robyn Wyness confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

This thesis focuses on the impact of elevated ozone (O_3) and/or nitrogen (N) on semi-natural vegetation, with an emphasis on C-partitioning within and between plant and soil. The project reports several studies allied to the exploration of the impacts of elevated O_3 and N employing short-term studies in laboratory-based controlled-environment chambers and solardomes plus long-term studies at free-air O_3 fumigation sites in the Swiss Alps and at Keenley Fell, Northumberland, UK. A solardome study indicated that both the grass *Dactylis glomerata*, and the forb *Ranunculus acris* exhibited increased senescence, and reduced C-allocation below-ground, when exposed to elevated [O_3]. Furthermore, N exacerbated the O_3-induced reduction in the root biomass of *D. glomerata*. This finding led to a mechanistic exploration of C-partitioning in response to short-term (three week) exposure of *D. glomerata* to a combination of elevated O_3 and N inputs in self-built fumigation chambers. Plants were pulse-labelled with ^{14}C, and the fate of the recent photosynthate then traced in nine plant and soil C-pools. The study revealed a reduction in below-ground respiration (incorporating root and soil microbial respiration) in high N treated plants, and a significant antagonistic interaction between O_3 and N effects on soil microbial biomass. To relate the findings to below-ground responses in an intact ecosystem, impacts of long-term O_3 and N exposure on soil microbial community diversity and C metabolism were investigated in a sub-alpine grassland. Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis and Community Level Physiological Profiling (CLPP) using ^{14}C labelled root exudate substrates and leaf litter, revealed no effects of O_3 and N on the soil bacterial diversity, and limited impacts on C substrate turnover. Moreover, in a long-term study on a traditional UK haymeadow, three years of elevated O_3 and N inputs did not result in significant changes in above-ground biomass of any plant functional group. However, a significant O_3 x N interaction on below-ground biomass of the sward was observed with reduced root biomass in high [O_3] plots. The variation in cover of individual plant species was not explained by either O_3 or N when analysed by redundancy analysis (RDA). Overall, this study suggests that N deposition subtly modifies vegetation responses to O_3 stress and highlights the potentially significant role played by rising levels of N deposition and O_3 as drivers of changes in carbon allocation in the natural environment.

Key words: Ozone; nitrogen; carbon allocation; grassland; microbial diversity

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Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

1.1 Tropospheric ozone (O_3)

1.1.1 Ozone formation, atmospheric concentrations and critical levels

Ozone (O_3) is formed naturally in the troposphere during a series of reactions between oxygen (O_2) and precursor compounds (principally volatile organic compounds (VOCs), carbon monoxide (CO) and nitrous oxides (NO_x)) driven by favourable temperatures and sunlight (Coyle et al., 2002). Emissions of the precursor gases have increased markedly over the past century due mainly to the rise in the combustion of fossil fuels for energy production. As a consequence, average background ground-level concentrations of O_3 have risen from pre-industrial levels of 10-20 parts per billion (ppb) to 20-45 ppb in the Northern hemisphere, and levels continue to rise at a rate of approximately 0.5 – 2% per year despite the introduction of increasingly effective controls on precursor emissions across Europe and North America (Vingarzan, 2004). The situation is expected to continue since the main reason for the current rise in background O_3 concentrations is the hemispheric transport of precursors from rapidly developing areas of the world, especially Asia (Dentener et al., 2006b). Sitch and colleagues (2007) predict that average ground-level O_3 concentrations may exceed 75 ppb, during the summer months, over much of Europe by 2100 (see Figure 1.1).

Precursor gas emissions are mainly associated with metropolitan areas, where there is reaction with many surfaces, or the gases are blown to the surrounding regions. As a consequence of the lack of scavenging gases, O_3 concentrations in rural areas are often higher than those in urban areas (Coyle et al., 2002). Here the distribution of the gas is affected by numerous variables not least vertical O_3 gradients within the vegetation canopy, reaction with soil and plant surfaces, and removal via stomatal uptake during gas exchange (Davison et al., 2003). In the model run by Sitch and colleagues (2007) using fixed pre-industrial atmospheric CO_2 concentration, global

gross primary productivity (GPP) is predicted to decrease by 14-23 % by 2100 due to O_3 induced plant damage (Figure 1.1).

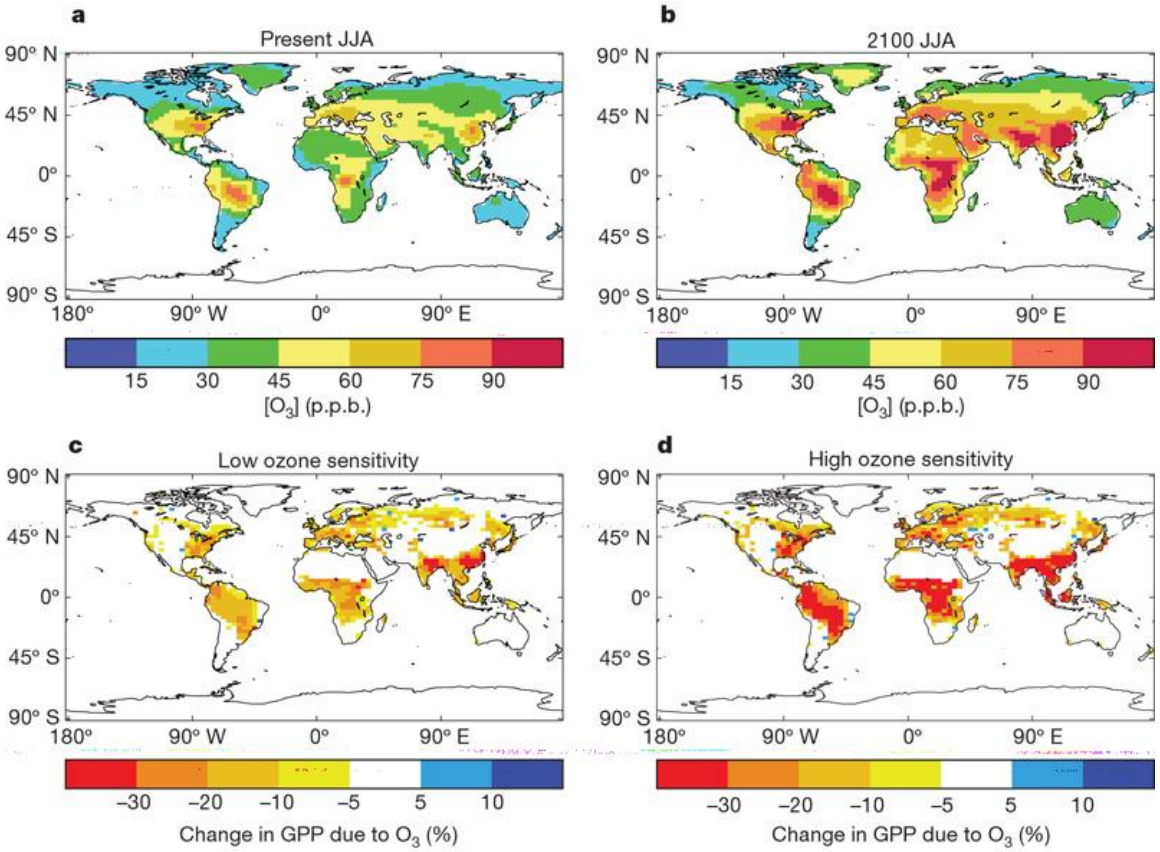

Figure 1.1. Temporal changes in modelled ozone concentrations and gross primary productivity. Modelled diurnal (24 h) mean surface [O_3] in ppb averaged over June, July and August (JJA) for the present day (a) and the year 2100 (b) under the Special Report on Emissions Scenario A2; Simulated percentage change in gross primary productivity (GPP) between 1901 and 2100 due to O_3 effects at fixed pre-industrial atmospheric [CO_2] for 'low' (c) and 'high' (d) ozone plant sensitivity (Sitch et al., 2007).

A threshold concentration of 40 ppb, above which negative effects on plants begins to occur, was recognized and agreed at the Kuopio UNECE Workshop in 1996 (Kärenlampi and Skärby, 1996), and a concentration-based long-term critical level of ozone for crops and (semi-) natural vegetation, was established based upon the AOT40 (the accumulated average hourly exposure to O_3 concentrations over a threshold of 40 ppb during daylight hours) to assist the development of pollution control policy and risk analysis at a European level. A level of 5 ppm.h, accumulated over 6 months, was determined from experimental data as the critical level for the protection of (semi-) natural vegetation communities dominated by perennials from

statistically significant impacts (LRTAP, 2010). Using such indices it has been possible to map potentially biologically-relevant O_3 exposure across the UK, as well as other parts of Europe. These maps, based on a network of 27 monitoring sites across the UK, illustrate the dependency of O_3 exposure on topography, climate and precursor levels in the troposphere (Coyle et al., 2002). Figure 1.2 illustrates the spatial variation in AOT40 during the growing seasons of 2006 (a high [O_3] year), and 2008 (an "average" [O_3] year), in the UK.

The limitations of exposure-based indices such as AOT40 are widely appreciated (e.g. Emberson et al., 2000, Pleijel et al., 2007, Mills et al., 2011) with the main problem centring on the fact that such indices take no account of the extent of uptake of O_3 into vegetation. As a consequence, stomatal flux-based approaches have been developed for targeted vegetation types, with robust models already available and in use for the determination and mapping of geographic O_3 risk to some crops, trees and clover as a representative species of grassland (LRTAP Convention (2010). The flux-based approach takes into account impacts of key drivers of stomatal conductance including, but not limited to, photosynthetic photon flux density (PPFD), temperature, vapour pressure deficit (VPD) and soil water deficit (Buker et al., 2007). The further development of ozone flux models, including models applicable to (semi-) natural communities and other vegetation types will facilitate better accommodation of canopy impacts on O_3 uptake plus more accurate temporal and spatial predictions of plant communities at risk (Mills et al., 2007).

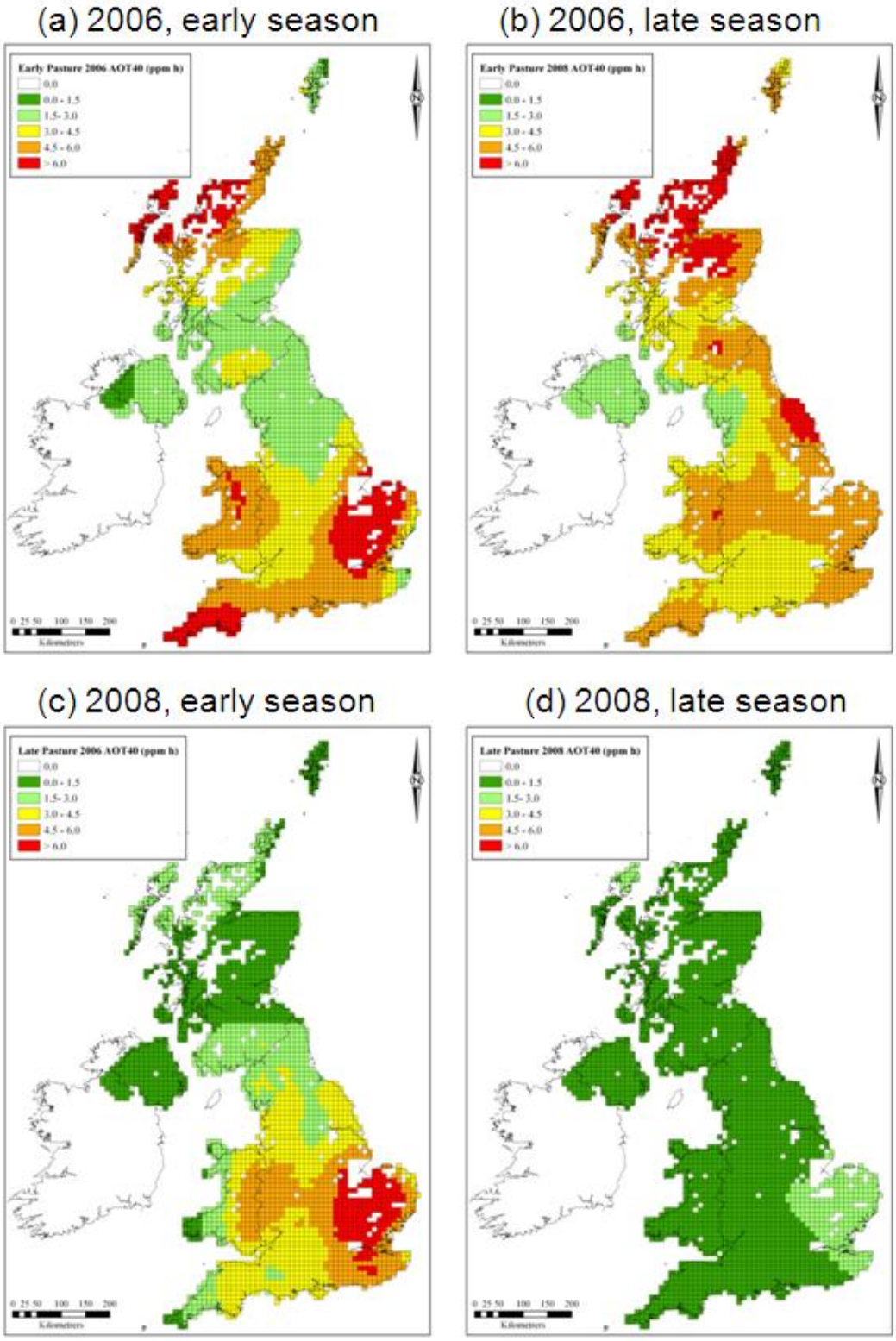

Figure 1.2. Ozone concentrations across the UK in 2006 and 2008 presented as the AOT40 (the accumulated average hourly exposure to O3 concentrations over a threshold of 40 ppb during daylight hours expressed in ppm.h) for early (April to June) and late (July to September) growing seasons (Mills et al., 2011)

1.1.2 Ozone uptake and detoxification

The dose of O_3 a plant receives is highly dependent on stomatal conductance (e.g. Buker et al., 2007; Tausz et al., 2007). In some cases during high O_3 episodes, the flux of O_3 into leaves will be relatively small due to stomatal closure instigated either directly or indirectly by the pollutant *per se* (Robinson et al., 1998; Lyons et al., 1999) (see Figure 1.3). In contrast, recent evidence indicates stomatal performance may be so impaired in some species by exposure to O_3 that plants are predisposed to drought stress, and stomata may 'gape' open enhancing O_3 uptake. For example, Mills and colleagues (2009) reported that a 20 week exposure to elevated O_3 reduced stomatal functioning in both cock's-foot grass (*Dactylis glomerata* L.). and rough hawkbit (*Leontodon hispidus* L.), with the mechanism underlying this response believed to be an O_3 induced disruption to the abscisic acid (ABA) cell-signalling pathway governing stomatal control.

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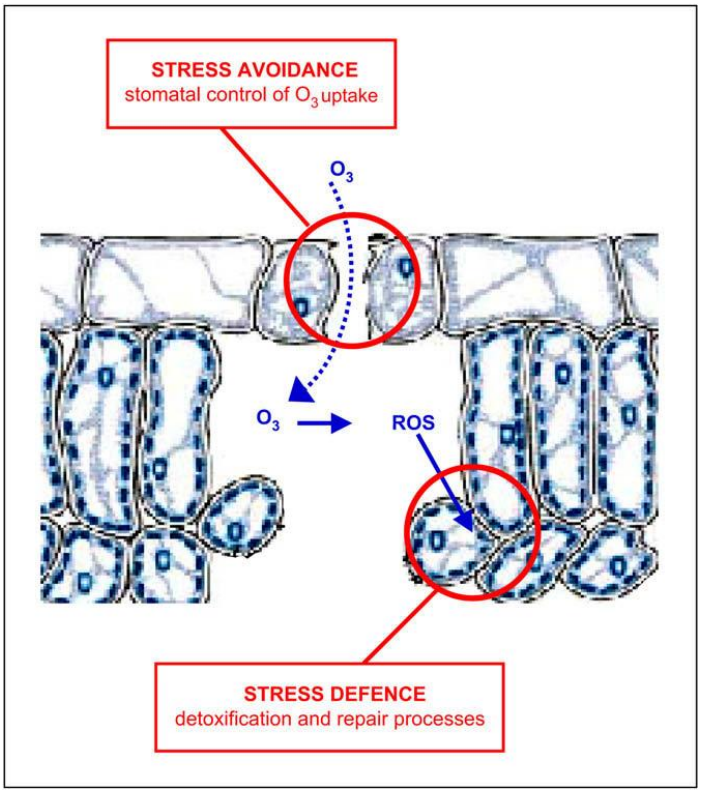


Figure 1.3. Schematic diagram illustrating the two principal control points governing plant cell responses to O_3 stress: (i) stomatal control of O_3 uptake (i.e. avoidance of ozone-induced oxidative stress) and (ii) cell defensive biological responses (tolerance of ozone-induced oxidative stress).

Once inside the leaf, and having penetrated the extracellular defences (Lyons et al., 1999) O_3 and its reactive products result in oxidative damage to plant cell membranes (Lyons et al., 1999; Heath, 2008). Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), the hydroxyl radical (HO•) and superoxide species (O_2^-) are formed when O_3 reacts with constituents of the extracellular matrix (e.g. Mehlhorn et al., 1990; Ron, 2002; Severino et al., 2007). To combat these ROS plant cells have evolved a sophisticated battery of anti-oxidative defences based around low molecular weight (LMW) compounds that are rapidly and efficiently recycled plus a range of enzymes that are capable of directly acting to remove ROS (Ranieri et al., 2000) (Figure 1.3). However, short-term exposure to high concentrations, such as during an ozone episode, can overwhelm the cellular defences and result in a measurable increase in the titre of ROS in plant tissues leading to visible leaf lesions and cell death (Ranieri et al., 2003).

The most widely investigated antioxidant synthesised by plants to counteract ROS in the apoplast is ascorbate (ASC) (see Barnes et al. 1999; Plochl et al., 2000). Leaf ASC accumulation has been shown to contribute to differences in O_3 sensitivity between plant species, including wheat (*Triticum aestivum* L..) (Feng et al., 2010) and tobacco (*Nicotiana tabacum* L.) (Sanmartin et al., 2003; Pignocchi et al., 2006). However the antioxidant efficiency of ASC is dependent on the cell's ability to maintain ASC in the reduced state (Castagna and Ranieri, 2009). Recent studies show that extracellular ASC is not solely responsible for governing ozone toxicity, and cannot account for the difference in O_3 susceptibility between clones of clover (*Trifolium repens* L. cv Regal) by D'Haese et al. (2005) or cultivars of soybean (*Glycine max* L. Merr.) (Cheng et al., 2007).

1.1.3 Ozone effects on plant physiology and carbon allocation

Ozone-induced oxidative stress has been shown to suppress photosynthesis in multiple species of tree, crop and (semi-) natural vegetation (Cardoso-Vilhena et al., 2004; Fiscus et al., 2005; Vandermeiren et al., 2005; Wittig et al., 2007). The mechanism of O_3-induced decline in photosynthesis is believed to be orchestrated by a depression in the expression of the small subunit of Ribulose 1, 5- bisphosphate carboxylase/oxygenase (Rubisco) (Heath, 2008). Reduced photosynthesis, growth (particularly above-ground) and premature senescence constitute typical plant responses to long-term exposure to relatively low-concentrations of O_3 (e.g. Hayes

et al., 2007; Hayes et al., 2010). Furthermore some of the cellular signals involved in reducing Rubisco concentrations have previously been associated with plant senescence mechanisms (Gielen et al., 2007). This highlights the fact that plant metabolic processes are interconnected, and therefore responses to different biotic and abiotic stresses are likely to overlap (Fujita et al., 2006).

There is strong evidence that phloem transfer of recently-assimilated carbon is restricted under elevated O_3 (Mortensen and Engvild, 1995; Grantz and Farrar, 2000) and this effect is exacerbated by enhanced rates of maintenance respiration associated with the repair of O_3-induced damage. Therefore, the effects of O_3 on plant growth consist not only of a reduction in net C input due to reduced photosynthesis, but also disruption of source-sink C partitioning (Andersen, 2003). Carbon and other nutrients may be retained in damaged leaves in order to repair O_3 injury, at a cost to the root system (Long and Naidu, 2002). In reality, it is likely to be a combination of these processes which lead to the changes in root:shoot ratio commonly observed in many species exposed to O_3 (Cooley and Manning, 1987; Grantz et al., 2006).

1.1.4 Responses of plant communities to elevated O_3

In general, legumes and forbs have been shown to exhibit more negative responses to elevated O_3 than graminoid species (Gimeno et al., 2004; Volk et al., 2006), although the reasons for this remain to be elucidated. In the natural environment, the responses of individual species within a plant community to ozone are dependent on several factors including the height, morphology, timing of growth, competitiveness and ozone sensitivity of the species in relation to those growing with it. Experiments using two species mixtures have shown how the canopy structure of one species can influence the response of another. For example, when the forb *Leontodon hispidus* was grown with sweet vernal grass (*Anthoxanthum odoratum* L.), it incurred increased O_3 damage and accelerated senescence compared to when grown with the more aggressive and stronger growing *Dactylis glomerata* (Hayes et al., 2011). This was attributed to the difference in canopy density, with the sward dominated by *A. odoratum* allowing greater levels of O_3 penetration and replenishment. The root biomass of all three species was negatively affected by exposure to 60 ppb above ambient [O_3] for 20 weeks. The effects of ozone on the dynamics of plant communities are illustrated by a study conducted by Fuhrer and colleagues (1994) in

which an artificially sown managed pasture was exposed to either charcoal filtered air (no O_3), non-filtered air (ambient O_3), or elevated O_3 using open top chambers (OTCs) installed in the field. The above-ground productivity of clover (*Trifolium pratense* L. and *Trifolium repens* L.) in the sward was reduced by 24% in the elevated O_3 chambers relative to those in charcoal filtered air after two years. This decline in *Trifolium* species was associated with an increase in grass productivity, principally *Dactylis glomerata*. Furthermore, Ashmore and colleagues (1995) reported species compositional changes with increased O_3 in mesocosms simulating calcareous grasslands and also in transplanted swards from a chalk grassland in an OTC experiment. When these communities were exposed to concentrations of O_3 above 40 ppb the proportion of forbs in the artificial communities decreased significantly. Elevated O_3 was shown to shift the species composition of the transplanted swards in favour of a community dominated by slower-growing vegetation types.

Bassin and colleagues (2009) proposed that functional traits such as greater specific leaf area (SLA) may predispose forbs and legumes to enhanced O_3 fluxes, but they found no significant correlations to that effect in 11 species of a subalpine grassland in Switzerland. As discussed by Körner (2003), alpine and subalpine plant communities may be relatively resilient to compositional change in the late successional stage, exemplified by the limited effects of O_3 on these species *in-situ.* Rämö and colleagues (2006) reported that only the forb and legume species harebell (*Campanula rotundifolia* L.), wild strawberry (*Fragaria vesca* L.), zigzag clover (*Trifolium medium* L.) and tufted vetch (*Vicia cracca* L.) exhibited reduced above ground biomass and/or visible injury symptoms out of seven-species mesocosms exposed to 40-50 ppb O_3 in OTCs. It has been proposed that grassland plants which employ low relative growth rates (RGR) and a stress tolerant lifestyle may be less susceptible to O_3 uptake and consequent damage (Bassin et al., 2007a).

There have been few studies in which the effects of elevated O_3 on grassland communities have been studied *in situ* (Wedlich et al., 2011). At one (of two) current European free air O_3 fumigation sites, Bassin and colleagues (2007b) have demonstrated that a subalpine grassland vegetation community seems relatively unaffected by 5 years' exposure of up to 1.6x ambient ozone concentrations (~74 ppb). Ozone did not cause significant changes in the above-ground productivity, or

species composition of the sward, suggesting impacts of elevated O_3 develop slowly in this ecosystem type. In contrast, Wedlich and colleagues (2011) report a significant negative effect of O_3 on above-ground forb productivity at a free-air O_3 fumigation site in Northumberland, UK, following exposure of an upland mesotrophic grassland to two years of moderately elevated O_3 (≤10 ppb above ambient); effects that mirror the reported impacts of O_3 on mesocosms containing a very similar MG3-B community during long-term OTC studies (ROTAP, 2011).

The sensitivity of individual species (in individual-species studies) does not always correlate with effects on the same species in a community context (Ashmore, 2005). Thus, considerable care needs to be taken when attempting to extrapolate individual species responses from short-term exposures to impacts on larger temporal and spatial scales (Fuhrer et al., 1994). Long-term effects of pollutants on species composition will depend both on the susceptibility of individual species to O_3-induced oxidative stress, and the impacts of the pollutant on the dominant species (Thwaites et al., 2006). The impacts of additional biotic and abiotic stress factors on plant community responses to elevated levels of ozone, for example drought stress and/or nitrogen deposition, which modify the scale and direction of O_3 effects on vegetation, are poorly understood (Fuhrer, 2007; Fuhrer, 2009).

1.1.5 Ozone effects on soil microbial communities

Ozone reacts readily with vegetation and the soil surface, and thus unless physically injected into soils it will have no direct affect on the soil microbial community (Blum and Tingey, 1977). However, due to the significant impacts of O_3 on plant physiology and in particular on C fixation and partitioning, the soil microbial community is likely to be affected by the altered quantity and/ or quality of C input below ground (Andersen, 2003; Chen et al., 2009). This can occur in three ways: through increased litter fall to the soil surface; changes to root growth and/or turnover rates; and/or the alteration of rhizodeposition. Since ozone may enhance senescence rates and lead to premature abscission of leaves, there is the potential that communities exposed to elevated levels of ozone will experience enlarged loads of organic matter entering the soil from above (Andersen, 2003).

There is some discrepancy in the literature as to the effect of O_3 on litter N content, with most attention paid to tree species. Scherzer and colleagues (1998) found that

O_3 reduced yellow poplar (*Liriodendron tulipifera* L.) litter N, whereas another study by Aneja and colleagues (2007) reported no significant effect on the C:N ratio or the decomposition rate of beech (*Fagus sylvatica* L..) or spruce (*Picea abies* L..) litter. The lignin and phenolic content of litter is also known to affect decomposition rates and therefore the rate of C turnover in the soil (Waldrop and Firestone, 2004). In a study on blackberry (*Rubus cuneifolus* Pursh.), Kim and colleagues (1998) found higher concentrations of permanganate lignin in litter from plants subject to elevated levels of O_3, and the litter also decomposed more slowly compared to non O_3-treated plant litter. Increased phenolic content of Eastern cottonwood (*Populus deltoids* Barn. ex. Marsh.) litter exposed to elevated O_3 resulted in decreased decomposition rate, and binding of available N (Findlay et al., 1996). On a community scale, if the pollutant has induced changes in the species composition of the sward, the balance of component nutrients and compounds in litter is also likely to be modified (Zell, 2011).

Reduced root growth in plants subject to O_3-stress has been widely reported in the literature (e.g. Hayes et al., 2011; Manninen et al., 2010; Winkler et al., 2009; McCrady and Andersen, 2000). However, very few studies to date have quantified alteration in rhizodeposition and/or root turnover in plants subject to O_3 stress, and results are inconsistent. In experiments using non-tree species, root exudation increased (e.g. in wheat (*Triticum aestivum*) (McCrady and Andersen, 2000), but McCool and Menge (1983) reported a decrease in amino acid exudation in tomato (*Lycopersicon esculentum* L.).

Secondary impacts of O_3 on root activity and rhizodeposition will affect the soil microbial community. In one of the few studies to date examining secondary O_3 effects on the soil microbial community, Kanerva and colleagues (2008) showed that ozone exposure altered microbial community structure after three growing seasons. Chen and co-workers (2009; 2010) demonstrated subsequently that microbial composition was altered (investigated by phospholipid fatty-acid analysis, PLFA), and species richness and Shannon diversity were significantly decreased by elevated O_3 in wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L. '3694-Fan').

1.2 Nitrogen deposition

1.2.1 Reactive N formation and atmospheric concentrations

Atmospheric Nitrogen (N) comprises reduced N compounds (NH_x, ammonia and ammonium), and oxidised N compounds (NO_X, particulate nitrates, nitrogen oxides and nitric acid) (RoTAP, 2011). In the process of dry deposition, gases and particles are directly transferred to surfaces and deposited on soil and plant surfaces, while wet deposition is a result of the element species being dissolved in water droplets and deposited in rainfall, snow or hail (Brimblecombe, 1996). The rate at which N is deposited to vegetation and soil surfaces depends on a variety of factors including precipitation and distance from point source (Lamarque et al., 2005). The past 150 years has seen global annual anthropogenic N inputs increase from ~15 Tg N in 1860, to ~187 Tg N in 2005 (Galloway et al., 2008) (see Figure 1.4), with the transformation of the global N cycle predominantly due to the dramatic increase in reactive N associated with agricultural practices and industrial processes.

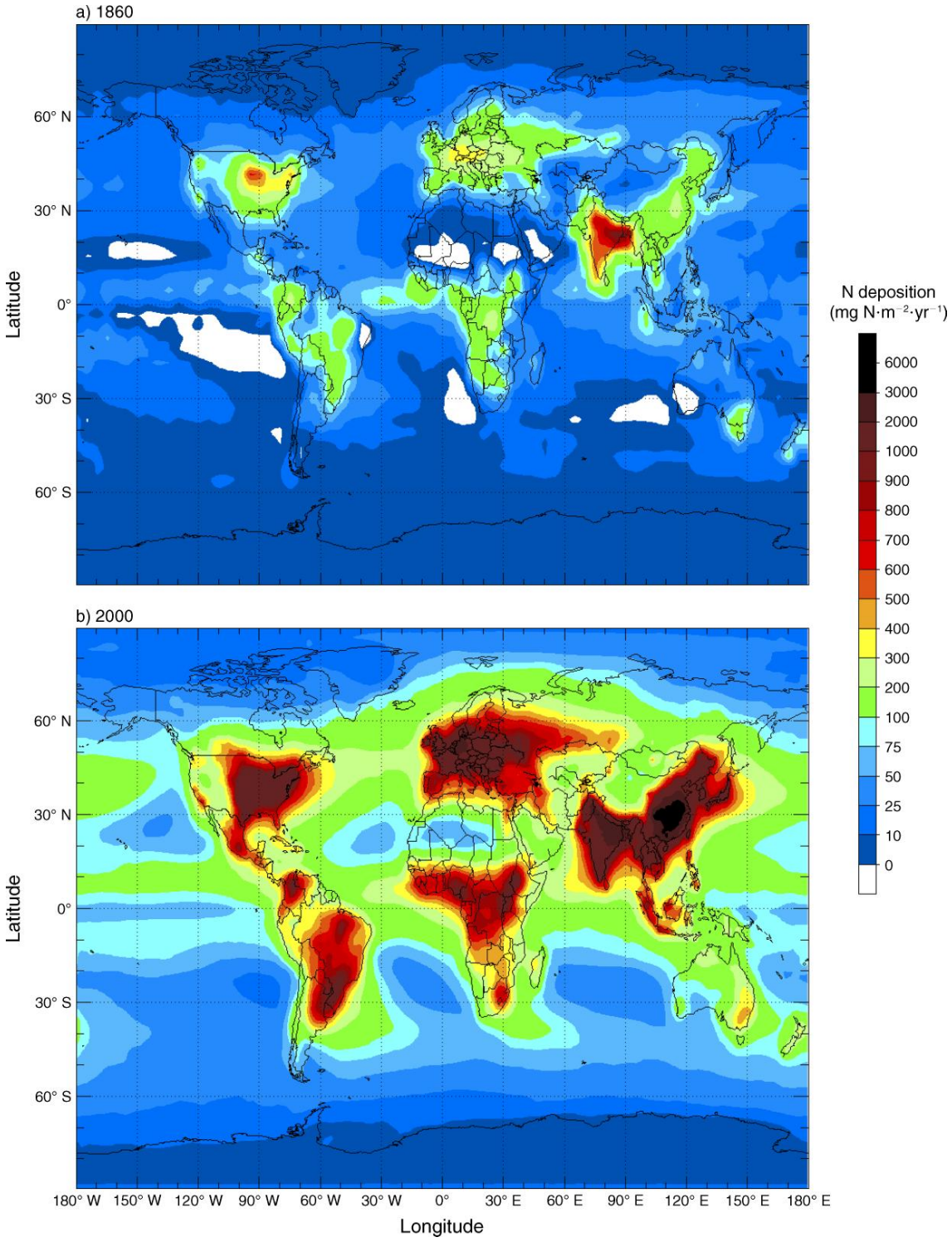


Figure 1.4. Computed wet + dry total nitrogen deposition rates (NH_x + NO_y) for (a) 1860 (Galloway et al., 2004) using the TM3 model and (b) the multi-model results for the year 2000 as described in Dentener et al.(2006a). The scale numbers may be converted to units of kg N ha^{-1} yr^{-1} by dividing by 100 (Bobbink et al., 2010).

Critical loads have been agreed to protect natural and semi-natural ecosystems from N deposition. The latest revision of these guidelines was undertaken in 2010 at the CLRTAP workshop in Noordwijkerhout (NL) (Hall et al., 2011). Calcareous grassland receiving 15-25 kg N ha^{-1} yr^{-1} or more are at risk of damage to sensitive species, and changes in plant community composition. Ninety-two percent of calcareous grassland in the UK is currently receiving loads ~6 kg ha^{-1} yr^{-1} above the critical load range (see Figure 1.5) (Hall et al., 2011). Alpine and subalpine grasslands in Central Europe currently receive approximately 5 kg N ha^{-1} yr^{-1}, but can reach up to 60 kg N ha^{-1} yr^{-1} in areas with intensive farming (Rihm and Kurz, 2001). The European empirical critical N load for these community types is 5-10 kg N ha^{-1} yr^{-1} (Bobbink et al., 2010), therefore subalpine grasslands may already be undergoing species diversity loss, and shifts in community composition. It is worthwhile to consider that environmental factors, for example, precipitation, base cation availability, or management, may influence where within a range the critical load should be set for some habitats (Hall et al., 2011).

a) b)

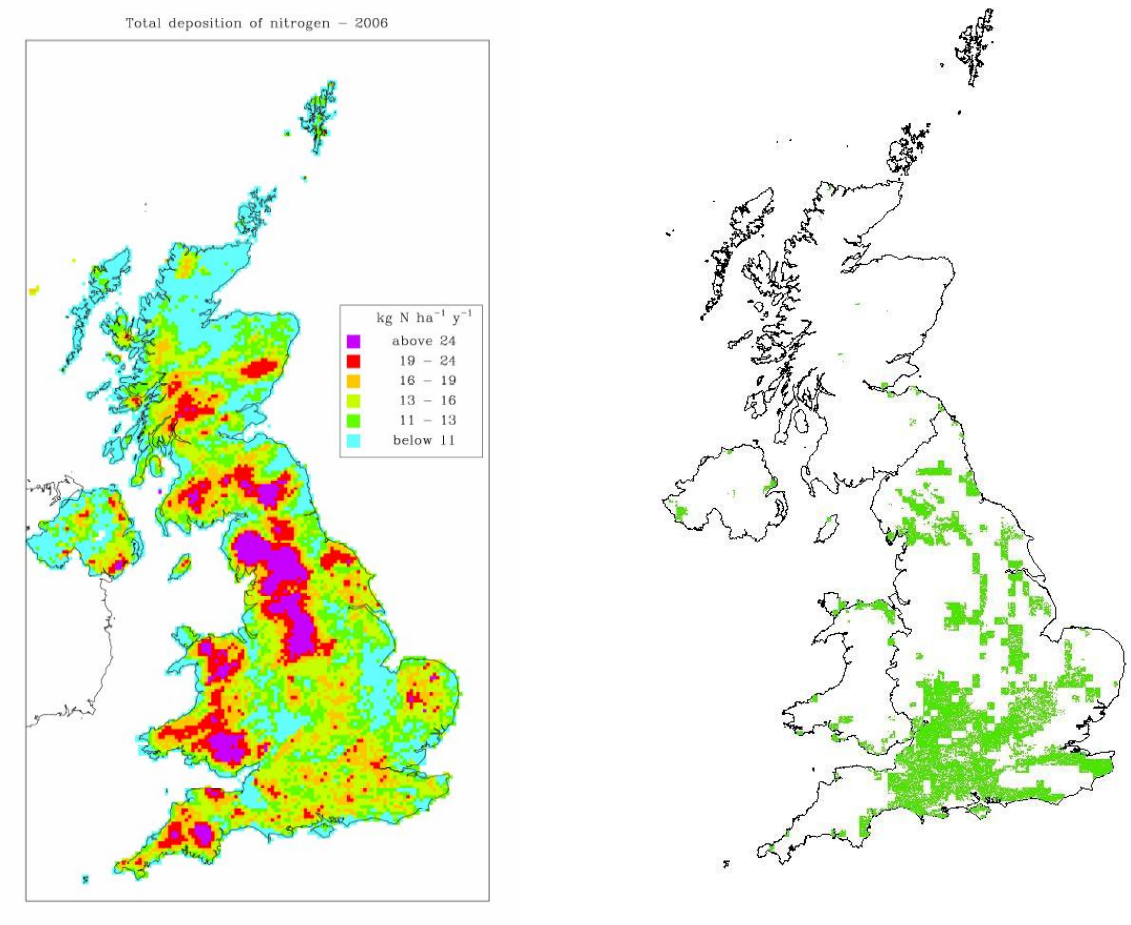


Figure 1.5. a) Total (oxidized +reduced) deposition of fixed nitrogen over the UK in 2006 (RoTAP, 2011), b) Habitat distribution maps for calcareous grassland in the UK (Hall et al., 2011).

1.2.2 Impacts of Nitrogen deposition on plant physiology and carbon allocation

Nitrogen is essential for the synthesis of amino acids, DNA and proteins within plant cells (Marschner, 1995). As such, additions of N via atmospheric deposition have been shown to have a significant fertilization effect on plant growth (e.g. Fangmeier et al., 1994; Achermann and Bobbink, 2003; Bassin et al., 2007b). Increased biomass induced by high N input, however, is not apportioned equally between above and below-ground plant parts. There is evidence in the literature for reductions in root to shoot ratio in a number of (semi-)natural species (Bobbink, 1998; Fenn et al., 2003). Several recent studies have demonstrated preferential C allocation above-ground in graminoid species, for example in *Dactylis glomerata* (Harmens et al., 2000), and sand sedge (*Carex arenaria* L.) (Jones et al., 2010). Hill and colleagues (2007) examined the combined effects of elevated [CO_2], grazing and N fertilization on C partitioning patterns in perennial ryegrass (*Lolium perenne* L.) swards using ^{14}C pulse labelling. This work showed that addition of 560 kg N ha^{-1} yr^{-1} reduced the ^{14}C root to shoot ratio by 14% compared to plants under lower N loads. Lee and Caporn (1998) and Carroll and colleagues (2003) demonstrated that increased N inputs to calcareous grassland communities significantly altered plant shoot tissue N concentration and decreased tissue C:N ratios. The physiological changes in plants exposed to elevated N deposition may increase susceptibility to other abiotic and biotic stresses, including herbivory (Bobbink et al., 2010). There is also the possibility that reduced root biomass may increase sensitivity to soil water deficit and reduce capability to acquire essential soil nutrients (Jones et al., 2010).

1.2.3 Responses of plant communities to elevated N deposition

Natural and (semi-)natural grasslands are typically oligotrophic (low in nutrients) or mesotrophic (of moderate inherent fertility), and are thus susceptible to change by increased N inputs (Wedin and Tilman, 1996; Stevens et al., 2006). Significant shifts in plant community composition have been observed in a number of ecosystem types, such as forests, heaths and grasslands across Europe and North America, as a direct result of increased atmospheric N deposition loads (Rowe, 2005; Hautier et al., 2009; Bobbink et al., 2010). Species which have adopted resilient traits to cope in low-nutrient habitats are often unable to respond to increased N availability and are at risk of shading and competition from nutrient-demanding species. Some

particularly sensitive species such as mosses and lichens can be eliminated from an ecosystem altogether by direct N toxicity (Lee and Caporn, 1998). There is a recognised pattern of N deposition-induced reduction in grassland species richness with grasses being particularly responsive to enhanced atmospheric N deposition at the detriment of forb and legume species (Stevens et al., 2006; Maskell et al., 2010). Key indicators of N critical load exceedance in grassland ecosystems have been identified as increases in nitrophilic graminoids, increased N leaching, increased N mineralization and change in biodiversity (Bobbink et al., 2010).

Clark and Tilman (2008) examined the effect of long-term chronic low-level N addition (≤10 kg N ha^{-1} yr^{-1} above ambient) on a Prairie grassland in Minnesota USA. They found relatively modest additional N input reduced plant species numbers by 17% relative to ambient N deposition (~6 kg N ha^{-1} yr^{-1}). Two long-term field experiments in which high N deposition treatments have been employed (one in Colorado, USA (Bowman et al., 2006), the other in the Swiss Alps (Bassin et al., 2007b)) have revealed no negative effects on biodiversity. The main response in both subalpine grasslands was an increase in the productivity of sedge species, but not at the expense of forb or legume species. Elevated N inputs at the Swiss Alp 'Flix' experiment enhanced sward productivity (Bassin et al., 2007b).

1.2.4 Nitrogen deposition effects on soil microbial communities

Nitrogen deposition effects on plants are mediated through impacts on soil chemical processes. Soil acidification caused by N deposition is a long-term process, leading to lower pH, base cation leaching and increased aluminium concentrations in the soil (which can be toxic to plants) (Ulrich, 1991; Vitousek et al., 1997; Goulding et al., 1998). The resulting nutrient imbalance induces soil microbial community stress, with negative feedbacks on plant growth (Emmett, 2007; Stevens et al., 2011). Acid neutralizing capacity varies between soil and bedrock types (Bell and Treshow, 2002; Bobbink et al., 2010). However, chronic N deposition can lead to significant reductions in buffering capability (Bowman et al., 2008). The form of reactive N input influences the net effect on soils and plants. Nitrate is typically immobilized by soil microbes and plants, or denitrified. Ammonium, however, can accumulate in the soil through adsorption to soil cations (Rowe, 2005; Stevens et al., 2011).

Nitrogen deposition can affect litter quantity and quality in two ways; via changes in plant sward species composition, and by tissue N changes within dominant species (Manning et al., 2006). Manning and colleagues (2008) experimentally tested both the direct (alteration of plant tissue N and soil N availability), and the indirect (community composition change) effects of N deposition on litter decomposition in artificial communities of eight annual herb species. They found direct effects of N deposition on litter input and decomposition. Other studies have reported initial stimulation of below-ground activity (Bowden et al., 2004) and decomposition of more labile portions of litter (Carreiro et al., 2000b), but high N content of litter and soil then suppresses the decomposition of lignin-rich recalcitrant litter (Berg and Matzner, 1997; Waldrop et al., 2004a). These findings indicate that litter decomposition is a complex process likely to be mediated by many phylogenetic and functional groups within the soil microbial community (Nielsen et al., 2011). When assessing N deposition effects on the soil microbial community as a whole, a useful meta-analysis conducted by Treseder (2008), reveals microbial biomass to decrease by an average of 15% in 82 published N-enrichment studies. Nitrogen deposition has been reported to both increase and decrease below-ground respiration rates in forest ecosystems (Bowden et al., 2004; Waldrop et al., 2004b), depending on the source and degree of recalcitrance of the C available to the microbial population (Carreiro et al., 2000a).

There is a trend in the literature for the soil bacterial communities to increase more than the fungal proportion of the microbial community in elevated N environments (Strickland and Rousk, 2010). High N inputs to *Schizachyrium scoparium* grassland for 18 years increased soil bacterial fatty acid methyl esters (FAMEs), and reduced fungal FAMEs. This indicates potential for N deposition to disrupt the symbiotic relationships between arbuscular mycorrhizal fungi and plant roots (Bradley et al., 2006). In contrast, eight years of addition of +30 kg N ha^{-1} yr^{-1} to a forest ecosystem in Michigan, revealed that soil microbial biomass was significantly decreased, but soil community composition was not altered (DeForest et al., 2004). An indirect effect of N inputs on the soil rhizosphere and decomposer communities may be alteration of labile C turnover rates. Currey and colleagues (2010), found that potential mineralisation of labile C was higher under NH_4 addition than under NO_3

addition in peat soils. In the same study it was also shown that consumption of the amino acid glutamic acid was higher under NH_4 than NO_3.

1.3 Tropospheric ozone and nitrogen deposition: potential interactive effects on plants and soil

Considering current knowledge of the effects of O_3 and N individually, there is much potential for greater than, or less than additive influences (i.e. positive interactive effects) when vegetation is exposed to the combination of stresses. The basis for this conclusion is summarized in Figure 1.6. Whereas the majority of studies state that an elevated level of O_3 reduces above-ground net primary productivity (NPP) (e.g. Hayes et al., 2007), increased N inputs have been shown to promote above-ground growth (Achermann and Bobbink, 2003). Additional N in the plant-soil system could provide resources for defence proteins in the leaves and for repair of O_3 damaged tissue, potentially ameliorating the negative effects of O_3 (Sanz et al., 2005). Jones and colleagues reported that N addition of 100 kg N ha^{-1} yr^{-1} reduced proportional senescence induced by O_3 treatment, and increased the O_3 dose (as AOT40) at which leaf damage occurred in the sedge *Carex arenaria*. The combined impacts of O_3 and N on above ground physiology and growth are likely to be complex and potentially antagonistic. The relative importance of each pollutant's impacts and the processes which the pollutants disturb will determine the overall influence on plant productivity and success in a natural community sward.

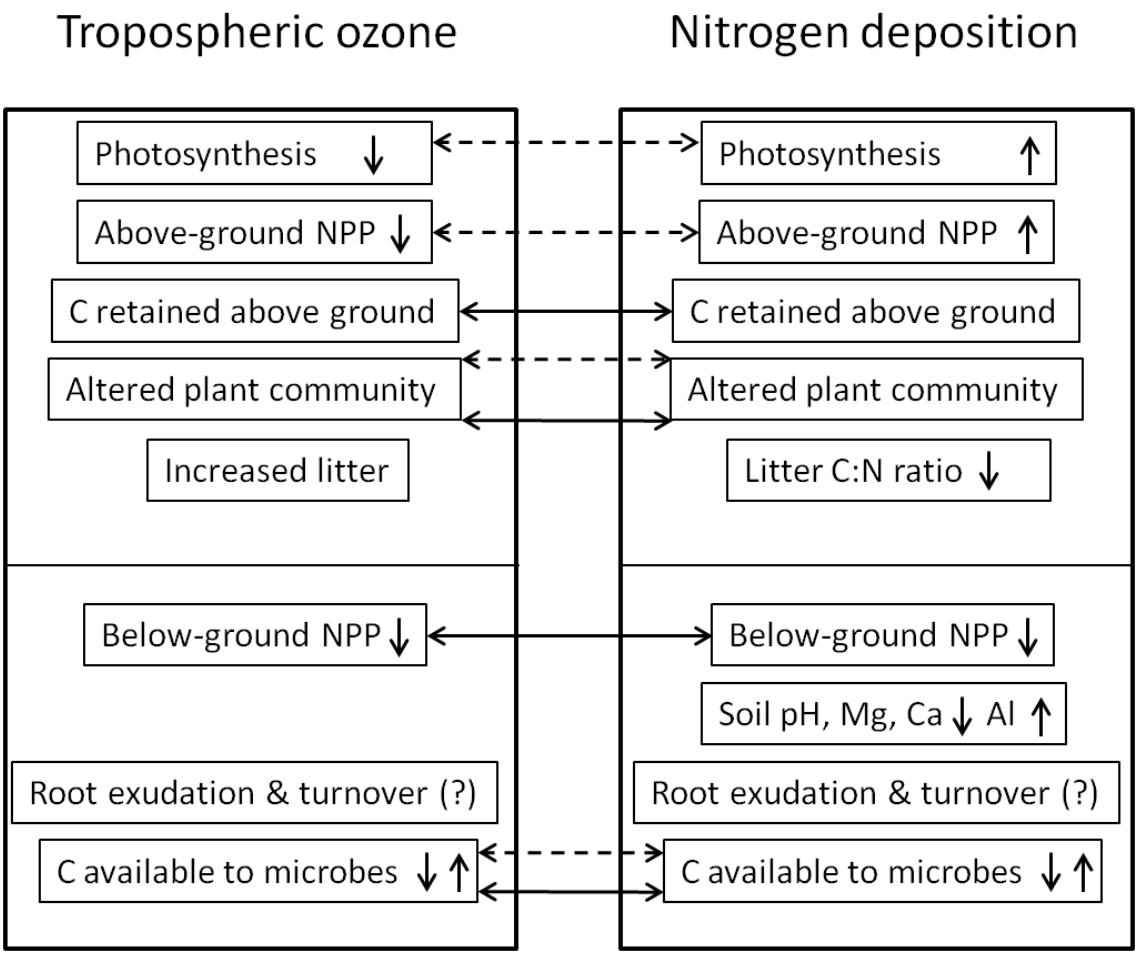


Figure 1.6. Schematic of tropospheric O_3 and N deposition effects on (semi-) natural vegetation and soil. Solid arrows between impacts indicate a potential synergistic interaction between effects as a result of combined exposure to the pollutants, dashed arrows indicate a potential antagonistic interaction on effects based on current knowledge in the literature.

There is extensive evidence in the literature for increased shoot:root ratio in response to both O_3 and N separately. The effect of combined exposure to these pollutants is likely to deliver greater than additive negative impacts on root growth, with potentially important secondary impacts on the soil microbial community. Furthermore, effects below-ground are likely to be cumulative in perennial species, as plants may be unable to gain benefit from excess N availability due to stunted root development. Pell and colleagues (1990) reported that O_3-induced depression in radish biomass (*Raphanus sativus* L. cv. Cherry Belle) was observed in plants grown in high N-supply, but not in those grown at limiting N. A study on subterranean clover (*Trifolium subteranneum* L.), demonstrated that N additions of 30 kg ha^{-1} yr^{-1}

enhanced detrimental effects of elevated O_3 on nutritive quality and on photosynthate allocation to roots (Sanz et al., 2005).

From evidence in the literature focused on separate effects of O_3 and N, plant litter quantity is likely to increase under the combined influence of these variables. Combined impacts on litter quality and decomposition are more difficult to predict, and, prior to this study, have I believe only been investigated in one preliminary study in the free-air O_3 and N experimental site at Alp Flix, Switzerland (Bassin et al., 2007b; Zell, 2011). Zell and colleagues (2011) found that litter lignin content increased in plots receiving 54 kg N ha^{-1} yr^{-1} compared to controls, and a significant antagonistic O_3 * N interaction was evident. Sanz and colleagues (2011) analysing the impact of O_3 and N on the nutritive quality of the grass *Briza media* reported that elevated O_3 increased foliar lignin content, decreasing nutritive quality and this counterbalanced the O_3-induced increase in senesced biomass but did not modify O_3 effects on nutritive quality. The findings of this study suggest the quality of litter is likely to change under combined influence of elevated O_3 and N, with the proportion of recalcitrant C potentially increasing. However, impacts are likely to be highly variable between species, and more investigation is needed to clarify these interactions.

There is a gap in current knowledge concerning the effects of O_3 and/or N on root turnover rates, and root exudation in grasslands. There is some evidence to suggest rhizosphere activity may be initially stimulated in response to O_3 stress, as stored C is mobilized for transport to the shoots, and 'use' genes are down- regulated in the root (Koch, 1996). McCrady and Andersen (2000) reported soluble root exudation of recent photosynthate to the rhizosphere increased in O_3 stressed wheat seedlings compared to controls. Although this could yield a transient increase in sugar availability for the soil microbial community, long-term effects of combined exposure to O_3 and N will likely result in reduced C input from roots to the soil, and a reduction in soil microbial biomass (Treseder, 2008; Chen et al., 2009). Levels of amino acids and reducing-sugar concentration in the root exudates of tomato (*Lycopersicon esculentum* L.) decreased under elevated O_3 (McCool and Menge, 1983). ^{14}C pulse labelling techniques have been employed to investigate rhizodeposition in Italian ryegrass (*Lolium multiflorum* Lam.) with N fertilization (Henry et al., 2005). They showed that high N availability increased the root soluble ^{14}C and the ^{14}C recovered

in the soil per unit of root biomass, suggesting a stimulation of root exudation. The direction and scale of potential O_3 and N interactive effects on soil microbial communities is uncertain, with few studies to date investigating the combined influence of more than one pollutant.

1.4 Aims of the thesis

The main aim of the work undertaken in this thesis was to assess the combined effects of elevated O_3 and N on growth, carbon allocation and species composition of (semi-)natural plant communities, and their associated soils. At the onset of this project, information on the combined effects of O_3 and N on wild plants was extremely scarce (Davison and Barnes, 1998; Black et al., 2000). The interactive effects of O_3 and N inputs both above ground and below ground have previously been assessed in relation to crops and forest trees (Grulke et al., 1998; Cardoso-Vilhena and Barnes, 2001; Grulke et al., 2005; Handley and Grulke, 2008), but there was scant information with regard to impacts on (semi-)natural plant communities despite the obvious importance of such considerations (Sanz et al., 2007; Bassin et al., 2007b; Jones et al., 2010; Sanz et al., 2011).

The present study of combined multiple stresses was intended to provide information on C allocation in meadow species/communities under the present and predicted future O_3 and N deposition loads. It includes an investigation of the effects of O_3, N, and their combination on C allocation patterns in individual species from different functional groups (i.e. grasses and forbs), the impacts on the principle C pools within the plant/ soil system, and the timing of any fluxes and changes in C partitioning. Furthermore, the study investigated whether the soil microbial community size, composition and function are affected by any plant response to above ground pollutant input. The components of the study were investigated using a variety of methods and scales of experiment, in order to observe and compare effects between pot-based controlled environment studies and longer-term, meadow community field experiments.

The general hypotheses tested were:

- Species of mesotrophic hay meadows are 'sensitive' to O_3 (measured as increased senescence, reductions in biomass and reduced allocation of carbon to the roots - Chapters 2, 3 and 5.

- Plant species vary in their response to O_3 and N. Forb species are more likely to be affected by O_3 than grasses, but grasses exhibit a greater response to N in terms of increased above ground growth - Chapters 2 and 5.

- Increased O_3 and N inputs cause changes in interspecific competition and consequently in plant community structure - Chapter 5.

- Nitrogen deposition ameliorates the above ground negative effects of O_3, but exacerbates below ground negative effects - Chapters 2, 3, 4 and 5.

- Elevated O_3 and N inputs induce an indirect negative effect on soil microbial biomass and below-ground respiration via retention of newly assimilated carbon in above ground tissues - Chapters 3 and 4.

- Exposure to elevated levels of O_3 pollution has no significant effect on soil chemical properties, but may reduce soil microbial biomass and turnover of soil C substrate *via* limitation of C introduction to the rhizosphere. High N inputs increase the labile soil C metabolism, but not the metabolism of more recalcitrant litter C, or microbial biomass - Chapters 3 and 4.

- Soil bacterial diversity (analyzed by terminal restriction fragment length polymorphism, T-RFLP) profiles are altered by N treatment but unaffected by O_3 - Chapter 4.

Chapter 2. Enhanced nitrogen deposition exacerbates the negative effect of increasing background ozone in *Dactylis glomerata*, but not *Ranunculus acris*.

2.1 Introduction

The precursors of airborne ozone (O_3) and nitrogen (N) pollution are generated in regions of high traffic density or industrialisation. These gases can be transported over thousands of kilometres before deposition to vegetation surfaces or soil, and many parts of Europe are commonly subject to the co-occurrence of these pollutants (Sitch et al., 2007; Bobbink et al., 2010; Volk et al., 2011). Many studies have reported negative effect of O_3 (McCool and Menge, 1983; Grulke et al., 1998; Grantz et al., 2006), or N (Marschner, 1995; Bobbink, 1998) on below-ground carbon allocation, but few have looked at the simultaneous impacts of these pollutants.

The concentration-based critical level (i.e. the AOT40; the accumulated exposure to O_3 concentrations over a threshold of 40 ppb during daylight hours) for the protection of (semi-)natural vegetation communities dominated by perennials is 5 ppm h, accumulated over 6 months (LRTAP, 2010). Across the U.K., 70% of the total land area covered by crops and (semi-)natural vegetation commonly exceeds the AOT40 based critical level (Coyle et al., 2002), and background concentrations are predicted to rise in the Northern hemisphere by as much as 20 ppb by 2100 (Prather et al., 2003). The negative effects of elevated atmospheric O_3 on plants are well established. Common impacts include chlorosis of the leaves, accelerated senescence, reduced biomass allocation below ground, enhanced rates of maintenance respiration and reduced capacity for photosynthesis (Ojanperä et al., 1998; Davison and Barnes, 1998; Flowers et al., 2007; Xu et al., 2007; Hayes et al., 2009). An O_3 induced reduction in assimilation rate (Fiscus et al., 2005) and increased respiration can lead to less carbon fixation, reductions in growth, and shifts in root:shoot biomass ratio (Andersen, 2003). Assimilate translocation from the shoot to the roots can also be disrupted by O_3, resulting in the retention of carbon in the leaves to repair damage (Grantz and Farrar, 2000; Vandermeiren et al., 2005). Sustained reduction in carbon allocation to roots over several growing seasons could

have important impacts on the plant's capability to compete for resources such as water and nutrients in a community context (Ashmore, 2005). Over 80 (semi-)natural species were included in a meta-analysis of O_3 effects on biomass by Hayes et al. (2007) and several of these have exhibited O_3 injury in ambient air in Europe (Mills et al., 2011). However this is a small fraction of the natural flora of grassland, heathland, wetland, and woodland understorey. These analyses highlighted inter-specific differences in O_3 sensitivity, and illustrated the need for further study of O_3 impacts on species from (semi-) natural vegetation communities. Anthropogenic N emissions have increased dramatically in parallel with rising atmospheric concentrations of O_3 over the second half of the last century. As a consequence the critical loads of N set to protect mesotrophic grasslands from change (15-20 kg N ha^{-1} yr^{-1}) are now widely exceeded (Achermann and Bobbink, 2003; Hall, 2004). Across the U.K., over 60% of sensitive habitat areas are currently in receipt of N loads above this range of values, with the area at risk expected to decrease to 49% by 2020 (RoTAP, 2011). Nitrogen fertilization is known to promote carbon allocation to above ground plant parts and to increase overall biomass production in many (semi-)natural plant species (Fangmeier et al., 1994; Bassin et al., 2007b). Accelerated shoot growth must be fed by recent photosynthate, resulting in reduced carbohydrate availability for storage and root growth, and a shift toward higher shoot to root ratios (Bobbink, 1998; Andersen, 2003). Moreover, there is growing recognition that long-term increases in N deposition are responsible for a significant reduction in community species richness over the past century with grasses being particularly responsive to enhanced atmospheric N deposition at the detriment of forb species (Stevens et al., 2006).

Where O_3 and N pollution co-occur, there are likely to be important combined impacts on plant communities, especially on carbon allocation and biomass partitioning in grassland species. So far, few studies have examined the simultaneous effects of N fertilisation and O_3 fumigation. Thomas and colleagues (2005) investigated the combined effect of elevated levels of O_3 and N on growth and carbon partitioning in young Norway spruce (*Picea abies* L.) trees employing a chamberless fumigation system, and found that N alleviated the negative effect of O_3 on root starch concentrations. In support of this finding, Ponderosa pine trees sited along an O_3 and N gradient in the San Bernadino mountains, California, have been

shown to exhibit decreases in root biomass (Grulke et al., 1998). In addition, Handley et al. (2008) showed that increasing N ameliorated the negative impact of O_3 on *Quercus kelloggii* Newb. Reports on the combined impacts of O_3 and N on non-tree species suggest that effects are complex and dependent on several factors, not least inter-specific differences in life-strategy and functional type. Some authors report synergistic effects. For example, Sanz et al. (2005) reported greater than additive effects of the individual pollutants on the nutritive quality of *Trifolium subterraneum*, and Whitfield et al. (1998) reported *Plantago major* plants grown in controlled conditions were more sensitive to O_3 when grown under a low N regime. Others report no significant interactions or antagonistic effects. For example, Cardoso-Vilhena and Barnes (2001) reported no significant interactions of O_3 and N on the growth and photosynthesis of young wheat leaves (*Triticum aestivum*). Nitrogen fertilization should alleviate visible symptoms of O_3 damage as more resources become available in leaf tissue to combat the oxidative burden imposed by O_3, as observed in *Briza maxima* (Sanz et al., 2011) and *Carex arenaria* (Jones et al., 2010). However, no ameliorating effect of N was observed in *Carex arenaria* root growth, indicating potential negative impacts of increased shoot to root ratio.

Inter-specific differences in sensitivity to O_3 (Hayes et al., 2007), and N fertilization (Bobbink, 1998) have been well documented. Phenotypic plasticity in growth rate and leaf area (Bassin et al., 2007a; Bassin et al., 2009), along with functional grouping (grass, forb or legume) plus edaphic and climatic factors are likely to influence the response to both O_3 and N pollution (Fuhrer et al., 1994; Volk et al., 2006). A recent study by Maskell et al. (2010) showed a significant increase in grass to forb ratio in calcareous grasslands across the UK with increasing N deposition. This effect was attributable to eutrophication, indicating that grasses may out-compete forbs in areas of rising N pollution. Bassin and colleagues (2009) hypothesised that alpine meadow species were relatively resistant to the effects of O_3 and N because of the restraints on growth from environmental factors such as temperature and the availability of other nutrients. If this was to be the case, then mesotrophic grassland species such as *D. glomerata* and *R. acris* may be more at risk from the combined impacts of rising background O_3 and N pollution, as growing conditions are more favourable and the growing season is longer than in alpine regions (Coyle et al., 2003). While there is increasing recognition of the impact of O_3

on carbon allocation, and feedbacks to climate regulation in Global Circulation Models (Sitch et al., 2007), there is relatively little information on how interactions between pollutants affect growth and carbon allocation. There is a particular need to understand interactions between the two commonest global pollutants, O_3 and N, on widespread species of temperate grasslands, both to understand potential impacts on biodiversity, but also to determine impacts on carbon allocation to further improve climate models.

In this study we examined the combined and interactive effects of increasing background [O_3] and N on growth, biomass and photosynthetic parameters of two common grassland species. We hypothesized that i) O_3 would increase senescence and cause reductions in biomass, especially below ground in both species, ii) N would ameliorate the negative impacts of O_3 on senescence and above ground biomass, but not ameliorate below ground effects, iii) there would be inter-specific differences in response to the treatments, with the grass *D. glomerata* being more responsive than the forb *R. acris* to the input of N fertilization, and would be better able to ameliorate the effects of O_3.

2.2 Methods

2.2.1 Plant material

"Plug-plants" of *R. acris* and *D. glomerata* of UK origin were supplied by 'British Wildflower Plants' complying with the Flora Locale code of practice (www.wildflowers.co.uk). They were planted in pots (13 cm diameter) containing 2 dm^3 of Levington organic topsoil (www.wynstaygroup.co.uk). Each pot was lined with perforated plastic to prevent root outgrowth and inoculated with 100 ml soil slurry from a natural meadow (Keenley Fell, Northumberland, 54°9'N, 2°32'E) to introduce and establish a soil microbial community. The soil slurry was made from approx 500 g sieved soil mixed with 6 dm^3 of water. Each pot contained one plant. *R. acris* plants were established for three weeks and *D. glomerata* plants for five weeks before commencing O_3 and nitrogen treatments.

2.2.2 Ozone exposure

Plants were exposed to elevated O_3 at the CEH solardome facility at Abergwyngregyn, North Wales, UK (53°14'N, 4°01'W). The solardome facility consists of eight hemi-spherical glasshouses (3 m diameter, 2.1 m high), see (Mills

et al., 2009). Briefly, oxygen (Workhorse 8 oxygen generator, Ozone Industries Ltd.) was passed through a G11 ozone generator (Ozone Industries Ltd.) to provide O_3 to the solardomes. Dosing was controlled by Lab-VIEW version 7 software using mass flow controllers (Celerity, Dublin, Ireland), with O_3 injected into charcoal filtered air via PTFE tubing to each dome. The O_3 concentration in one dome was continuously sampled using a Model 49C analyser (Thermo Electron, Reading, UK) for feedback control, and O_3 release to the other seven solardomes was adjusted accordingly. The O_3 concentration in each dome was measured on a 30 min cycle by two API400 UV monitors (Envirotech, UK) with matched calibrations.

The eight O_3 treatments simulated an observed ambient O_3 episode over one week based on data (31st May to 6th June 2006) at an upland O_3 monitoring site (Marchlyn Mawr, 510m a.s.l., 53°8′12″N, 4°4′9″W) 12 km from Abergwngregyn. Each treatment used the same O_3 profile, but with a different starting point. The treatments were: simulated ambient (AA), AA-20 ppb, AA+12 ppb, AA+24 ppb, AA+ 36 ppb, AA+48 ppb, AA+60 ppb, and AA+72 ppb (see Mills et al., 2009), with treatments randomly allocated to the solardomes at the start of the experiment. The experimental design therefore provided a range of background O_3 concentrations starting below current ambient levels, to those above 40 ppb, and reflecting predicted future O_3 climates in Europe. Solardomes with O_3 concentrations of AA+36 and higher were turned down to 50 ppb on Tuesdays to allow safe access for plant assessments.

2.2.3 Experimental design

R. acris plants were exposed for 14 weeks from 13th May to 19th August, 2008 and *D. glomerata* plants for 9 weeks from 6th August to 8th October. The young plants were ranked according to size and split into three increasing size categories. Four plants of each size group were randomly allocated to eight solardomes comprising O_3 and N treatments (six plants/ N treatment/solardome) in a nested design. The plants were kept well watered and in addition received doses of either 0, or 75 kg ha^{-1} yr^{-1} nitrogen in the form of NH_4NO_3 (concentration 1.37x10^{-3}M, 50 ml per plant per week).

2.2.4 A-C_i measurements

A-C_i curves were constructed for plants of both species subject to the following treatments: AA, AA +24, AA+48 and AA+60. These O_3 concentrations were selected due to time constraints to provide a representative sample from the O_3 gradient.

Measurements were made on a minimum of 3 independent plants per treatment on each day of measurement. *A*-C_i curves (net CO_2 assimilation rate, *A*, versus calculated sub-stomatal CO_2 concentration, C_i) were used to determine effects on photosynthetic efficiency and capacity of *D. glomerata* leaves after 9 weeks and *R. acris* after 8 and 13 weeks of O_3 exposure. Leaves of a similar size with no visible chlorosis or senescence were chosen for measurement, and a different plant was used for each *A*-C_i curve.

A-C_i curves were measured by infra-red gas analysis using a PP-Systems CIRAS-I fitted with an automatic cuvette using the method described in Hayes et al. (2009). The rate of net photosynthesis (*A*, μmol m^{-2} s^{-1}) and intercellular CO_2 concentration (C_i, ppm) were calculated using the equations of Von Caemmerer and Farquhar (1981). An estimate of the activity of RubisCO within the leaves ($V_{c,max}$) was calculated by fitting the equations of Farquhar et al. (1980), modified to include the temperature dependency of the kinetic constants of RubisCO, K_c and K_o as described in Stirling et al. (1997) and Wullschleger et al. (1993) . The photosynthetic capacity of the leaves (A_{max}), was calculated from the upper asymptote of the *A*-C_i curves using the equation:

$$A = (1 - \Gamma^* / Ci).A_{max}$$

Where *A* (μmol $m^{-2}s^{-1}$) is the instantaneous CO_2 assimilation rate and Γ* is the CO_2 compensation concentration in the absence of light and was calculated from the empirical observation of Farquhar et al. (1988) that Γ* =1.7T where T = temperature (°C).

2.2.5 Biomass

After the exposure periods, the plants were destructively harvested. The above ground plant parts were cut to soil level and sorted by hand into senesced and non-senesced material. A leaf, stalk, or petiole consisting of 25% or more senesced material was defined as senesced and the senesced portion was removed with scissors. The roots were washed free of soil, and all parts were dried to constant weight at 60°C, then weighed.

2.2.6 Statistical analysis

Comparisons of biomass, and photosynthetic parameters were made using R Version: 2.12.0 taking into account the split plot design of the experiment. Data were first checked for normal distribution and homogeneity of variance, and were transformed prior to analysis where required. Analyses were based on the value means within N treatment. A linear term for O_3 was extracted, and the remaining 6 degrees of freedom were used as if they represented true replication, when in fact they represent “lack of fit”. This effectively gives a regression analysis within an analysis of variance. In the nitrogen stratum, the lack of fit interaction provides an error term for testing N and N * O_3 effects.

2.3 Results

2.3.1 Ozone exposure

Ozone treatment	*D. glomerata* mean [O_3] (ppb)	*D. glomerata* AOT40 (ppb h)	*R. acris* mean [O_3] (ppb)	*R. acris* AOT40 (ppb h)
AA-20	15.5	0.0	16.4	0.0
AA	32.5	1.7	34.1	0.8
AA+12	40.6	7.7	45.5	8.0
AA+24	46.8	13.7	52.2	14.7
AA+36	54.5	22.3	65.4	27.4
AA+48	65.7	30.2	75.8	36.1
AA+60	83.3	45.8	91.0	51.3
AA+72	82.5	46.0	92.7	52.0

Table 2.1 Mean O_3 concentrations calculated over 24 h and mean AOT40 for *D. glomerata* and *R. acris* exposure periods.

Ozone concentrations for the period of the exposure are shown in Table 2.1. The concentration-based critical level for O_3 is an AOT40 of 5 ppm h, based on accumulated ozone exposure during daylight hours only, whereas the O_3 treatments in this study were designed to reflect small fluctuations in background O_3 and were maintained high at night as occurs in upland areas (Mills et al., 2009). The aim of the experiment was to investigate the impact of a large gradient of increasing background O_3 including values below 40 ppb. Therefore, all O_3 treatments are presented as 24 h mean concentrations in parts per billion (ppb). AOT40 values are included in Table 2.1 for comparison. *D. glomerata* O_3 exposure ranged from a 24 h mean of 15.5 ppb to 82.5 ppb and that for *R. acris* from a 24 h mean of 16.4 ppb to 92.7 ppb.

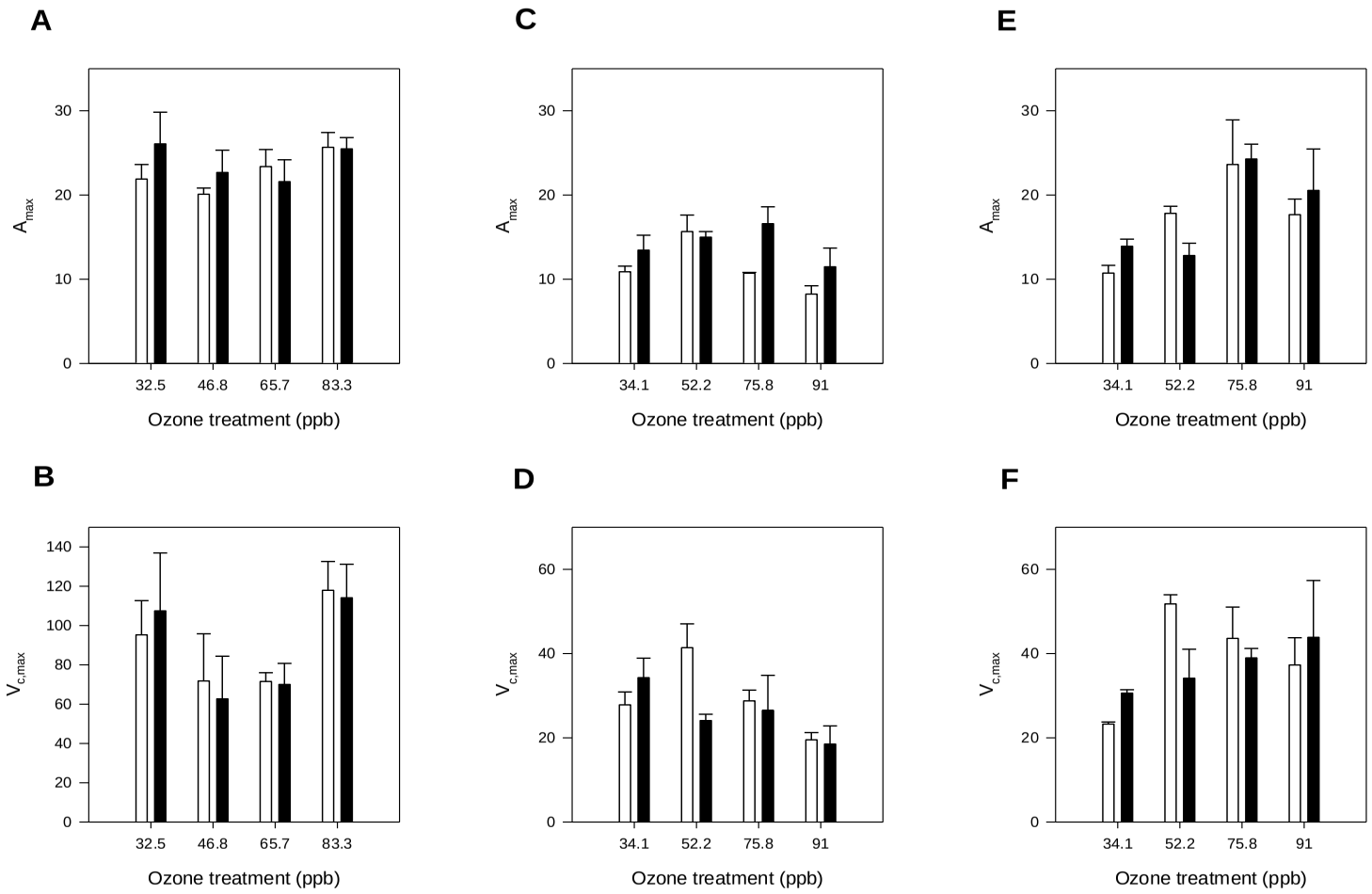


Figure 2.1 (A) *D. glomerata* A_{max}, (B) *D. glomerata* $V_{c,max}$, (C) *R. acris* week 8 A_{max}, (D) *R. acris* week 8 $V_{c,max}$, (E) *R. acris* week 13 A_{max}, (F) *R. acris* week 13 $V_{c,max}$. White bars= low nitrogen treatments, black bars= high nitrogen treatments. Values are means ±SE n=4 (*D. glomerata*) and n=3 (*R. acris*). Treatment effects on A_{max} and $V_{c,max}$ were not statistically significant for either *D. glomerata* or *R. acris*.

2.3.2 Photosynthesis parameters

Analysis of the *A*-C_i curves produced from measurements on *D. glomerata* leaves after 9 weeks of O_3 and N treatment showed no significant change in leaf-level photosynthetic capacity (A_{max}) or rate of RubisCO carboxylation ($V_{c,max}$) (Fig. 2.1 A and B).

There was no effect of O_3 or N on *R. acris* A_{max} or $V_{c,max}$ at either week 8 or 13 when values from all four of the measured domes are included in the analyses (Fig. 2.1 C-F).

2.3.3 Biomass partitioning

High N treatment significantly increased the biomass of both healthy and senesced above-ground *D. glomerata* material compared to low N plants (Fig 2.2 A $p \leq 0.001$ and Fig 2.2 B $p \leq 0.001$ respectively); and therefore the total above ground biomass was also increased (Data not shown, $p \leq 0.001$). Increasing background O_3 reduced the biomass of healthy leaves and increased senesced leaf biomass (Fig 2.2 A $p \leq 0.005$ and Fig 2.2 B $p \leq 0.0005$ respectively), but not total above ground biomass. *R. acris* plants showed a trend towards a reduction in healthy above ground biomass with increasing O_3 concentration, but this was not statistically significant (Fig 2.2 C). Rising background O_3 concentration increased senesced above ground biomass in this species (Fig 2.2 D $p \leq 0.05$).

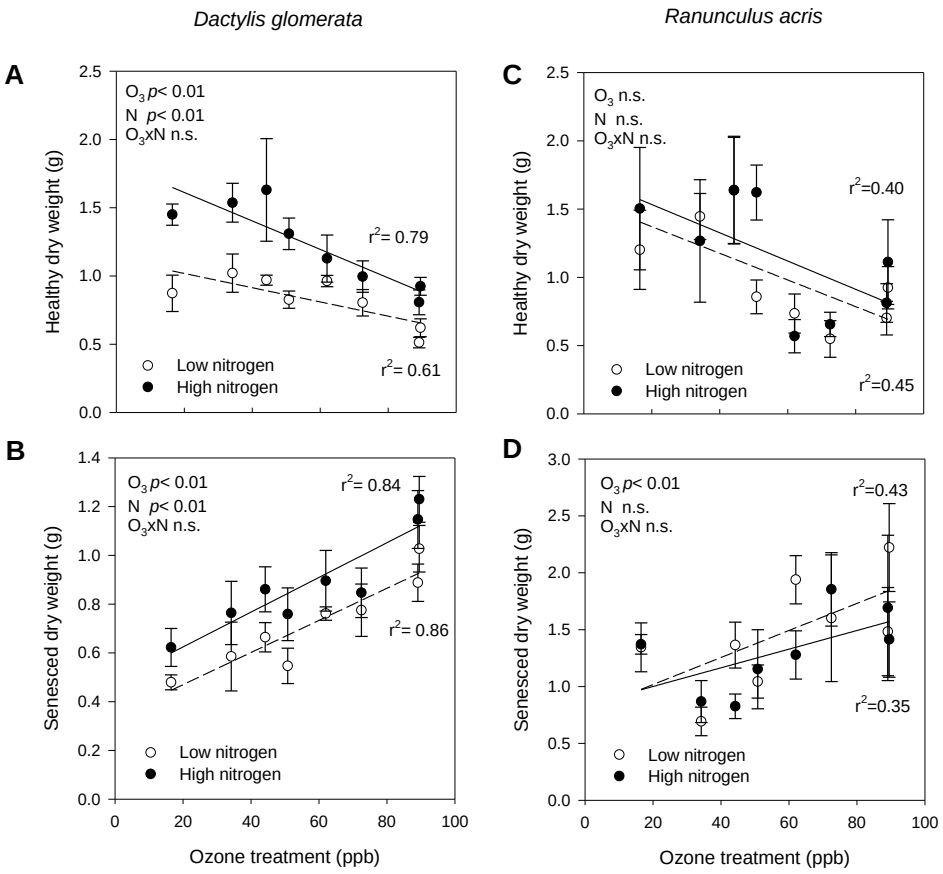


Figure 2.2 Above ground biomass at destructive harvest (A) *D. glomerata* healthy biomass (B) *D. glomerata* senesced biomass (C) *R. acris* healthy biomass (D) *R. acris* senesced biomass. Six plants per treatment. Bars are standard errors. Solid line = high nitrogen regression, dashed line = low nitrogen regression.

Figure 2.3 A shows that there were significantly greater than additive suppression of root biomass in *D. glomerata* exposed to the combination of elevated O_3 and N ($p \leq 0.05$). However, overall root biomass remained greater in the high N plants even at the highest O_3 treatment. In contrast to the results for *D. glomerata*, increasing O_3 treatment significantly reduced *R. acris* root biomass, irrespective of N treatment (Fig 2.3 B $p \leq 0.005$).

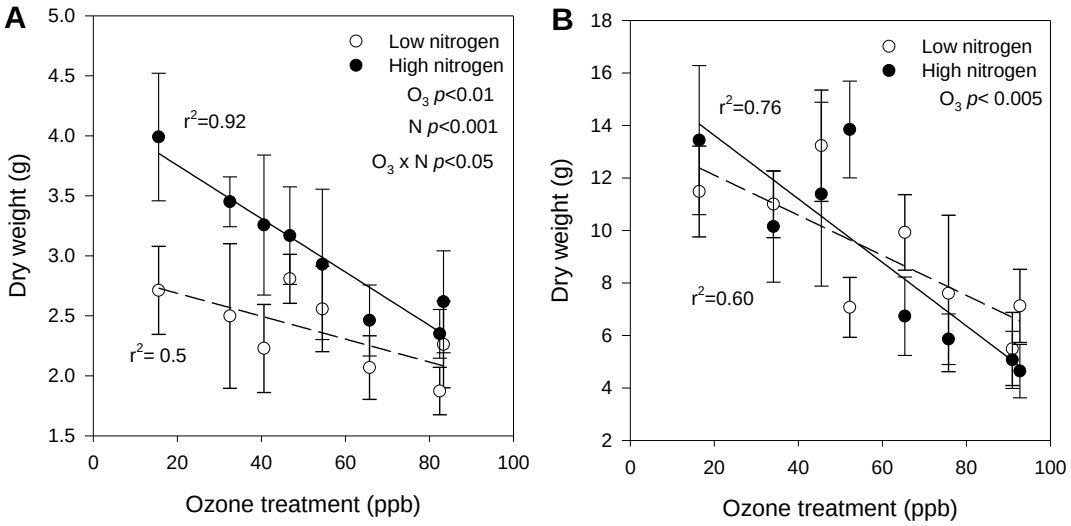


Figure 2.3 Root biomass at destructive harvest (A) *D. glomerata* (B) *R. acris*. Six plants per treatment. Bars are standard errors. Solid line = high nitrogen regression, dashed line = low nitrogen regression.

There was a trend for the *D. glomerata* root to shoot ratio to decrease with increasing O_3, but there was no statistically significant $O_3 * N$ interaction (Figure 2.4 A). *R. acris* root to shoot ratio indicated a larger negative impact of increasing O_3 $p \leq 0.005$ (Fig 2.4 B), but there was no clear effect of N treatment.

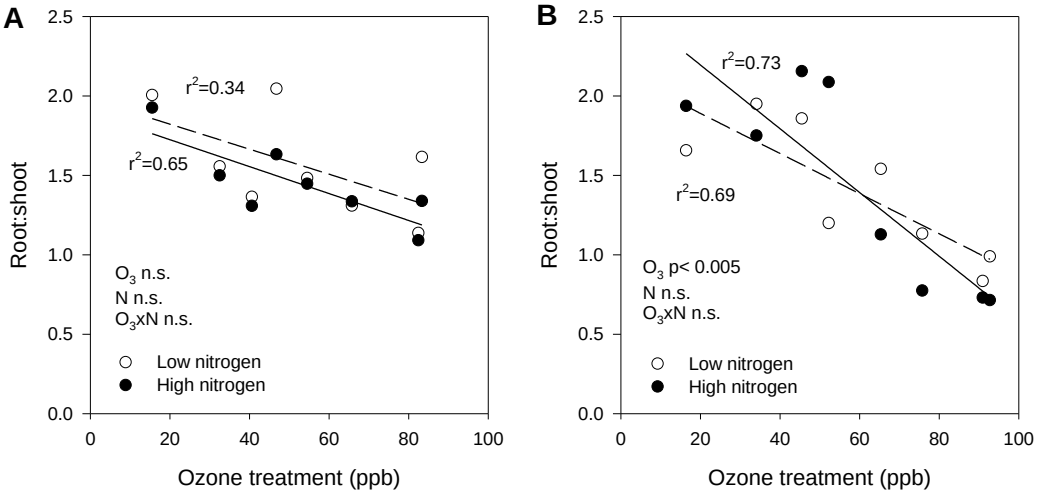


Figure 2.4 Root to shoot ratio at destructive harvest (A) *D. glomerata* (B) *R. acris*. Six plants per treatment. Solid line = high nitrogen regression, dashed line = low nitrogen regression.

2.4 Discussion

There is much information about the impacts of O_3 exposure or N deposition on grassland vegetation, but few studies have considered the combined impacts of these common pollutants. The findings presented here suggest that N modifies vegetation responses to O_3 stress and highlights interactive effects of N and O_3 as an important consideration when constructing regional and global carbon models.

Although significant O_3 and N effects on root biomass were evident, there was no treatment effect on A_{max} and $V_{c,max}$ in *D. glomerata* suggesting that individual leaves not yet showing visible signs of O_3 damage retain 'normal' photosynthetic capacity. Hayes and colleagues (2009) reported that the newest fully expanded leaf of *Lolium perenne* plants exposed to O_3 for 10-11 weeks showed no significant effect of O_3 on photosynthetic capacity or carboxylation efficiency. This provides support for the results seen in *D. glomerata,* and suggests that allocation of carbon resources may be up-regulated in new leaves without O_3 injury. Any differences in carbon allocation and biomass parameters at the whole plant level may be due to loss of photosynthetic capacity in older leaves which have increased O_3 damage and do not provide sufficient sink strength for defence and repair of photosynthetic machinery (Andersen, 2003). As shown by Morgan and colleagues (2004), leaf age modifies the effect of O_3 on photosynthetic capacity and rate of RubisCO carboxylation, with young leaves of soybean (*Glycine max* L.) shown to be more resistant to O_3 stress. Cardoso-Vilhena and Barnes (2001) studied the modifying effect of N availability on photosynthesis of spring wheat (*Triticum aestivum* cv. Hanno) leaves. They reported *c.* 20% reduction in A_{max} and $V_{c,max}$ in leaves of plants supplied with low levels of N (1.5 m*M* NO_3^-) compared to those with high N availability (14 m*M* NO_3^-); but no effects of O_3 exposure for upto 12 days after leaf expansion.

D. glomerata A_{max} and $V_{c,max}$ values were comparable to those reported by Harmens and co-workers (2000a), where hydroponic plants were grown in ambient or elevated CO_2 subject to four N treatments. These researchers found that increasing N had a positive effect on RubisCO content of the leaves, and subsequently, on photosynthetic capacity. However, although RubisCO continued to accumulate at N inputs above 0.6 m*M*, $V_{c,max}$ did not increase similarly at lower N concentrations.

The aim of the present study was to examine the effects of simulated high N deposition on plants usually found in grasslands of moderate inherent fertility. Although some beneficial effects of high N treatment were detectable on biomass, previously reported protective effects of high N on the photosynthetic machinery of O_3 stressed plants was not observed, suggesting that increasing nutrient availability above a certain threshold may not increase defence and repair of damaged tissue, or promote photosynthesis. However enhanced N inputs did affect the sink strength of leaf tissue, and apparently starved the roots of recent photo-assimilate (see Figs. 2A and 3A).

The O_3 and N interaction on root biomass reported here for *D. glomerata,* was similar to that reported for *Trifolium subterraneum* exposed in open top chambers for one month, with N additions of up to 30 kg ha^{-1} yr^{-1} (Sanz et al., 2005). Nitrogen did not affect above ground biomass in *Ranunculus acris*, and no O_3 - N interactions were found for this species. For both species, exposure to increasing O_3 decreased biomass allocation to the roots. This was probably due to an increase in the retention of carbon in source leaves to fuel repair processes, and a combination of reduced carbon assimilation, and increased sink strength in older leaves. This negative effect on root:shoot ratio has been widely reported in crops and (semi-)natural vegetation (Cooley and Manning, 1987; Andersen, 2003).

D. glomerata has previously been shown to alter dry matter partitioning in response to N availability (Harmens et al., 2000b). In the current study, there was no significant N effect on root : shoot ratio, but the beneficial effect on root biomass of added N seen at low O_3 concentrations was lost at the highest O_3 concentrations. Indeed, the slope of the regression for the high N treatments was substantially steeper (y= -0.02x + 4.20) than for the low N treatments (y= -0.0095x + 2.88), indicating that the O_3 response is magnified by elevated N. This corresponds with observations made by Pell and colleagues (1990) who reported that O_3 reduced biomass of radish (*Raphanus sativus* L. cv. Cherry Bell) plants grown at high N supply but not those grown at limiting N, but are in contrast to the observation of Jones et al. (2010) for a rhizotamous sedge *Carex arenaria*.

Specific O_3 effects on reproductive development of natural species in an open top chamber community mesocosm experiment, showed *R. acris* to be one of the least

responsive to treatment (Rämö et al., 2007). However changes in carbon allocation patterns and root development as shown in the current experiment, may be important for long term survival, especially in the case of increasing background O_3 exposure over many seasons, as is predicted for rural areas (Mills et al., 2009). *R. acris* is highly variable in its physiology and appearance with a large capacity for phenotypic plasticity (Harper, 1957). This inherent variability reduces the likelihood of seeing a consistent response to applied treatments, and is one of the main challenges when studying pollutant effects on (semi-)natural vegetation (Davison and Barnes, 1998; Bergmann et al., 1999).

In the current study we exposed two commonly occurring temperate grassland species to O_3 concentrations typical of pre-industrial to predicted post-2010 levels. Not only did these two species show linear responses to O_3 in terms of enhanced senescence and diminished root growth, but these responses were modified by the co-occurrence of simulated N deposition. Inter-specific differences in carbon allocation patterns could affect the competitive balance between species and drive species diversity and community composition change. This is already observed to be a consequence of rising background O_3 (Ashmore et al., 1995) and N deposition (Stevens et al., 2006; Bobbink et al., 2010; Vries et al., 2010). Therefore our findings could be of concern for the long term stability of grassland ecosystems subject to the combination of rising levels of O_3 and N pollution.

2.5 Conclusions

This study has highlighted the importance of researching the combined effects of atmospheric pollutants on (semi-)natural vegetation. Increased N input has been shown to have synergistic effects on the response of *D. glomerata* to O_3 stress and exacerbates O_3-induced reduction in carbon allocation below ground in this species. Grasslands store approximately 34 percent of the global stock of carbon in terrestrial ecosystems (Olson, 1994) and are a dynamic source and sink for CO_2. Ozone and N may modify the rate of carbon fixation in plant biomass and carbon sequestration in the soil, with long term consequences for these ecosystems.

D. glomerata and *R. acris* responded differently to the combined treatment of O_3 and N, highlighting important potential effects on community composition in (semi-)natural grasslands. Although it is difficult to translate these results to potential changes at the community level because of the modifying influence of field conditions such as soil characteristics and climate, and competitive interactions between plant species, a recent study by Hayes and colleagues (2011), showed that effects of the same O_3 treatments on roots of *D. glomerata* were carried over to the following spring. More long term field experiments are needed to expand our knowledge of synergistic effect of pollutants on natural vegetation.

This work has been written-up and published as a peer-reviewed paper:

Wyness, K., G. Mills, et al. (2011). "Enhanced nitrogen deposition exacerbates the negative effect of increasing background ozone in *Dactylis glomerata*, but not *Ranunculus acris*." Environmental Pollution **159**(10): 2493-2499.

Chapter 3. Short-term effects of ozone and nitrogen exposure on carbon partitioning in the common grassland species *Dactylis glomerata* plant-soil system using ^{14}C pulse labelling.

3.1 Introduction

Reactive nitrogen compounds and tropospheric ozone (O_3) are currently inducing changes in the ecophysiology of vegetation across diverse habitats on a global scale (Fuhrer, 2009; Bobbink et al., 2010). Grasslands cover approximately 40% of the land surface of the planet (Olson, 1994), and provide important ecosystem services including grazing, habitat for pollinators, refuge for biodiversity of plants and animals, and C sequestration (Loreau et al., 2001; de Groot et al., 2002). The combined impacts of O_3 and N have the potential to disrupt the carbon-capture to carbon-emission ratio of these systems, with marked shifts in speciation commonly observed in grasslands subjected to elevated inputs of these pollutants (Davison and Barnes, 1998; Zavaleta et al., 2003; Ashmore, 2005; Bassin et al., 2007). However, few studies have investigated the likely synergistic effects of these common pollutants.

Although peak O_3 episodes are becoming less frequent in developed European countries (RoTAP, 2011), rising background concentrations of O_3 are causing the exposure-based critical level set to protect (semi-) natural vegetation (AOT40: 5 ppm.h.), to be widely exceeded during the summer months in many parts of Europe and North America (Mills et al., 2011). The pollutant reduced European crop value by 6.7 billion in 2000 (Holland et al., 2006), is known to have profound impacts on natural ecosystems (Davison and Barnes, 1998) and rising levels have been shown to threaten human health (Cisneros et al., 2010). On a global scale, rising background levels of O_3 represent an important driver of climate change through indirect radiative forcing (Ramaswamy et al., 2001; Sitch et al., 2007).

Ozone primarily affects C capture and partitioning in vegetation via oxidative signal-induced depressions in photosynthesis (Fiscus et al., 2005; Flowers et al., 2007; Hayes et al., 2009), compounded by the impacts of programmed cell death manifested as accelerated senescence of tissues exposed to O_3 (Vandermeiren et

al., 2005; Hayes et al., 2010). Enhanced maintenance respiration has also been reported in many species (Landolt et al., 1997; Biswas et al., 2008). Grantz and Farrar (2000) showed that O_3 has the potential to restrict phloem loading and promote the retention of newly assimilated C in primary photosynthetic tissues; shifts in the root:shoot ratio (with shoot gaining at the expense of the root) are a widely reported consequence of exposure to O_3 (Cooley and Manning, 1987; Grantz et al., 2006). Although knock-on effects below ground have been identified as being important, these are often problematic to study effectively without disturbance to the system due to experimental methodology (Andersen, 2003).

Increased C demand for defence and repair of O_3 damaged leaf tissue, leading to root starvation, has secondary implications for the microbial community inhabiting the rhizosphere and bulk soil (McCool and Menge, 1983; Chung et al., 2006; Esperschutz et al., 2009). Chen and colleagues (2009; 2010) report significant O_3-induced changes in soil microbiota, with the fungal population being the most depleted group. There is also a widely held belief that O_3 negatively influences mycorrhizal associations and this may reduce nutrient availability to plants (Landeweert et al., 2001), and retard the decomposition of litter (Perez-Moreno and Read, 2000).

O_3 formation is dependent on anthropogenic emissions of reactive N compounds (Coyle et al., 2002; Ashmore, 2005; Dentener et al., 2006), some of which are deposited on terrestrial systems as either wet or dry deposition (Asman et al., 1998; Galloway et al., 2008), often many miles from their source of origin (Lamarque et al., 2005). Naturally oligotrophic or mesotrophic ecosystems such as grasslands are known to be susceptible to damage from increased N inputs (Stevens et al., 2004; 2006) resulting in marked shifts in plant community composition (Rowe, 2005; Hautier et al., 2009; Bobbink et al., 2010). Long term, N deposition can also lead to soil acidification and nutrient imbalance resulting in plant and soil microbial community stress (Emmett, 2007; Stevens et al., 2011). The critical N load for calcareous grassland is 15-25 kg ha^{-1} yr^{-1} (Hall, 2004), Currently more than 60% of sensitive habitats in the U.K. receive N deposition loads in excess of this (RoTAP, 2011).

The fertilizing effect of N deposition on (semi-) natural vegetation has been extensively studied (Achermann and Bobbink, 2003; Bobbink et al., 2010), with reductions in root to shoot ratios being one of the most widely reported findings (Bobbink, 1998; Fenn et al., 2003). Hill et al (2007) examined the combined effect of CO_2, grazing and N fertilization on C partitioning patterns in *Lolium perenne* swards using ^{14}C pulse labelling. This work showed that N reduced the ^{14}C root to shoot ratio by 14% and the root free soil ^{14}C activity by 51% compared to plants receiving lower N input. Nitrogen deposition has been reported to both increase and decrease below-ground respiration rates in forest ecosystems (Waldrop et al., 2004; Bowden et al., 2004), depending on the source and degree of recalcitrance of the C available to the microbial population (Carreiro et al., 2000). In a *Calluna vulgaris* and *Eriophorum vaginatum* peatland receiving elevated N for 5 years, ^{13}C fixation increased, and below-ground respiration remained constant compared to control plots indicating enhanced C translocation below ground (Currey et al., 2011). High N inputs to *Schizachyrium scoparium* grassland for 18 years increased soil bacterial fatty acid methyl esters (FAMEs), and reduced fungal FAMEs, suggesting the potential for N deposition to disrupt the symbiotic relationships between arbuscular mycorrhizal fungi and plant roots (Bradley et al., 2006).

Results from Chapter 2 suggest that exposure of the grass *Dactylis glomerata* to increasing background concentrations of ozone caused the retention of carbon in above-ground tissues, to the detriment of root growth. Indeed the combined influence of ozone and high nitrogen treatment resulted in a significant reduction in root biomass after nine weeks in solardomes.

The use of C isotopes in pulse chase studies is a widely practiced method to investigate C partitioning dynamics in vegetation and soil (Swinnen et al., 1994; Kuzyakov and Domanski, 2000; Domanski et al., 2001; Hill et al., 2007; Werth and Kuzyakov, 2008). One potential caveat in characterizing whole plant C budgets from pulse labelling, is the assumption that the transport and allocation of older unlabelled C pools is the same as newly assimilated C (Paterson et al., 2009). Nonetheless the approach is extremely useful for the tracking and characterization of the fate of newly derived photosynthate.

It is increasingly important to consider the combined influence, and interactions between, drivers of ecosystem change, in order to understand and predict their joint effects on vegetation (Ollinger et al., 2002). This study takes a mechanistic approach to investigate the impacts of short-term exposure to environmentally relevant concentrations of O_3 and/or N on C allocation within the grass *Dactylis glomerata*. The hypotheses under exploration are i) O_3 and N will alter carbon allocation via retention of newly assimilated carbon in above ground tissues, causing a reduction in partitioning of recent photosynthate to the roots, and ii) O_3 and N treatment will induce an indirect negative effect on soil microbial biomass and below-ground respiration. This is the first study, to our knowledge, to derive a complete C budget for a grass-soil system under O_3 and N exposure.

3.2 Methods

3.2.1 Plant material

Individual *Dactylis glomerata* plants were grown from seed (Naturescape British Wild Flowers, Nottinghamshire, UK) in circular pots (9 cm diameter, 14 cm depth) containing 0.6 dm^3 Levington topsoil (www.wynstaygroup.co.uk). Each pot was inoculated with 10 ml soil slurry from a natural meadow (Keenley Fell, Northumberland, 54°9'N, 2°32'E) to introduce and establish a soil microbial community. The soil slurry was made from approx 500 g sieved soil mixed with 6 dm^3 of water. The pots were freely draining, and leached soil solution was collected at the base of each pot. The young plants were ranked according to size and split into 2 size groups 5.5 weeks after sowing. Equal numbers of each size group were randomly allocated to O_3 and N treatments.

3.2.2 Experimental design

The experimental design consisted of six experimental chambers (20 x 40 x 55 cm) arranged in three replicate blocks. Each chamber was constructed of 5 mm thick clear Perspex to allow maximum strength and light penetration. A mixing fan was positioned in the centre of each chamber roof, and an extractor fan situated at the exhaust of each chamber. The average air-flow was sufficient to deliver approximately one air change per minute in each chamber. Each replicate block consisted of one ambient O_3, and one elevated O_3 chamber. Four pots of *D. glomerata* per N treatment were randomly allocated to each chamber (8 plants per

O_3 chamber). There were 4 positions within each chamber; A, B, C or D relating to distance from the chamber door. One low N and one high N plant was placed at each position; assignment to which side of the chamber was assigned at random.

Ozone was supplied by a G11 O_3 generator (Dryden Aqua, UK), using oxygen from a Workhorse 8 oxygen generator (Dryden Aqua, UK). The O_3 dose was controlled using a PTFE microbore tube (Cole-Parmer Instrument Co. Ltd., London, UK) feed system into the main air-flow after the point at which the pipe was split between ambient and elevated O_3 chambers. Ozone delivery to the system was directly related to the length of the microbore tube, and the ozonated air flowed through a mixing pipe before feeding into the chambers. The O_3 concentration was monitored in one chamber at a time, and a PTFE sampling tube autochanged between chambers regularly to monitor concentrations within individual chambers. The sampling tube was positioned at canopy height in each chamber and the O_3 concentration was monitored for five minutes every 30 minutes with a UV photometric O_3 analyser (Environmental Technology Services 400A) connected to a computer running a LabView controller system (LabView version 6.0). Pairs of chambers were illuminated with 600 W daylight ballast HPS lamps fitted with reflective aero-wings (www.aquaculture-hydroponics.co.uk), providing a photosynthetic photon fluence rate (PPFR) of ~700 μmol m^{-2} s^{-1} at canopy height over a photoperiod of 12 h (0700-1900 hours). Temperature was 26±5 °C during the photoperiod and 14±7 °C during the dark period. Air humidity was controlled by means of a two-stage Mitsubishi MUE16V dehumidifier/B125 humidifier. Measurements were continually logged every minute with radiation-shielded thermistors (Skye Instruments Ltd, Llandrindod Wells, Powys, UK) linked to a Delta-T2 Data Logger (Delta-T Devices Ltd, Cambridge, UK). Ozone and N treatments were introduced for 3 weeks, commencing on 27th May 2010 (when plants were 5.5 weeks old), and ending on the 18th June 2010. The elevated O_3 treatment aimed to reflect projections for 2100 in the Northern Hemisphere (Sitch et al., 2007). All six chambers were supplied with unfiltered ambient air from within the building housing the chambers (average 9.4ppb ±0.17 SE); O_3 was injected into three of the chambers providing a mean concentration of 79.4ppb ±0.87 SE for 10h d^{-1}, from 0730-1730 hours.

Soil moisture was assessed at the start of ozone exposure and each pot weighed. The pots were re-weighed daily, and a rain solution (Emmett and Poskitt, 2007) added to replace water lost via transpiration. In addition, the plants received either 1 ml extra rain solution per plant; or 50 kg ha^{-1} yr^{-1} nitrogen in the form of NH_4NO_3 solution (concentration $1.37x10^{-3}$M in 1 ml per plant per day) throughout the O_3 exposure period. These treatments represent 'low nitrogen' and 'high nitrogen' treatments, respectively. The rain solution contained NaCl, $CaCl_2$, $CaSO_4$, $MgSO_4$ and H_2SO_4 but no N compounds.

3.2.3 ^{14}C pulse labelling

Each pot was pulse labelled with $^{14}CO_2$ at a constant specific activity on 10th June 2010; 2 weeks after commencement of ozone and nitrogen treatments. ^{14}C-labelled CO_2 was applied using clear airtight plastic domes of 1.8 litre volume. Prior to applying the dome, a small (*c.*10 ml volume) reaction vessel was positioned under a re-sealable 20 mm diameter hole at the highest point of the dome. 50 µl of 0.492 MBq ml^{-1} (0.246 MBq total) $Na_2{}^{14}CO_3$ soln. was added to each reaction vessel. This was followed by 2 ml 1 M H_2SO_4. Immediately after adding the acid, the hole was sealed and plants were allowed to fix the $^{14}CO_2$ for 2 h. Labelling was performed outside the chambers at a photosynthetic photon fluence rate (PPFR) of ~200 µmol m^{-2} s^{-1} at canopy height. All plants were labelled within 10 minutes of each other.

After 2 h, 20 ml of air was drawn from each dome with a gas-tight syringe and domes were removed. The 20 ml gas sample was slowly bubbled through 15 ml of Oxosol scintillant (National Diagnostics, Atlanta, GA, USA) to capture any unfixed $^{14}CO_2$. The ^{14}C activity was measured by liquid scintillation counting in a Wallac 1404 scintillation counter (Perkin-Elmer Life Sciences, Boston, MA). The plants were then placed back in their original positions within the chambers.

Measurements of below-ground respiration and soil solution commenced immediately following the removal of the domes. Below-ground respired $^{14}CO_2$ was captured passively by pushing a 27 mm diameter, capped polypropylene tube a few mm into a bare patch of soil beside each plant. $^{14}CO_2$ was captured in tubes containing 1 ml 1 *M* NaOH which were placed inside the larger tube. NaOH traps were changed after 4 h, 24 h, and then daily until the 18th of June 2010, and analysed by liquid scintillation counting in HiSafe 3 scintillant (Fisher Scientific,

Loughborough, UK). Soil solution was sampled using 5 cm Rhizon soil solution samplers (Rhizosphere Research Products, Wageningen, Netherlands) inserted 3 cm from the edge of each pot at a depth of 7 cm. Soil solution was collected in sterile Venoject vacuum tubes (Terumo Corp., Tokyo, Japan) which were changed every 24 h. 1ml of soil solution was sampled at each time point and acidified with 200 μl 1 *M* HCl to drive off any dissolved CO_2 before analysis by liquid scintillation counting as described previously.

1 ml of 0.5 M K_2SO_4 extract obtained from non fumigated and chloroform fumigated soil samples were analysed by liquid scintillation counting to quantify ^{14}C in the extractable soil C pool and the microbial biomass pool. Subsamples of dried ground soil and plant biomass, were oxidized in an OX400 Biological Oxidiser (RJ Harvey Instrument Corp., Hillsdale, NJ, USA), with $^{14}CO_2$ collected in Oxosol scintillant (National Diagnostics, Atlanta, GA, USA) and ^{14}C activity measured by liquid scintillation counting in a Wallac 1404 scintillation counter (Perkin-Elmer Life Sciences, Boston, MA, USA) as described in (Hill et al., 2007). Hereafter, the C pool referred to as the 'Soil (total)' refers to the oxidised soil subsamples; and includes the extractable soil ^{14}C, non-extractable soil ^{14}C, microbial biomass ^{14}C and the soil solution ^{14}C.

3.2.4 Harvest and soil analysis

All plants were destructively harvested at the end of the experiment, 8 days after ^{14}C pulse labelling. Senesced material was separated, roots were washed and sieved to remove soil particles and capture fine roots. All plant material was then dried at 60°C, weighed, ground to a powder and stored for subsequent analysis. Soil LOI and pH (H_2O extraction) were assessed for fresh soil shaken from the roots. Microbial biomass C was measured using the chloroform method described by Vance et al. (1987) and Wu et al. (1990). Soil total extractable nitrogen (TN) was quantified from 0.5 M K_2SO_4 extracts, which were centrifuged and filtered before analysis on a Shimadzu TOC-V-TN analyzer (Shimadzu Corporation, Kyoto, Japan). NO_3^- was determined colorimetrically by the Cu–Zn–hydrazine reduction method of Downes (1978) and NH_4^+ by the salicylate–hypochlorite procedure of Mulvaney (1996).

3.3 Statistical analysis

Comparisons of biomass and ^{14}C pool sizes were made using R Version: 2.12.0 analysis of variance, taking into account the split plot design of the experiment. Data were first checked for normal distribution and homogeneity of variance, and transformed prior to analysis as required. Analyses were based on the value means within nitrogen treatment. Curve fitting in Fig.1a was performed in SigmaPlot 11.0 using Exponential Rise to Maximum, Single, 2 Parameter f=a*(1-exp(-b*x)).

3.4 Results

No effect of O_3 or N was found on the total amount of ^{14}C fixed by the plants during labelling (Table 3.1). This was taken as a proxy for photosynthetic capacity showing that after two weeks; plants from all four treatments assimilated equivalent quantities of $^{14}CO_2$. Table 3.2 shows that bulk allocation of C within the *D. glomerata* plants (both labelled and unlabelled) was not influenced by O_3 and/or N after three weeks of treatment.

	Treatment						
	Ambient ozone		Elevated ozone		Change due to treatment (%)		
	Low nitrogen	High nitrogen	Low nitrogen	High nitrogen	Ozone	Nitrogen	O_3 x N
Total ^{14}C fixed (kBq)	245.54±0.08	245.61±0.09	245.61±0.07	245.60±0.04	ns	ns	ns
Healthy shoot ^{14}C content (kBq 100 mg^{-1})	3.30±0.27	3.52±0.30	3.58±0.24	3.80±0.32	ns	ns	ns
Senesced shoot (kBq 100 mg^{-1})	2.28±0.27	2.18±0.26	2.21±0.16	2.33±0.17	ns	ns	ns
Root (kBq 100 mg^{-1})	1.89±0.19	2.08±0.24	1.92±0.26	2.07±0.23	ns	ns	ns
Soil (total)(Bq 100 mg^{-1})	4.06±0.41	3.68±0.34	4.05±0.33	3.89±0.34	ns	ns	ns
Soil (extractable)(Bq g^{-1})	0.84±0.12	0.90±0.12	0.88±0.12	1.0±0.11	ns	ns	ns
Microbial biomass (Bq g^{-1} soil)	6.78±1.12	8.63±1.00	8.89±0.87	7.40±0.78	ns	ns	**Interaction 0.041**
Root ^{14}C (kBq 100 mg^{-1}): shoot ^{14}C (kBq 100 mg^{-1})	0.71±0.18	0.59±0.04	0.52±0.04	0.54±0.03	ns	ns	ns
Root ^{14}C (kBq total): shoot ^{14}C (kBq total)	0.38±0.06	0.47±0.05	0.46±0.04	0.54±0.03	ns	ns	ns

Table 3.1 Measurements of gross ^{14}C concentrations in *Dactylis glomerata* plants and soil, 8 days after ^{14}C pulse labelling. Values are mean ±SE (n=12). Changes due to treatment are shown with *P*-values for statistical significance shown underneath.

	Treatment						
	Ambient ozone		Elevated ozone		Change due to treatment (%)		
	Low nitrogen	High nitrogen	Low nitrogen	High nitrogen	Ozone	Nitrogen	O_3 x N
Leaf count at destructive harvest	111.3±7.4	103.5±6.1	108.5±3.1	98.3±6.5	ns	ns	ns
Healthy shoot biomass (g)	2.81±0.10	2.76±0.17	2.51±0.18	2.52±0.21	ns	ns	ns
Senesced shoot biomass (g)	0.18±0.05	0.13±0.02	0.28±0.02	0.36±0.06	ns	ns	ns
Total shoot biomass (g)	2.99±0.10	2.89±0.19	2.79±0.18	2.88±0.23	ns	ns	ns
Root biomass (g)	2.28±0.24	2.26±0.26	2.32±0.24	2.53±0.24	ns	ns	ns
Root biomass : shoot biomass	0.76±0.07	0.78±0.07	0.82±0.06	0.87±0.04	ns	ns	ns
Whole plant biomass (g)	5.27±0.30	5.15±0.40	5.11±0.37	5.41±0.45	ns	ns	ns

Table 3.2 Measurements of *Dactylis glomerata* dry plant biomass after 3 weeks of O_3 and/or N exposure. Values are mean ±SE (n=12). Changes due to treatment are shown with *P*-values for statistical significance shown underneath.

The flux of $^{14}CO_2$ from below-ground respiration (the combined respiration of roots and soil microbes) declined over the 168 h period following pulse labelling (Figure 3.1a). There was a trend for elevated O_3 to decrease below ground respiration although this effect was not significant. High N treatment, on the other hand, significantly (P = 0.04) reduced below ground respiration 8 days into the pulse chase experiment (Table 3.3). The percentage of the total fixed ^{14}C estimated to be in the soil solution pool over time, is shown in Figure 3.1b. After 4 h, plants in all treatments leached approximately 0.045 % of the ^{14}C pulse. Between 4 h and 24 h, leaching increased to a maximum of 0.8 % of the total fixed, and the quantity of ^{14}C in the soil solution then returned steadily to the experimental start point (0.05 % after 168 h). All *D. glomerata* pots showed a similar pattern of decline in soil solution ^{14}C over time, with no significant impacts of O_3 and/or N treatment.

The proportions of ^{14}C (of the total fixed ^{14}C), in the measured and derived C pools in the plant-soil system at destructive harvest are shown in Table 3.3 There were no significant treatment effects on the amount of labelled C sequestered in the plant biomass, soil, or soil solution pools, eight days after the ^{14}C pulse. There was also no effect of O_3 or N on the flux of above ground $^{14}CO_2$ respiration.

A significant antagonistic interaction between O_3 and N effects on ^{14}C sequestered in microbial biomass as a proportion of total ^{14}C fixed, was evident 168 h after labelling (P = 0.034, see Table 3.3). Figure 3.2 shows the O_3 x N interaction on microbial biomass ^{14}C when expressed as a proportion of the fixed ^{14}C allocated below-ground (P =0.015). The same result is seen when expressed as microbial biomass ^{14}C specific activity Bq g^{-1} soil (P = 0.041, see Table 3.1).

There were no other significant treatment effects on the ^{14}C gross activity of the measured C pools (see Table 3.1) and no treatment effects on pH, total soil nitrogen (TN), nitrate or ammonium content of the soil (see Table 3.4).

Figure 3.3 provides a schematic summary of ^{14}C partitioning in the *Dactylis glomerata* plant-soil system across O_3 and N treatments. The total soil C pool represents the soil solution ^{14}C, microbial biomass ^{14}C, extractable ^{14}C, and non-extractable ^{14}C. Generally 68% of the total ^{14}C remaining in the plant biomass was retained above ground and 32% in the roots. This ratio of 2.2 (above-ground C to below-ground C) is maintained when C fluxes associated with respiration and soil

leaching are included in the scheme. Little ^{14}C was leached in the soil solution. The ^{14}C lost from the system, which was not accounted for in the quantified C pools or in below-ground respiration, was assumed to be shoot respiration.

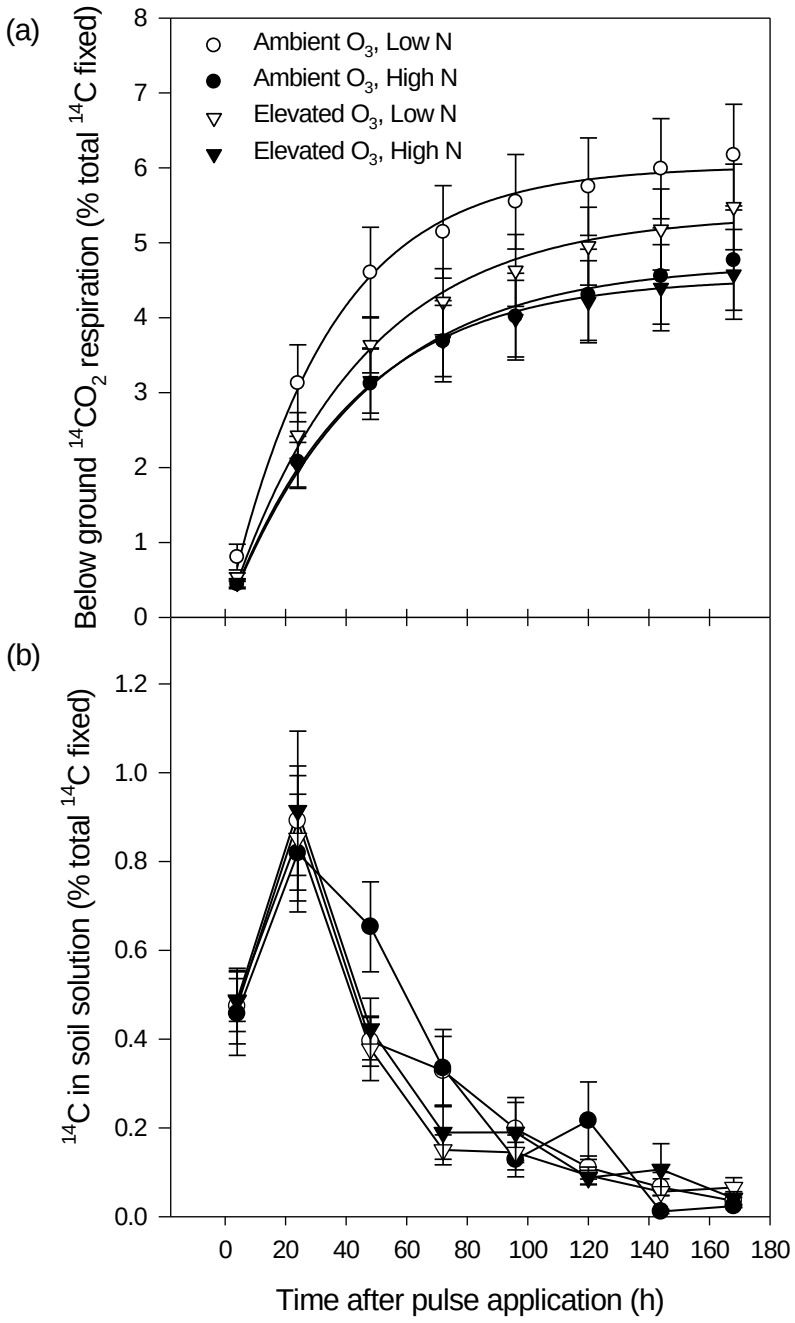


Figure 3.1 (a) Cumulative below-ground $^{14}CO_2$ respiration, as a % of total fixed, with time after pulse application. Data points are mean ±SE (n=12); Ambient O_3, Low N r^2=0.998, Ambient O_3, High N exponential rise to maximum curve fit: r^2=0.994, Elevated O_3, Low N r^2=0.995, Elevated O_3, High N r^2=0.997. (b) ^{14}C in the soluble soil carbon pool with time after pulse application. Data points are mean ±SE (n=12). Figure key applies to both (a) and (b).

	Treatment						
	Ambient ozone		Elevated ozone		Change due to treatment (%)		
	Low nitrogen	High nitrogen	Low nitrogen	High nitrogen	Ozone	Nitrogen	O_3 x N
Healthy shoot	0.402 ±0.01	0.376 ±0.02	0.349 ±0.01	0.364 ±0.02	ns	ns	ns
Senesced shoot	0.021 ±0.008	0.011 ±0.001	0.025 ±0.002	0.03 ±0.008	ns	ns	ns
Root	0.15 ±0.02	0.17 ±0.01	0.16 ±0.01	0.19 ±0.01	ns	ns	ns
Soil (total)	0.09 ±0.01	0.08 ±0.01	0.09 ±0.01	0.08 ±0.01	ns	ns	ns
Soil (extractable)	0.002 ±0.0003	0.002 ±0.0003	0.002 ±0.0003	0.002 ±0.0002	ns	ns	ns
Microbial biomass	0.015 ±0.002	0.019 ±0.002	0.019 ±0.002	0.016 ±0.002	ns	ns	**Interaction 0.034**
Soil solution	0.0004 ±0.0001	0.0002 ±3.43E-05	0.0007 ±0.0002	0.0004 ±0.0002	ns	ns	ns
Below ground respiration	0.06 ±0.007	0.05 ±0.007	0.05 ±0.006	0.046 ±0.006	ns	**-19.8 0.038**	ns
Shoot respiration	0.296 ±0.03	0.315 ±0.02	0.336 ±0.02	0.279 ±0.021	ns	ns	ns

Table 3.3 ^{14}C pools and fluxes as a proportion of fixed ^{14}C in *Dactylis glomerata* plants and soil 8 days after pulse labelling. Values are mean ±SE (n=12). Where significant, percentage changes due to treatment are shown with *P*-values for statistical significance shown underneath.

	Treatment						
	Ambient ozone		Elevated ozone		Change due to treatment (%)		
	Low nitrogen	High nitrogen	Low nitrogen	High nitrogen	Ozone	Nitrogen	O_3 x N
pH	6.7±0.03	6.7±0.03	6.7±0.03	6.7±0.02	ns	ns	ns
TN (mg g-1 dry soil)	0.02±1.0E-03	0.02±3.0E-03	0.02±1.5E-03	0.02±1.3E-03	ns	ns	ns
NO_3^- (mg g-1 dry soil)	5.19E-03±7.56E-05	5.11E-03±6.73E-05	5.25E-03±8.14E-05	5.44E-03±2.01E-04	ns	ns	ns
NH_4^+ (mg g-1 dry soil)	2.03E-03±4.93E-05	2.02E-03±2.31E-05	1.99E-03±4.11E-05	2.11E-03±7.94E-05	ns	ns	ns

Table 3.4 Measurements of soil characteristics in *Dactylis glomerata* pots after 3 weeks of O_3 and/or N exposure. Values are mean ±SE (n=12). Changes due to treatment are shown with *P*-values for statistical significance shown underneath.

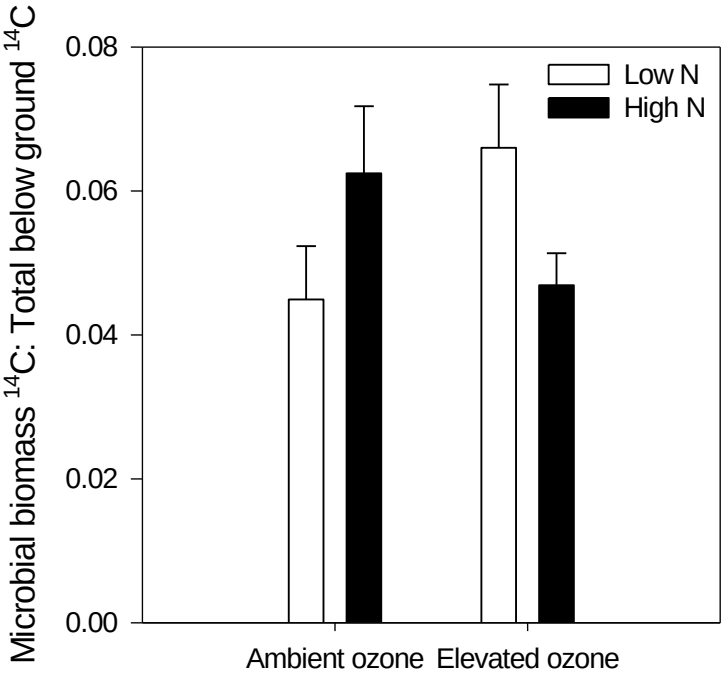


Figure 3.2 ^{14}C in soil microbial biomass 168 h after pulse labelling, as a proportion of the total ^{14}C recovered from below ground pools. Data are mean ±SE (n=12) Interaction between O_3 and N treatment P = 0.015.

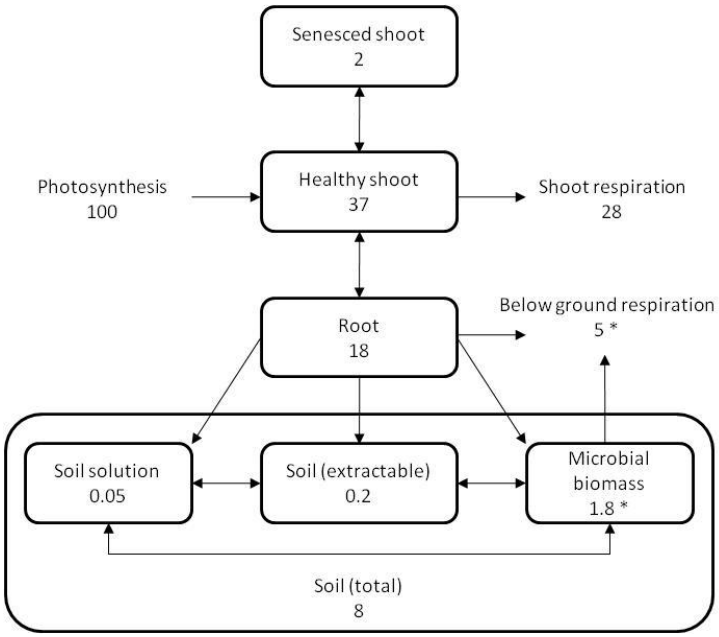


Figure 3.3 Schematic diagram of C pools and fluxes in the *Dactylis glomerata* plant and soil system. All values are presented relative to an input from photosynthesis of 100. Fluxes are cumulative for the period 0-8 d. Pool sizes are those remaining 8 d after ^{14}C labelling. * indicates a significant treatment effect. Adapted from Hill et al. (2007).

3.5 Discussion

Impacts of O_3 and N pollution on C allocation within plants and soils are a growing area of study given the implications for C sequestration. This study aimed to elucidate the timing and extent of C allocation changes following short-term exposure to elevated levels of O_3 and/or N pollution, as distinct from longer-term changes mediated by cumulative effects on growth (as described in Chapter 2).

Dactylis glomerata is a common grass species with a range spanning most of Europe, North Africa, the USA, and temperate Asia (Hubbard, 1984). Studies on the effect of O_3 on above ground biomass suggest *D. glomerata* to be relatively insensitive compared to some other grasses such as timothy grass (*Phleum pratense* L.) or forbs such as clover (*Trifolium spp*.) (Hayes et al., 2007). However, root biomass of *D. glomerata* has been shown to be significantly decreased by elevated O_3 and N after 9 weeks during a solardome study (Chapter 2), and Mills and co-workers (2009) used *D. glomerata* as a model to study O_3-induced changes in the ABA-induced signal transduction pathway resulting in stomatal closure. Moreover, enhanced N inputs have been shown to exacerbate the negative effects of increasing background ozone on the root biomass of *D. glomerata* (see chapter 2). These findings imply that this species may be prone to changes in C allocation patterns under the combined influence of O_3 and N stress. Vegetation responses to environmental pollutants such as O_3 and N accumulate throughout the growing season (Davison and Barnes, 1998; Bobbink et al., 2010), and there is also evidence for carry-over effects of pollutants from one season to the next (Hayes et al., 2006).

Plants in the present study received and assimilated the same quantity of the ^{14}C pulse, irrespective of applied O_3 and N treatments. Prolonged O_3 exposure has been shown to reduce photosynthetic capacity and efficiency in many species (Fiscus et al., 2005; Wittig et al., 2007; Xu et al., 2007; Hayes et al., 2009), and high N fertilization can promote leaf growth and increase fixation rates providing other nutrients are not limiting (Marschner, 1995; Lee and Caporn, 1998; Aerts and Bobbink, 1999). After 2 weeks' exposure to elevated O_3 and/or N the plants in the present study did not exhibit significantly increased levels of visible leaf injury or reduced rates of C fixation. Therefore, any differences within the measured ^{14}C pools and fluxes observed between treatments were more likely to be due to alteration of C partitioning, storage and/or metabolism.

The soil solution C pool is dynamic, with C constantly removed and replenished by the soil microbial community biomass (Grayston et al., 1997; Boddy et al., 2007). The initial maximum flux of 0.8 % of the fixed ^{14}C leached to the soil solution pool at 24 h is relatively small compared with other fluxes because C is quickly captured by microbes, or adsorbed to soil particles after exudation (Jones and Murphy, 2007; Hill et al., 2008). After 160 h only a small percentage of the total ^{14}C fixed was detected in the soil solution. This is probably attributable in most part to the lysis and recycling of soil microbial cells rather than new rhizodeposition, as previous studies in wheat (*Triticum aestivum* L.) have found that exudation of recent photosynthate is greatest 3 h after assimilation (Dilkes et al., 2004), and declines significantly thereafter. Root exudation has been demonstrated to be sensitive to the restriction of C input to the roots in wheat (*Triticum aestivum* L., var. Consort) (Hill et al., 2007a), indicating that in the current study, high levels of N input to the system may have constrained exudation by promoting retention of C in above ground plant parts. Soil solution was sampled from 0-7 cm depth, and a uniform distribution of ^{14}DOC in the soil was assumed. The estimates of the soil solution C pool presented here are consistent with results for wheat presented by Hill et al. (2007), where 0.1% of the total fixed ^{14}C was recovered in the DOC pool.

Henry et al. (2005) reported that elevated N supply decreased the partitioning of ^{14}C to roots of Italian ryegrass *Lolium multiflorum* Lam., but increased the root soluble ^{14}C and the ^{14}C recovered in the soil per unit of root biomass. This suggests that N fertilization stimulated root exudation. However, this effect did not induce changes in the rhizosphere microbial population. Although rhizodeposition was not measured directly in the current study, we hypothesise that the observed reduction in below ground respiration was due to reduced root and exudation induced by N input.

The effect of increased N deposition on soil respiration has been studied in temperate forest ecosystems, showing that after 13 years of fertilization, soil respiration was reduced by 41% (Bowden et al., 2004). However, other reported results vary in scale and direction with species and conditions. Andersen and colleagues (2000) show that below-ground respiration initially increases with elevated O_3 in pine trees (*Pinus ponderosa* Laws.). In non-tree systems, Currey and colleagues (2011) report increased fixation of ^{13}C label with high N and increased allocation below-ground in hare's-tail cottongrass (*Eriophorum vaginatum* L.), and

Gavrichkova and Kuzyakov (2008) show that N input to maize (*Zea mays* L.) and white lupin (*Lupinus albus* L.) increased below ground respiration. These results contrast with our observations of a decrease in below-ground respiration in high N treated *D. glomerata* plants, indicating the need for longer-term studies on a variety of species/systems to increase our understanding of C partitioning in response to rising levels of N and O_3 deposition.

The observed interactive effects of O_3 and N on ^{14}C partitioning into microbial biomass is worthy of further detailed exploration. Microbial community composition may be changing under the impact of O_3 and N treatment, which in turn alters soil (and possibly root) respiration.

Short-term controlled environment studies are useful to investigate mechanisms of plant C allocation, but care needs to be taken when endeavouring to scale-up from pot based studies. In natural ecosystems, litter (Lee and Caporn, 1998; Andersen, 2003), plant species interactions (Ashmore et al., 1995; Stevens et al., 2006) and edaphic factors will undoubtedly influence the strength and even direction of pollution effects (Ashmore and Bell, 1991).

3.6 Conclusions

C fluxes through the plant-soil system studied here were dynamic. Retention times within certain C pools are likely to be affected by nutrient status and environmental stresses such as N deposition and O_3 pollution. This is the first study, to the best of our knowledge, to derive a complete C budget for plants and soil subjected to simulated (albeit short-term) O_3 and N pollution. The approach adopted in the present study allowed the differentiation of impacts on C partitioning mechanisms, from long term effects mediated by altered growth patterns. ^{14}C was used to trace the fate of recently- fixed carbon in pot-grown *Dactylis glomerata* plants. It has been demonstrated that effects of N inputs on C partitioning are more immediate than those of O_3 pollution, at least for the studied model system, with reduced root exudation and below-ground respiration amongst the primary impacts. It is hypothesised that exposure to elevated O_3 would exacerbate these effects longer-term by reducing photosynthetic capacity and promoting the retention of new assimilate in the shoots.

Chapter 4. Limited effects of long-term exposure to elevated ozone and nitrogen inputs on soil microbial diversity and function in a subalpine grassland soil.

4.1 Introduction

Global background concentrations of tropospheric ozone (O_3) are increasing due to anthropogenic emissions, and the detrimental effects of this phytotoxic pollutant on crops and semi-natural vegetation have been well documented over the past few decades (Davison and Barnes, 1998; Ashmore, 2005). Impacts of ozone pollution on plants are broadly similar and include visible leaf injury, accelerated senescence (Hayes et al., 2010), suppressed rates of net photosynthesis (Cardoso-Vilhena et al., 2004), enhanced rates of maintenance respiration (Amthor, 1988) and changes in carbon partitioning (Andersen, 2003). However, the scale of responses to ozone-induced oxidative stress differs between species, depending on detoxification capacity (Fuhrer and Booker, 2003) and adaptations to climate and other stresses (Lyons et al., 1997; Yoshida et al., 2001; Bassin et al., 2009). Bassin and colleagues (2007a) indicate that on a grassland community scale, factors such as the length of the growing season, nutrient availability, species composition, balance between functional groups, and management regime will all influence community susceptibility to ozone. It has been predicted that due to the slow growing, low specific leaf area, and stress tolerant phenotype of many plants growing in alpine areas, they should be less susceptible to injury from ozone than (semi-) natural species in lowland ecosystems (Bassin et al., 2007b). But this view is at odds with that expressed by Mills and colleagues (2007) who suggest that many species common to alpine grasslands maybe sensitive to O_3 damage.

The diversity and metabolic activity of any given plant community is closely coupled to the soil supporting its foundation and *vice versa* (Jones et al., 2009). Ozone pollution has been shown to disrupt the recycling of soil C through reduced detrital inputs, increased decomposition losses, or both (Loya et al., 2003). This recycling relies strongly on the microbial community, made up of bacteria, archaea, and fungi. In particular, rhizosphere community diversity and function is closely coupled to the

photosynthetic rate of the associated vegetation (Dilkes et al., 2004), and can be negatively affected by O_3 (Chen et al., 2009; Chen et al., 2010).

Atmospheric nitrogen (N) deposition, on the other hand, has the potential to modify vegetation response to O_3, and the synergistic effects of these two commonly occurring pollutants are an emerging area of research with important consequences for sensitive plant communities (Fuhrer, 2007). As an essential nutrient, reactive nitrogen is utilised by plants to synthesise amino acids and proteins. The reactive forms of N are made up of reduced N (NH_x, ammonia and ammonium), and oxidised N (NO_y, particulate nitrates, nitrogen oxides and nitric acid) (RoTAP, 2011a). Levels of reactive N deposition in Europe and North America have increased dramatically over the past half century primarily due to industrial activities and agricultural practices (Galloway et al., 2008), and the European empirical critical N load set for Alpine and subalpine grasslands of 5-10 kg N ha^{-1} yr^{-1} is predicted to be exceeded by 2030 (Bobbink et al., 2010). The key indicators of exceedance of the critical load are an increase in nitrophilic graminoids and change in biodiversity (Bobbink et al., 2010). However, two long-term field experiments in which high N deposition treatments have been employed (one in Colorado, USA (Bowman et al., 2006), and the other in the Swiss Alps (Bassin et al., 2007b)) have not revealed negative effects on biodiversity. The main response in both subalpine grasslands was an increase in the productivity of sedge species, but not at the expense of other non-graminoid species. Elevated N inputs at the Swiss Alp Flix experiment enhanced sward productivity, O_3 had no significant impact, and no interactive effects between O_3 and N were observed above-ground (Bassin et al., 2007b). Yet, in a reciprocal litter decomposition study on the same experimental site, root material from unfertilized plots decayed 22% faster in ambient plots compared to other treatments, and both leaf and root tissue composition were altered by O_3 and N (Zell, 2011). Previous research has highlighted the retardation of recalcitrant C in litter by increased N deposition (e.g. Berg and Matzner, 1997), and a meta-analysis conducted by Treseder (2008), revealed microbial biomass to decrease by an average of 15% in 82 published N-enrichment studies. It is likely therefore that combined effects of O_3 and N on plants may have secondary effects on the microbial community and C turnover in soils.

There have been numerous investigations which demonstrate that high N can lead to acidification of soils (Ulrich, 1991), and alter carbon allocation patterns in vegetation with the detriment to roots (Marschner, 1995). Thus, subalpine soil systems may incur secondary effects as a result of modification of litter quantity and/or quality plus changes in plant carbon allocation patterns induced by O_3 and N. The combined impact of O_3 and N below ground has previously been investigated in individual (semi-)natural plant species. A study on *Trifolium subteranneum*, demonstrated that N additions of 30 kg ha^{-1} yr^{-1} enhanced detrimental effects of elevated O_3 on photosynthate allocation to roots (Sanz et al., 2005). Jones and colleagues (2010) suggest that N mediates above-ground impacts of O_3 but not impacts on below-ground resource translocation in the sedge species *Carex arenaria*. Further research is required to elucidate effects on roots and soil of intact plant communities in the field.

The use of molecular methods to investigate soil microbial ecology has become increasingly popular in the last three decades (Hartmann et al., 2005). Terminal restriction fragment length polymorphism (T-RFLP) analysis is one such cultivation-independent method providing a tool for describing complex microbial communities at high resolution via specific PCR amplification of phylogenetic marker genes such as the bacterial small ribosomal subunit. The subsequent detection of laser-induced fluorescence and the digital output can readily be converted into numeric data which enables subsequent statistic analyses (Hartmann et al., 2005). T-RFLP has been shown to be more discriminatory than physiological and biochemical approaches such as Biolog plates and phospoholipid fatty-acid analysis (PLFA) (Singh et al., 2006), and is a sensitive technique for monitoring environmental and anthropogenic effects on microbial community structures. For example, Lazzaro and colleagues (2008) have shown that changes in the bacterial community of soils contaminated with the heavy metal cadmium were detected using T-RFLP analysis, and Wolsing and Priemé (2004) used the approach to demonstrate that soils treated with mineral fertilizer or cattle manure showed different T-RFLP patterns (and thus different communities) of nirK-containing denitrifying bacteria.

In this study we report the combined and interactive effects of five years' exposure to elevated O_3 and N on soil chemical properties, soil bacterial community composition and soil function of a sub-alpine grassland. Soil function was investigated by quantifying the turnover of sixteen different low molecular weight (LMW) C substrates and grass (perennial ryegrass: *Lolium perenne* L.) leaf litter. It was hypothesized that i) long-term exposure to elevated levels of O_3 pollution will not have a significant effect on soil chemical properties, but may reduce the soil microbial biomass and C substrate turnover rate *via* limitation of C input to the rhizosphere ii) High N inputs will increase the labile soil C compound metabolism, but not the metabolism of more recalcitrant litter C, or microbial biomass. iii) Combined injections of elevated O_3 and N will have antagonistic effects on soil C substrate turnover. iv) Soil bacterial terminal restriction fragment (T-RF) profiles may be altered by N treatment but would be unaffected by O_3.

4.2 Methods

4.2.1 Study site and experimental design

The experimental field site was constructed and managed by the Air Pollution and Climate Group, Agroscope Research Station ART, Switzerland (Bassin et al., 2007b). It is located in the Central Alps at Alp FlixSur (9°39'N/46°32'E), Switzerland 2000 m above sea level, and encompasses a species-rich *Geo-Montani-Nardetum* pasture subject to low intensity management. Approximately one-half of the sward cover consists of *Festuca violacea*, *Nardus stricta* and *Carex sempervirens*. The remaining half is composed of > 70 forb and a few legume species. The site has permanent snow cover in winter and annual precipitation of 1200 mm. See Bassin et al. (2007b) for a more detailed description of the site and experimental design. Briefly, the ozone fumigation system consists of nine rings of 7 m diameter arranged in 3 linear blocks on a small ridge (length 150 m). Three ozone fumigation treatments ambient (control), 1.2 x ambient, and 1.6 x ambient concentration were randomly assigned to the three rings in each block (Bassin et al., 2007b). The average ambient ozone concentration during the growing season is approximately 45-47 ppb. In 2006, the mean ozone fumigation treatments were 42.1, 54.9 and 74.2 ppb respectively. Fumigation continued for 24 h daily during the growing season to simulate rising global background O_3 concentrations.

Plots within the fumigation rings incorporated monoliths of 40 cm length, 30 cm width and 20 cm depth. The monoliths had been excavated from a nearby montane pasture in 2003 and placed in plastic boxes with drainage holes. The monoliths were randomly assigned to fumigation rings, and put in shallow pits flush with the surrounding vegetation. To replace cattle grazing, plots were cut once per year at peak canopy development, to a height of 2 cm (Volk et al., 2011).

Nitrogen treatments were applied to the monoliths twice-weekly during the growing season as 200 ml solutions of NH_4NO_3 in well water (< 0.05 kg N ha^{-1} $year^{-1}$). Taking account of the background level of N deposition at the field site (4 kg ha^{-1} $year^{-1}$), the N treatments administered to plots was equivalent to 4, 9, 14, 29 or 54 kg ha^{-1} $year^{-1}$. Results are reported for four replicate N treatment monoliths in each fumigation ring.

The reported study was restricted to samples from the ambient O_3 N0 (zero addition of N), ambient O_3 N50 (50 kg ha^{-1} y^{-1} N), 1.6 x ambient O_3 N0 and 1.6 x ambient O_3 N50 treatments, *n*= 48.

4.2.2 Soil sample collection

Soil samples were collected in the first week of May 2009 during snow melt using a soil corer cleaned with ethanol between samples. Four replicate samples were collected at random positions within each selected monolith using a 2 * 30 cm soil corer, pushed into the soil to a depth of 15 cm. Duplicate samples were collected for DNA analysis, snap frozen and transferred in dry ice. Additional duplicate samples were recovered for soil property and function analysis. These were stored at 4°C until analysis. Soil samples were bulked and thoroughly mixed according to the following protocol. Monoliths receiving the same nitrogen treatment within an ozone ring were paired according to soil Mg and Ca content (as quantified in 2003 by the Air Pollution and Climate Group, Agroscope Research Station ART, Switzerland). Initial soil samples of each duplicate were bulked, then second samples and so on. Samples for DNA analysis were bulked, mixed and analysed within 24 h of thawing. After bulking there were 24 samples, the level of replication of nitrogen treatments was *n* = 6, and for ozone *n* = 3.

4.2.3 Soil property analysis

Field moist soil pH was measured using a Corning 220 pH meter in a 1 : 2.5 mixture with deionised water (Avery, 1974). Soil microbial C content was analysed using the

chloroform fumigation method described by Vance et al. (1987). Fumigated samples and control samples (aliquots of 5g field moist soil) were extracted using 0.5 *M* K_2SO_4. For calculating the microbial biomass carbon, a k_{EC}-factor (extractable fraction of microbial biomass C) of 0.45 was used (Wu et al., 1990) The extracts were diluted 1:10 with deionised water and analysed using a Shimadzu TOC–V–TN analyser (Shimadzu Corp., Kyoto, Japan). Kjeldahl N was measured using a Tecam OG-1 Block digester and 2300 Kjeltec Analyzer Unit (Foss Tecator v1.14 1997). Ammonium and nitrate were determined from *M* KCl extractions according to Page (1982) and analysed using a SEAL AQ2+ Automated discrete analyzer. Na, K, Ca, and Mg were determined from 0.5 *M* NH_4Cl extractions, with determinations performed using a Perkin-Elmer Atomic Absorption Spectrophotometer.

4.2.4 Soil community-level physiological profiling (CLPP)

Soil samples for the carbon substrate mineralization studies were kept at 4°C in the dark to minimize soil microbial activity. All were analysed within 10 days of collection. 1 g of field moist soil was placed in a 50 ml container with an airtight lid, and incubated at 10 °C for 24 h to allow soil microbial metabolism to stabilize at the new temperature. The mineralization of sixteen different ^{14}C labelled carbon substrates known to be present in roots, and/or components of root exudates was investigated. These included six amino acids; Arginine, Aspartic acid, Glycine, Lysine, Phenylalanine, and Valine, five carbohydrates; Fructose, Glucosamine, Glucose, Starch and Sucrose, and five carboxylic acids; Oxalic acid, Salycilic acid, Succinic acid, Sodium acetate, and Malate. 100 μl of a 10 mM solution of each labelled substrate was pipetted onto separate soil samples (one substrate per soil sample). 10 mM solution of each substrate was chosen to reflect the likely concentration in soil following the lysis of a root cell (Jones et al., 2004). A polypropylene scintillation vial containing 1 ml M NaOH was immediately suspended above the surface of the soil sample to trap the respired $^{14}CO_2$, and the airtight lid securely replaced on the container. The NaOH trap was replaced 4 h and 72 h after the addition of the substrate. Immediately on being removed from the centrifuge tube, 4 ml of Optiphase scintillation fluid (Wallac Optiphase 3 scintillation fluid (EG&G Ltd) was added to the NaOH trap and ^{14}C content was then determined by liquid scintillation counting in a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK). The ^{14}C activity of the substrate initially added to the soil samples was determined by adding 100 μl

of substrate stock solution to 4 ml Optiphase scintillation fluid then a count conducted in the scintillation counter. After 72 h, the amount of ^{14}C remaining in the soil, which was not assimilated into microbial biomass was quantified by extraction in 5 ml 0.5 M K_2SO_4 and counted using a scintillation counter as described previously.

4.2.5 Community level physiological profiling (CLPP) analysis

Carbon substrate mineralization in most soils has a biphasic pattern of CO_2 production (Oburger and Jones, 2009; Boddy et al., 2007). The initial rapid phase is attributable to the process of assimilation of low-molecular-weight (LMW) C compounds from the soil solution into microbial cells, and their subsequent catabolic respiration. The slower second phase approximates to anabolic processes; the synthesis of secondary metabolites within the microbial biomass, and turnover within the soil microbial community. In the current study, $^{14}CO_2$ respired during the initial 4 h following substrate addition was categorized as catabolic respiration, and the rate of catabolic ^{14}C respiration (expressed as mmol $kg^{-1}h^{-1}$) was derived from equation:

$$R = \left(\frac{a}{b}\right)/c$$

where R represents the rate of catabolic respiration, a is the cumulative amount of ^{14}C respired at the end of the catabolic respiration chase period, b is the quantity of ^{14}C added in the substrate at time 0 h, and c is the chase period for the initial catabolic respiration phase (4 h). The total amount of ^{14}C taken up by the soil microbial community and partitioned into catabolic and anabolic processes is described by the microbial yield, and is derived from the equation:

$$Y = A/(b - d)$$

where Y represents the microbial yield, A is the amount of ^{14}C in the microbial biomass, b is the quantity of ^{14}C added in the substrate at time 0 h, and d is ^{14}C bound to soil particles (^{14}C extracted by 0.5 *M* K_2SO_4).

This approach is acknowledged as a simplification of the soil metabolism system, as, *in vivo*, there are many dynamic C pools, with varying degrees of connectivity. However, the aim was to provide a summation for medium-term C pathways, and

despite the limitations, catabolic respiration rate and microbial yield provide a valuable estimation of C mineralization and therefore represent useful tools for comparing treatment effects on soil function (Olsen et al., 2010).

4.2.6 Soil community leaf litter mineralization

Mineralization rates of ^{14}C-labelled leaf litter from ryegrass (*Lolium perenne* L.) with an initial activity of 12.3 kBq g^{-1} were assessed. The leaf litter was labelled with $^{14}CO_2$ as described in Hill et al. (2007). 100 mg of ^{14}C-labelled leaf litter (0.02 kBq g^{-1} soil) was added to 1 g of soil in a 50 ml container with an airtight lid, then placed in an incubator at 10 °C. Mineralized $^{14}CO_2$ was trapped in 1 M NaOH as described previously. Traps were changed after 1, 3, 7, 14 and 28 days, 4 ml of Optiphase scintillation fluid (Wallac Optiphase 3 scintillation fluid (EG&G Ltd) was added to the NaOH trap and the ^{14}C content was determined using a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK). After 28 days, the amount of ^{14}C remaining in the soil, which was not assimilated into microbial biomass was quantified by extraction in 5 ml 0.5 M K_2SO_4 and counting using the scintillation counter as described previously.

4.2.7 Molecular analysis of the soil bacterial community

DNA extractions were performed using the method described by Griffiths and colleagues (2000). Nucleic acids were extracted from 0.25 g soil by bead beating with hexadecyltrimethylammonium bromide (CTAB) extraction buffer (Sigma-Aldrich, Dorset, UK) and phenol-chloroformisoamyl alcohol (Sigma-Aldrich, Dorset, UK). Total nucleic acids were then precipitated using polyethylene glycol 6000 (Sigma-Aldrich, Dorset, UK) and washed with ice-cold 70% ethanol (vol/vol).

Terminal restriction fragment length polymorphism (T-RFLP) analysis was carried out according to a method described in (Thomson et al., 2010) Briefly, 1 µl of 1:10 diluted extracted nucleic acids for total community analysis was used as a template for amplification using high-performance liquid chromatography (HPLC)-purified forward primer 63F- (5'-CAGGCCTAACACATGCAAGTC-3') labelled at the 5' end with D4 blue fluorescent dye (Sigma-Proligo, Dorset, UK) and reverse primer 530R (5'-GTA TTA CCG CGG CTG CTG-3') (MWG Biotech, London, UK). Following amplification, fluorescently labelled amplicons were purified using PureLink 96 PCR Purification kit, version B (Invitrogen). Subsequently, 3 µl purified PCR product, was

digested with 0.3 μl of restriction enzyme *Msp*I (New England Biolabs Inc., Ipswich, MA, USA). Subsequent fragment analysis was carried out using a Beckman Coulter CEQ 2000XL capillary sequencer (Beckman Coulter Corporation, California, USA). Resulting data were analysed by peak height analysis using the binning option within the Beckman Coulter CEQ 8000 software. Binning analysis parameters were set to analyse fragment sizes ranging from 50 to 500 nt with a repeat unit length of 1 nt and a minimum of one data point per bin. Relative abundances of amplicons were estimated as the ratio between the integrated fluorescence of each of the terminal restriction fragments (T-RFs) and the total integrated fluorescence of all T-RFs. (Thomson et al., 2010)

4.2.8 Statistical analysis

Analyses of O_3 and N effects on soil chemical properties and on C substrate mineralization were made using R Version: 2.12.0 analysis of variance, taking into account the split plot design of the experiment. Data were transformed where necessary to meet assumptions of normality and homogeneity of variance. Analyses were based on the value means within nitrogen treatment.

The relationship between soil bacterial T-RF profiles and explanatory variables (including soil chemical properties, microbial biomass C, Shannon's diversity index, and the applied ozone and nitrogen treatments) was analysed by redundancy analysis (RDA) in CANOCO (CANOCO, version 4.54, Plant Research International, Wageningen, the Netherlands). Redundancy analysis allows the direct analysis of soil bacterial diversity of the samples in relation to specific environmental variables (Ter Braak and Smilauer, 2002). For the RDA ordination, samples were classified into their ozone and nitrogen treatments. All canonical axes were tested for significant contribution to the explanation of the variation in the T-RF profile data with the Monte Carlo permutation test ($P<0.05$). The permutation scheme incorporated the split plot design of the experiment.

4.3 Results

4.3.1 Soil chemical properties

The only significant effect of long-term elevated O_3 and N inputs on soil chemical properties was on soil K content, which was decreased in plots subject to high levels of N injection ($P \leq 0.01$, Table 4.1).

Soil Property	Control O_3		Elevated O_3	
	Control N	High N	Control N	High N
pH	5.63 ± 0.07	5.72 ± 0.15	5.72 ± 0.10	5.71 ± 0.08
LOI (%)	17.62 ± 1.44	18.47 ± 0.79	20.97 ± 2.46	19.65 ± 1.64
Soil microbial C (mgC g^{-1})	1.09 ± 0.11	1.16 ± 0.10	1.21 ± 0.13	1.28 ± 0.12
Kjeldahl N (g kg^{-1})	5.82 ± 0.31	5.87 ± 0.28	6.15 ± 0.26	6.51 ± 0.27
Ammonium (mg g^{-1})	1.59E-02± 9.44E-03	1.56E-02± 1.91E-03	4.03E-03± 1.07E-03	2.39E-02± 5.85E-03
Nitrate (mg g^{-1})	1.22E-04± 3.65E-05	4.08E-04± 1.34E-04	1.98E-04± 5.34E-05	9.33E-04± 5.47E-04
Total Min N (mg g^{-1})	1.60E-02± 9.46E-03	1.60E-02± 2.00E-03	4.23E-03± 1.10E-03	2.48E-02± 5.92E-03
PO_4-P (mg g^{-1})	6.14E-03± 7.17E-04	4.67E-03± 5.06E-04	4.50E-03± 3.45E-04	4.13E-03± 2.63E-04
Na (meq $100g^{-1}$)	4.66E-02± 2.38E-03	4.20E-02± 3.52E-03	4.55E-02± 4.79E-03	4.59E-02± 5.36E-03
K (meq $100g^{-1}$) **	2.93E-01± 8.44E-03	2.31E-01± 1.63E-02	2.97E-01± 2.06E-02	2.48E-01± 1.34E-02
Ca (meq $100g^{-1}$)	5.82± 6.55E-01	6.37± 8.41E-01	7.73± 9.46E-01	6.18± 9.25E-01
Mg (meq $100g^{-1}$)	3.35± 5.45E-01	4.19± 8.64E-01	4.98± 1.01	3.77± 8.48E-01

Table 4.1 Soil chemical properties and microbial biomass C from soil samples collected at the Alp Flix free-air O_3 fumigation site in May 2009. Values are sample means within N treatment ± SE (n =6). ** indicates significant N effect ($P \leq 0.01$). With the exception of pH and LOI%, all soil property results are per g or per 100 g dry weight.

4.3.2 Community level physiological profiling (CLPP) and leaf litter mineralization

There were significant differences in the microbial yield and rate of catabolic respiration between soil samples with different ^{14}C substrates added to them (Tables 4.2, 4.3 and 4.4). However, O_3 and N treatments exerted limited statistically significant effects on soil microbial metabolism. The microbial yield from ^{14}C labelled oxalic acid and sucrose showed a significant interaction between O_3 and N effects ($P \leq 0.05$ and $P \leq 0.05$ respectively). Microbial yield was higher in plants exposed to high-O_3 and ambient-N treatment compared to those with high-O_3 high-N. The same O_3 * N interactive effect was observed in the catabolic respiration rate of salycilic acid ($P \leq 0.05$). O_3 significantly reduced the catabolic respiration rate of soils inoculated with ^{14}C labelled oxalic acid substrate ($P \leq 0.01$).

No significant effect of O_3 or N on the mineralization of ^{14}C-labelled ryegrass leaf litter (*Lolium perenne* L.) was evident throughout the chase period (Figure 4.1). At the end of the chase period (28 d), 22.3% ± 0.46 (SE) of the ^{14}C labelled substrate had been metabolized by the soil microbial community.

	Control O_3				Elevated O_3			
	Control N		High N		Control N		High N	
Substrate	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)
Amino acids								
Arginine	5.69E-01± 7.44E-02	1.51E-02± 1.39E-03	6.15E-01± 9.94E-03	1.71E-02± 1.73E-03	6.61E-01± 1.45E-02	1.46E-02± 7.94E-04	6.20E-01± 1.19E-02	1.69E-02± 8.60E-04
Aspartic acid	5.11E-01± 1.58E-02	9.39E-02± 3.63E-03	5.17E-01± 1.18E-02	9.36E-02± 3.38E-03	4.97E-01± 7.86E-03	9.85E-02± 1.39E-03	5.23E-01± 1.69E-02	9.17E-02± 3.06E-03
Glycine	6.03E-01± 8.00E-02	1.91E-02± 1.46E-03	6.20E-01± 1.86E-02	2.02E-02± 2.75E-03	6.60E-01± 1.45E-02	2.31E-02± 2.44E-03	6.28E-01± 1.58E-02	2.44E-02± 2.17E-03
Lysine	7.24E-01± 7.28E-03	2.15E-02± 8.61E-04	7.26E-01± 7.40E-03	1.99E-02± 9.33E-04	7.22E-01± 4.22E-03	1.92E-02± 1.89E-03	7.14E-01± 3.32E-03	2.13E-02± 6.17E-04
Phenylalanine	6.54E-01± 7.56E-03	2.19E-02± 5.94E-04	6.82E-01± 2.70E-02	1.94E-02± 2.22E-03	6.76E-01± 9.01E-03	2.02E-02± 9.93E-04	6.52E-01± 3.35E-03	2.28E-02± 5.07E-04
Valine	7.27E-01± 5.65E-03	3.95E-03± 4.10E-04	7.46E-01± 1.46E-02	3.73E-03± 4.14E-04	7.26E-01± 1.52E-02	3.63E-03± 2.80E-04	6.98E-01± 1.33E-02	4.10E-03± 3.31E-04

Table 4.2 ^{14}C amino acid substrate mineralization in the soil samples. Values are sample means within N treatment ± SE (*n*= 6).

	Control O_3				Elevated O_3			
	Control N		High N		Control N		High N	
Substrate	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)
Carbohydrates								
Fructose	8.06E-01± 6.34E-03	2.76E-02± 8.73E-04	8.15E-01± 1.12E-02	2.57E-02± 2.36E-03	8.18E-01± 3.59E-03	2.94E-02± 9.91E-04	8.06E-01± 5.03E-03	2.90E-02± 1.24E-03
Glucosamine	6.74E-01± 6.01E-03	4.77E-03± 2.38E-04	6.95E-01± 1.36E-02	4.29E-03± 2.62E-04	6.82E-01± 6.63E-03	4.69E-03± 2.17E-04	6.76E-01± 1.67E-02	4.82E-03± 3.53E-04
Glucose	7.24E-01± 9.74E-03	4.37E-02± 4.77E-03	7.39E-01± 1.40E-02	4.60E-02± 2.50E-03	7.29E-01± 5.75E-03	4.80E-02± 1.73E-03	7.10E-01± 7.75E-03	5.04E-02± 2.26E-03
Starch	7.03E-01± 1.62E-02	4.56E-02± 3.63E-03	7.21E-01± 1.12E-02	4.32E-02± 1.52E-03	7.44E-01± 1.52E-02	3.92E-02± 2.07E-03	6.94E-01± 8.25E-03	4.65E-02± 1.80E-03
Sucrose (**ab**)	8.10E-01± 5.61E-03	3.04E-02 ± 6.15E-04	8.25E-01± 9.05E-03	2.70E-02± 1.89E-03	8.26E-01± 9.21E-03	2.53E-02± 1.83E-03	8.15E-01± 4.26E-03	2.93E-02± 6.10E-04

Table 4.3 ^{14}C carbohydrate substrate mineralization in the soil samples. Values are sample means within N treatment ± SE (*n*= 6). Letters in parentheses next to the substrate name indicate degree of significance of treatment effect calculated by split-plot ANOVA, ab= O_3 x N interactive effect on microbial yield $P \leq 0.05$.

	Control O_3				Elevated O_3			
	Control N		High N		Control N		High N	
Substrate	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)	Microbial yield	Rate of catabolic respiration (mmol $kg^{-1}h^{-1}$)
Carboxylic acids								
Sodium acetate	6.90E-01± 3.77E-02	3.20E-02± 6.68E-03	6.99E-01± 8.56E-03	3.31E-02± 6.15E-03	7.06E-01± 1.99E-02	3.68E-02± 6.42E-03	6.79E-01± 1.25E-02	3.73E-02± 3.95E-03
Malate	5.67E-01± 1.09E-02	7.12E-02± 2.94E-03	5.51E-01± 1.24E-02	7.38E-02± 5.14E-03	5.97E-01± 2.39E-02	6.60E-02± 8.96E-03	5.47E-01± 7.95E-03	8.00E-02± 4.56E-03
Oxalic acid (**ab, C**)	1.75E-01± 1.32E-02	4.28E-02± 2.93E-03	2.67E-01± 6.99E-02	4.31E-02± 2.87E-03	2.61E-01± 4.80E-02	3.58E-02± 5.21E-03	1.98E-01± 1.70E-02	4.25E-02± 4.61E-03
Salicylic acid (**AB**)	1.19E-01± 1.17E-02	4.26E-03± 2.82E-04	1.24E-01± 1.71E-02	4.46E-03± 4.35E-04	1.22E-01± 9.60E-03	4.73E-03± 4.48E-04	1.63E-01± 1.44E-02	4.41E-03± 9.76E-04
Succinic acid	9.03E-01± 2.03E-02	7.26E-03± 1.60E-03	8.97E-01± 7.00E-03	7.97E-03± 6.10E-04	9.09E-01± 1.61E-02	6.18E-03± 1.42E-03	8.73E-01± 8.15E-03	9.55E-03± 7.29E-04

Table 4.4 ^{14}C carboxylic acid substrate mineralization in the soil samples. Values are sample means within N treatment ± SE (*n*= 6). Letters in parentheses next to the substrate name indicate degree of significance of treatment effect calculated by split-plot ANOVA, ab= O_3 x N interactive effect on microbial yield $P \leq 0.05$, AB= O_3 x N interactive effect on the rate of catabolic respiration $P \leq 0.05$, and C= O_3 effect on the rate of catabolic respiration $P \leq 0.01$.

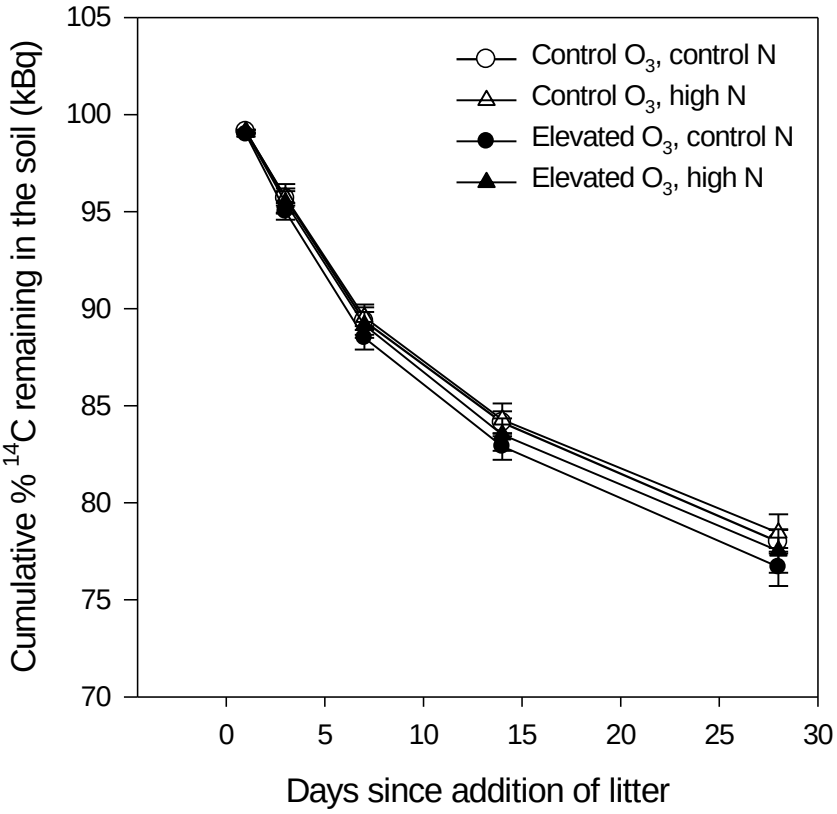


Figure 4.1 Cumulative % ^{14}C remaining in the soil with time, after addition of ^{14}C-labelled ryegrass leaf litter (*Lolium perenne* L.). Values are sample means within N treatment ± SE (*n* =6).

4.3.3 Molecular analysis of the soil bacterial community

There were no significant effects of O_3 or N treatment on the T-RF profiles of the soil samples, and no clear grouping of samples was evident when the relationship between T-RF profiles and explanatory variables was analysed by redundancy analysis (RDA) (Figure 4.2). The cumulative percentage variance explained in the first and second axes by the species data and by the species-environment relationship was 47.8% and 64.5%, respectively. The canonical axes were not significant when tested together using the Monte Carlo test of significance (F ratio= 3.95, *P*= 0.11). Although not statistically significant, the variables which explained most of the variation in the samples were soil Mg (meq 100 g^{-1} dry soil), Ca (meq 100 g^{-1} dry soil), and pH . The regression of soil pH with the RDA axis 1 scores is shown in Figure 4.3 (r^2= 0.39, not statistically significant at the 5% level).

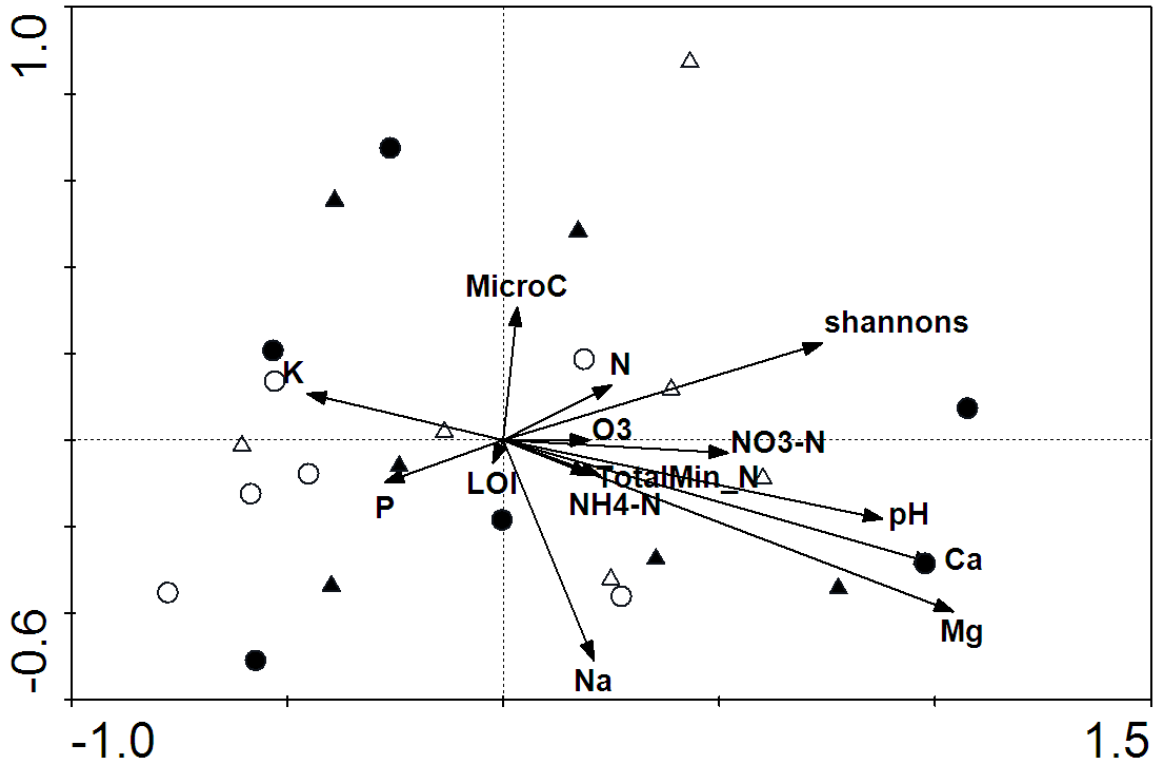


Figure 4.2 Redundancy analysis (RDA) of T-RF profiles and explanatory variables. Open symbols represent samples from control O_3 plots, filled symbols represent samples from elevated O_3 plots. Circles represent samples from low N plots; triangles represent samples from high N plots.

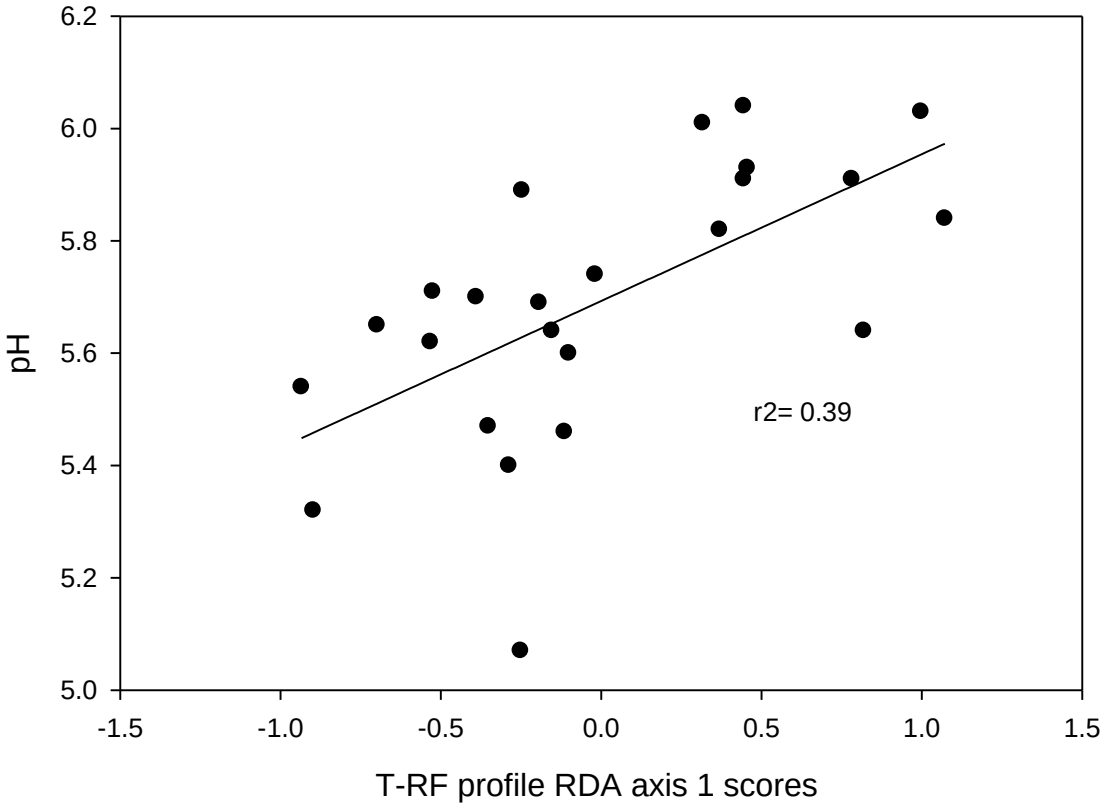


Figure 4.3 Scatter plot of T-RF profile RDA axis 1 scores vs pH. r^2 of regression shown on graph.

4.4 Discussion

Soil microbial communities drive fundamental ecosystem processes, such as decomposition and nutrient cycling (Garland, 1997). Ozone pollution, or increased N deposition will affect the quality and/or quantity of plant derived C input to the rhizosphere and below-ground C pools (Andersen, 2003). There are several C pools and fluxes which are likely to change under the influence of these environmental variables including root biomass, rhizodeposition, root turnover, litter loads and litter chemistry (Andersen, 2000). Long-term changes in root growth may also cause changes in the soil structure and moisture content, which may have consequences for soil microbial population size and species composition (Torsvik and Øvreås, 2002).

While O_3 pollution is highly unlikely to affect soil properties or bacterial communities directly (Andersen, 2003), accelerated senescence of above ground plant parts may increase leaf litter, with the potential to boost soil organic matter (SOM) input. However, as observed in many species, exposure to O_3 pollution reduces C allocation below-ground, and increases the shoot : root ratio (Cooley and Manning, 1987; Pell et al., 1994; Grantz et al., 2006). It was not possible to harvest the roots in the present study, and thus the implications of potential effects of long-term ozone exposure on soil properties and function require further exploration. The impact of N deposition on soil chemistry and microbial communities has been more widely studied (eg. Carreiro et al., 2000; Lilleskov et al., 2002; Nemergut et al., 2008; RoTAP, 2011b), with soil acidification (Vitousek et al., 1997), shifts in the ratio of fungi, bacteria and archaea (e.g. Bradley et al., 2006; Strickland and Rousk, 2010), and reduced capacity for decomposition of lignin-rich litter (Waldrop et al., 2004) among the most widely reported consequences. How (semi-)natural grasslands, and their soils, respond to the combined influence of O_3 and N, is an important question to consider, as global atmospheric chemistry models predict rising levels and the co-occurrence of these pollutants over much of the UK and Europe during the course of this century (Dentener et al., 2006).

In the current study on slow-growing alpine vegetation, there were marginal effects of O_3 and/or N on soil after 5 years of treatment. This result is possibly not unexpected since N may be taken up and rapidly immobilized by plants and the soil microbial biomass, with the main impact likely to be the increased growth and

dominance of N-tolerant species. It may take many decades before the effects of increased levels of reactive N are noticeable in the environment, due to the large soil organic matter C and N pools, and the excellent buffering capacity of many soils (Rowe and Hellsten, 2005; Emmett, 2007). The total soil N pool is largely inactive (Tamm, 1991) and so is not a good indicator of changes in N over the time scale of this experiment. Increased ammonium in the soil can interfere with potassium uptake by the roots (e.g. Topa and Jackson, 1988) and this process may explain the slightly higher potassium levels observed in high N treated plots.

The difference in rate of catabolic respiration and microbial yield from soils receiving different ^{14}C substrates is consistent across many studies (e.g. Boddy et al., 2007; Jones and Murphy, 2007; Olsen et al., 2010), and reflects the diverse pathways of assimilation and metabolism associated with different compounds within the microbial cells (Jones and Murphy, 2007; Oburger and Jones, 2009). In general, carboxylic acids and sugars were metabolized faster than amino acids (with the exception of aspartic acid, salycilic acid, succinic acid and glucosamine). In a study conducted by Grayston and colleagues (1998), rhizosphere microbial communities from individual plant species were demonstrated to utilize different quantities of carbohydrates, carboxylic acids and amino acids on Biolog plates. This suggests that soil bacteria and fungal populations adapt to the specific root exudates from plants. The limited effect of O_3 and N on the metabolism of labelled root exudate compounds observed in the Swiss soils was perhaps not surprising given the minor impacts on plant species composition above-ground (Bassin et al., 2007b). The main interactive effect of O_3 and N treatment was an increase in microbial yield with high-O_3 in ambient-N plots, but a decrease in high-O_3 high-N plots with the addition of sucrose and oxalic acid. The same interaction was observed for the rate of salycilic acid catabolic respiration. The only significant independent O_3 effect was a reduction in oxalic acid catabolic respiration in high O_3 treated plots. The mechanism behind these results cannot be clearly defined in the current study. However, reductions in microbial yield and low molecular weight (LMW) carbon substrate utilization in soils under combined O_3 and N indicate potential changes in some soil processes. Additional research employing ^{13}C labelled substrates found that LMW root exudate compounds were used by a wide range of the soil microbial community across varying soil types (Waldrop and Firestone, 2004; Paterson et al., 2008). Furthermore,

Waldrop and Firestone (2004) demonstrated that the microbial community responsible for the decomposition of more recalcitrant C compounds was more specific to soil and ecosystem type. Many soil organisms are metabolically capable of utilizing a specific C source, and equally, a particular bacteria or fungi may be important to a number of soil processes; therefore the degree of functional redundancy in soil microbes is considerable (Jones and Murphy, 2007). This suggests that only a large plant-mediated change in soil C input from rhizodeposition, or indeed a significant alteration in the composition and quantity of recalcitrant C (e.g. through plant litter and root turnover changes), would induce a measurable change in the metabolism of soil C pools. It is likely the case that soil processes are altered as a function of the relative activities of the component microbial populations rather than the presence or absence of individual species (Paterson et al., 2009). With regards to potential effects of increased N on the turnover of soil C pools, an investigation by Currey and colleagues (2010) revealed that labile pools were turned over faster with N input, whereas the activity of enzymes involved in the breakdown of recalcitrant C (*N*-acetyl-glucosaminidase and cellobiohydrolase) were decreased. The present study indicated little increase in labile C metabolism with high N with any significant effects confined to ambient O_3 plots.

The breakdown of litter will likely be more complex than for LMW carbon compounds. However, Vaieretti et al. (2005) found that leaching of LMW compounds from solid plant material into the soil solution causes a rapid phase of CO_2 production, and that the more recalcitrant carbon compounds such as cellulose, hemi-cellulose and lignin are mineralized in a slow second phase incorporating assimilation into the microbial biomass, and microbial turnover in the soil. The pool of labile C estimated to be available from the ^{14}C-labelled ryegrass (*Lolium perenne* L.) leaf litter was approximately 37%, based on fractions that were extractable in water and ethanol (Olsen et al., 2010). It was hypothesised that high N would retard the turnover of litter in the current study, as discussed by Berg and Meentemeyer (2002). However, there was no indication of differences in the use of this C pool by soils from the different treatments in the present study.

There was very little variation in bacterial diversity between treatments (Shannon index 3.45 ± 0.03 SE.). The small level of variation evident in the T-RF profiles was best explained by soil chemical properties such as Mg, Ca, and pH. Although not

significant, the trend for soil bacterial diversity to change with pH is consistent with other studies in grassland soils (Fierer and Jackson, 2006; Cookson et al., 2007; Rousk et al., 2010). Soil chemistry, and in particular pH, has been previously reported to control soil microbial diversity and the decomposition of labile soil organic C (Leifeld et al., 2008). During a free air CO_2 and O_3 enrichment experiment on aspen (*Populus tremuloides* Michx.) in Wisconsin, USA, fungal sporocarp production and community structure was reported to be changed during the course of a four year study (Andrew and Lilleskov, 2009). Similarly, Kanerva and co-workers (2008) went on to show that the structure of the microbial community was altered, and soil fungal and bacterial biomass had decreased in meadow mesocosms exposed to O_3 after three growing seasons. Conversely, soil from the rhizosphere of beech trees grown in an elevated ozone environment had an increased microbial biomass compared to controls (Esperschutz et al., 2009).

There is some discrepancy in the literature concerning the level of influence particular plant species litter exerts on decomposition rates and microbial diversity in soil (Ayres et al., 2006). A number of studies demonstrate that the species of plant affects soil microbial community structure (Bardgett et al., 1999) and function (Grayston et al., 1998) via root exudation. Hossain and colleagues (2010) showed that N content of litter directly influences the rates of N mineralization and the relative dominance of bacteria or fungi on litter samples. Litter from semi-natural grassland is richer in fungi and actinomycetes compared to improved grasslands (receiving yearly input of chemical fertilizers). However, the increase in sedge species biomass and cover with N treatment (Bassin et al., 2007b) was not enough to cause a shift in soil function or diversity at the Alp Flix site in the present study. The fungal: bacterial ratio of soil samples was not investigated in the current study - this may have been influenced by N treatment and is worthy of further exploration.

The lack of change observed in the microbial biomass and diversity between O_3 treatments, is likely due to the stability and resistance of the aboveground plant community (Bassin et al., 2007b; Bassin et al., 2009); and is in line with results from an open top chamber experiment on soil bacterial communities in O_3 tolerant and O_3 sensitive grassland species (Dohrmann and Tebbe, 2005). Other studies which observed decreases in microbial biomass attributed the effect to reduced above and

below-ground plant biomass with elevated O_3 (Kanerva et al., 2008) and plant mediated changes in the rhizosphere (Chen et al., 2009).

Soil microbial diversity and functioning varies throughout the year and is affected by temperature, soil moisture and the quantity of plant derived C entering the soil (Grayston et al., 2001; Lipson et al., 2002; Avramides et al., 2009). Research into the effects of elevated O_3 on soil microbial communities in a rice paddy, revealed that significant changes in the microbial diversity occurred only during plant flowering, suggesting a temporal link between rhizosphere microbes and plant activity (Chen et al., 2010). As soil sampling in the current study was conducted very early in the growing season in the Swiss Alps, our measurements may not represent the potentially larger O_3 and N effects on rhizodeposition during peak summer.

The rate of amino acid mineralization has been shown to decrease with soil depth in three unfertilized sites in the UK (Kemmitt et al., 2008), and Chen and colleagues (2009) reported significant differences in the rhizosphere soil microbial biomass of wheat seedlings, but not bulk soil. Soil cores in the current study were taken within the rooting depth of the plant sward, and by mixing the soil from 0 cm to 15 cm depth, any variation in the microbial function and/or diversity with depth was controlled, providing an integrated view of any potential treatment effects on bulk soil microbial communities and function.

4.5 Conclusions

The present study reports the effects of 5 years' exposure to elevated O_3 and/or N on the soil supporting a species-rich subalpine grassland. Microbial biomass was not affected by O_3 and/or N, and the study revealed a limited impact of the pollutants on soil function (investigated by Community Level Physiological Profiling, CLPP). Only the turnover rates of three of the sixteen ^{14}C labelled compounds agreed with the hypothesis that O_3 pollution reduces labile C metabolism and that turnover is accelerated by high N inputs. Soil microbial diversity (quantified by Terminal Restriction Fragment Length Polymorphism, T-RFLP) was apparently unaffected by long term exposure to elevated N inputs and this mirrored the lack of plant community responses to the treatments reported by Bassin and colleagues (2007b). Overall this study suggests that sub-alpine grassland soil systems maybe strongly buffered against (i.e. resistant to) the impacts of rising ground level ozone concentrations and N inputs.

Chapter 5. Ozone and nitrogen effects on the productivity and community composition of a species-rich grassland after 3 years of treatment.

5.1 Introduction

Ecosystems are currently under pressure from land-use, climate change, and atmospheric pollutants, all of which can contribute to reductions in biodiversity, changes in ecosystem function, and impact negatively on the ability of ecosystems to adapt to future environmental pressures (Sala et al., 2000). Grasslands cover approximately 40% of global landscapes, and are important for conservation of biodiversity, carbon sequestration and in many cases agricultural use (Olson, 1994). The recent UK-DEFRA commissioned Review of Transboundary Air Pollution (RoTAP) (2011) indicates that both elevated tropospheric ozone (O_3) and nitrogen deposition (N) have the potential to reduce the conservation value of sensitive priority habitats, but detailed knowledge is lacking in this subject area. As a consequence, the present study employed free-air fumigation of an established mesotrophic upland hay-meadow at Keenley Fell, Northumberland, UK, to explore impacts in the field and cocnsistency of effects with the prescriptive management regime employed under the UK's Higher Level Stewardship Scheme (HLS) for the creation, maintenance and restoration of species-rich semi-natural grassland. As discussed in previous chapters, rural areas are prone to elevated tropospheric O_3 and N deposition due to the emission of precursor compounds from anthropogenic sources (Coyle et al., 2002). Ozone is known to reduce plant productivity and alter species composition of plant communities in both mesocosm and field studies (e.g. Nussbaum et al., 2000; Thwaites et al., 2006). However, few chamberless experimental O_3 fumigations have been undertaken on intact grasslands *in situ* (Wedlich et al., 2011).

The effects of nitrogen deposition on plants and soil have been widely investigated in the past few decades (Dupre et al., 2010). Fertilization, soil acidification, biodiversity

loss and species composition change are the most frequently reported consequences of increasing N input to (semi-)natural ecosystems (Achermann and Bobbink, 2003; Maskell et al., 2010; Hall et al., 2011; Stevens et al., 2011). The question remains as to what the combined effects will be of rising background [O_3] and N inputs on grassland communities (Bassin et al., 2007a). There are likely to be antagonistic effects on above ground growth, with O_3 reducing productivity (as demonstrated in the meta-analysis by Hayes and colleagues (2007)), and N promoting productivity (e.g. Achermann and Bobbink, 2003). However, variation in the response of species from different functional groups is to be expected (Mills et al., 2007). Legume cover and diversity was shown to decline with increasing [O_3] in a study by Volk and colleagues (2006), and both species richness and cover of forbs declined strongly with increasing N deposition in UK acid grasslands (Stevens et al., 2006). In a recent study conducted in parallel with the work reported in this thesis at the Keenley Fell free-air O_3-enrichment site, Wedlich and colleagues (2011) reported significant O_3-induced reductions in herb biomass, and changes in the species composition of the herb and legume components of the sward. The experiment reported here focused on the combined influence of N deposition and elevated O_3 on species composition and biomass of plant functional groups. Recent research in the literature indicates the potential for O_3 and N to cause synergistic negative effects on C allocation to roots (Sanz et al., 2005), with the potential for alteration of the microbial biomass, and C turnover in soils (Andersen, 2003; Kanerva et al., 2008; Treseder, 2008). But, secondary impacts of combined O_3 and N exposure on below-ground biomass in field based experiments are rarely quantified (See Bassin et al. , 2007b).

In this study, the impacts of three years of exposure to elevated O_3 and/or N on productivity (above and below-ground) and species composition of an upland species-rich haymeadow were studied using a free-air fumigation facility situated at Keenley Fell, Northumberland, UK.. The hypotheses tested were i) O_3 and N exert antagonistic influences on above-ground productivity; O_3 reducing above-ground biomass, whereas N promotes above-ground growth. ii) The relative proportions of the plant functional groups (i.e. grasses, forbs and legumes) will be altered by the combined influence of O_3 and N, with grasses increasing in dominance to the

detriment of forbs and legume species biomass and cover. iv) Increased O_3 and N will exert synergistic negative effects on below-ground biomass.

5.2 Methods

5.2.1 Field site

The study site at Keenley Fell, Northumberland, UK (54°9'N, 2°32'E), is located 360 m above sea level, on mesotrophic species-rich hay meadow, with predominantly silty-clay soils (Wedlich et al., 2011). The meadow is managed under the Higher Level Stewardship Scheme (HLS), which involves a cut in early autumn (once a year) to a height of approximately five cm. Sheep are grazed on the site in October/November and again for a defined perioid in early spring, to open-up the sward. The site receives one farmyard manure treatment per annum at a maximum load of 12.5 t ha^{-1}. This management plan was superimposed in the current study by a cutting and nitrogen treatment regime introduced on experimental plots in January 2008. The plots are excluded from sheep grazing and the yearly meadow cut using 1.2 m^2 domed wire exclusion cages.

5.2.2 Ozone and nitrogen application

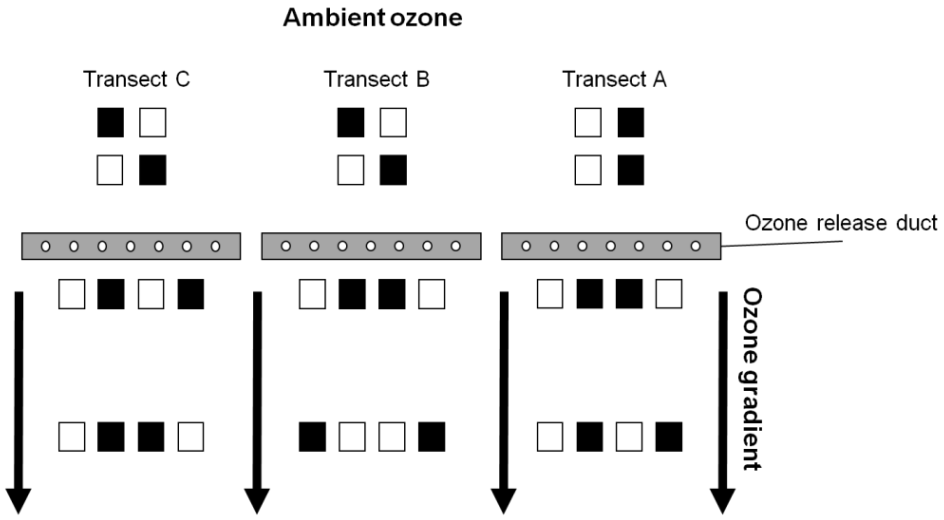


Figure 5.1 Schematic diagram of experimental design at the Keenley Fell free-air O_3 fumigation site. White squares are low nitrogen 1.2 m^2 plots and black squares are high nitrogen 1.2 m^2 plots (not to scale).

The ozone fumigation system at Keenley Fell is described in detail by Wedlich and colleagues (2011). Briefly, the system comprises three 'replicate' ozone fumigation transects (A, B and C). Ozone is generated from pure O_2 by electrical discharge, and released gradually from a 6 m long pipe within each transect when prevailing winds are between 180° and 270° (SW) and exceeding 0.3 m s^{-1}. Limiting fumigation to these conditions ensured the controlled dispersion of O_3 across the experimental plots. The level of O_3 fumigation was increased by 50% in August 2008, due to lower than anticipated exposure during the early part of this year. The O_3 concentrations are sampled from 10 m upwind of the release pipe (ambient plots), and 10 m downwind in each transect, sequentially (on a 12 min cycle). Ozone montors (UV monitors calibrated at weekly intervals against a unit in turn calibrated against a US_EPA-approved source) controlled the release of O_3 via a LabView computer software control system, with the aim to maintain [O_3] ~20-30 ppb above ambient at a distance of 10 m downwind of the release pipes. Duplicate passive (integrating) sampling devices (Gradko, UK) were employed to monitor the O_3 concentrations at several points along the gradient in each transect throughout the year. Passive samplers were exposed for 2-4 week periods and the cumulative dose over that

period was quantified. Co-location of the passive samplers with the four sites of active monitoring by the control system was used to calibrate the passive samplers under the local conditions (Wedlich et al., 2011).

The current study consisted of 36 experimental plots receiving O_3 and nitrogen treatments in a split plot design. Each transect comprised four plots receiving ambient ozone (10 m upwind of ozone release), four plots receiving high ozone (< 1 m downwind of the release pipe), and four plots receiving low ozone (10 m downwind of the release pipe), see Figure 5.1. Each plot was 1.2 m^2 and all measurements were recorded from a central 1 m^2 quadrat, to negate possible edge-effects. Nitrogen treatments were randomly assigned to 2 plots within each ozone treatment in each transect. Nitrogen applications commenced in January 2008 and continued on a monthly basis representing 5 L of NH_4NO_3 in rain water (high N) or 5 L rain water (low N) which equated to N treatments superimposed on a background level of N deposition at the field site of 22 kg $ha^{-1}year^{-1}$ and 72 kg $ha^{-1}year^{-1}$.

5.2.3 Species composition surveys

The percentage cover of individual plant species was recorded in August 2008 and 2010 using a 1 m^2 quadrat placed in the centre of each experimental plot. The Shannon-Wiener diversity index (H') was derived according to the following:

$$H' = \sum_{i=1}^{s} (Pi\ lnPi)$$

(s, species number; Pi, relative abundance of the ith species). Shannon-Wiener diversity index takes into account the number of individuals as well as number of taxa. H' varies from 0 for communities with only a single species to high values for communities with many species, each with few individuals. The dominance index (D) was derived from the following equation:

$$\mathrm{D} = \sum i \left(\frac{n_i}{n}\right)^2$$

(n_i is number of individuals of taxon i). The dominance value ranges from 0 (all species are equally present) to 1 (one species dominates the community completely).

Cover weighted Ellenberg values adjusted for British plants (Hill et al., 1999) were calculated for each plot. Ellenberg values provide an indicator of a plants realized niche and can help to interpret differences in species composition based on a species preference in terms of moisture (CWF), acidity (CWR), and nutrient level (CWN) (Hill et al., 1999). Four species of *Agrostis* were recorded in the plots. *Agrostis capillaris* was clearly identifiable in the field, however the cover of *Agrostis stolonifera*, *Agrostis gigantea* and *Agrostis canina* were combined for analysis due to higher levels of uncertainty. The combined *Agrostis* group is labelled *Agro_sg*.

5.2.4 Above-ground biomass of functional groups

Plants were cut from a central 50 cm^2 quadrat at 5 cm above the surface once each year in Aug 2008, 2009 and 2010. Plant material was stored at 4 °C until separation into functional groups (grasses forbs and legumes). Samples were dried at 60 °C to constant weight. The remainder of each plot was cut to 5 cm and removed to simulate a hay cut.

5.2.5 Below ground biomass

Soil cores were taken in August 2010 directly after the vegetation cut. Two soil cores (5 cm diameter, 15 cm depth) were taken at random positions within each plot. Each core was halved lengthways; one half was used for soil property analysis, and the other for root biomass determination. Half cores from each plot were bulked for the respective analysis. Root biomass was determined by washing in a sieve to collect fine plant roots and samples were dried at 60 °C to constant weight.

5.2.6 Soil property analysis

In 2008, 15 cm depth soil cores (two per plot, bulked and thoroughly mixed) were used to quantify soil moisture, loss on ignition (LOI), electrical conductivity (EC), and pH. In 2010 soil cores were taken as described previously in section 5.2.5. Moisture content and loss on ignition (LOI) were assessed using field moist soil within 10 days of collection by oven drying at 105 °C and 375 °C respectively. The remainder of each soil sample was air dried and ball milled before subsequent analysis. Soil pH (De-ionised H_2O) was measured using a Corning 220 pH meter (Avery, 1974), and soil conductivity (EC) quantified using Jenway 4320 EC meter, (cell constant 0.92) (Avery, 1974). Kjeldahl N was measured using a Tecam OG-1 Block digester and 2300 Kjeltec Analyzer Unit (Foss Tecator v1.14 1997). Ammonium and nitrate were

determined from M KCl extractions according to (Page, 1982) and analysed using SEAL AQ2+ Automated discrete analyzer. Na, K, Ca, and Mg were determined from 0.5 M NH_4Cl extractions, analysed using the Perkin-Elmer Atomic Absorption Spectrophotometer. Phosphate (PO_4-P) was analysed at 880 nm by continuous flow colorimetry using a Skalar Autoanalyzer, phosphomolybdate-blue method.

5.2.7 Statistical analysis

To assess any variation in selected soil properties, species diversity, and root biomass due to O_3 and/or N treatment, analysis was carried out in Genstat (version 10, VSN International Ltd.) using split-plot analysis of variance. Transect was included as 'block', ozone treatment was analysed at the whole-plot level, and nitrogen treatment was nested within ozone treatment. The same method was used to assess changes in the percentage cover of each individual species due to O_3 and/or N treatment. Due to large heterogeneity in species cover across plots, only species with at least 80 % constancy were included. Data were transformed (where necessary) to meet assumptions of normality and homogeneity of variance.

To characterize variation in species cover near the beginning of O_3 and N treatments (August 2008), an unconstrained analysis was executed using principal components analysis (PCA) in CANOCO (CANOCO, version 4.54, Plant Research International, Wageningen, the Netherlands), including pH, loss on ignition (LOI), soil moisture, (MOIS), electrical conductivity (EC), ozone (O_3), nitrogen (N), cover weighted Ellenberg acidity (CWR), cover weighted Ellenberg moisture (CWF) and cover weighted Ellenberg nutrients (CWN) as explanatory variables. A redundancy analysis (RDA) was then run in CANOCO, allowing direct analysis of vegetation composition in relation to specific environmental variables (Ter Braak and Smilauer, 2002). All variables except for O_3, N and CWN were tested for significant contribution to the explanation of the variation in the vegetation composition data using the Monte Carlo permutation test ($P \leq 0.05$) associated with the forward selection subroutine in CANOCO. Permutations were conditioned on the split-plot design of the experiment. To investigate if a shift in vegetation community composition had occurred after three years of elevated O_3 and N exposure (August 2010), RDA was run in CANOCO including all explanatory variables (O_3, N, CWR, CWN, CWF, soil properties measured in 2010: K, Mg, Ca, Na, NO_3, NH_4, MOIS, LOI, Kjeldahl N and pH), and taking account of the split-plot experimental design. Cover weighted Ellenberg

moisture (CWF) and cover weighted Ellenberg acidity (CWR), were included as covariates in the RDA, due to their significant contribution to the underlying variation in species cover of the plots (identified by the 2008 PCA results, see Table 5.3). The RDA was then re-run and O_3 and N were tested for significant contribution to the explanation of the variation in the vegetation composition data with the Monte Carlo permutation test ($P \leq 0.05$) associated with the forward selection subroutine in CANOCO.

5.3 Results

The O_3 exposure measured by active UV monitoring during the peak growing period - May, June and July in 2008, 2009 and 2010 is shown in Table 5.1. In 2008, the average [O_3] at 10 m was elevated by 3 ppb, 2 ppb and 3 ppb in transects A, B and C respectively. The level of fumigation increased in 2009, and in 2010 the [O_3] at 10 m averaged 6 ppb 5 ppb and 5 ppb above ambient in transects A, B and C respectively. There was no fumigation between September 2008 and January 2009 owing to a combination of snow cover and systems failure (Wedlich et al., 2011). Between March and August 2008, the wind direction was within the appropriate sector 27% of the time, and fumigation was achieved 18% of the time. Due to the modest increase in O_3 exposure achieved during 2008 as a whole, measurements taken in this season were considered suitable as a baseline, and used to inform the analysis of data taken in subsequent seasons. Figure 5.1 shows the average ambient O_3 concentration during May, June and July in 2010, recorded using duplicate passive diffusion samplers along the gradient in transect A. The O_3 concentration at 1.5 m was extrapolated from the measured values using a 5^{th} order polynomial regression.

	Mean ppb				Mean AOT40			
	Ambient	A	B	C	Ambient	A	B	C
2008	27.93	30.94	29.92	30.52	0.22	2.04	1.21	1.41
2009	32.15	36.72	34.61	34.57	1.24	4.87	2.90	2.88
2010	27.30	33.40	32.18	32.21	0.47	3.84	2.10	2.03

Table 5.1 Ozone fumigation during the peak growing-season (May, June and July) of 2008, 2009 and 2010. Ambient O_3 measurements were taken 10 m upwind of the ozone release pipes and 10 m downwind of the release pipes in transects A, B and C by UV absorption spectroscopy, with sequential sampling from the three transects and ambient air on a 12-min cycle. Mean [O_3] represents the average O_3 concentration over 24 h. Mean AOT40 represents hourly average data where total solar radiation > 50 W m^{-2}

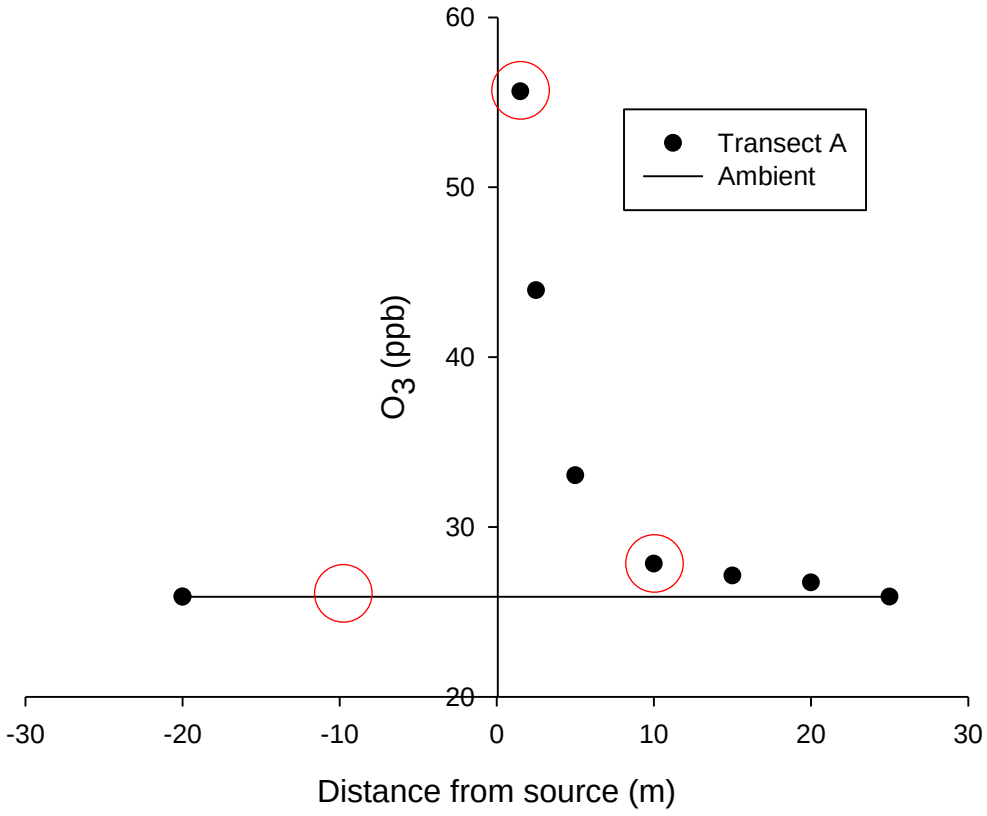


Figure 5.2 Average O_3 concentrations for May, June, July at the Keenley Fell free-air fumigation site in 2010 (quantified by duplicate passive diffusion samplers).The O_3 concentration at 1.5 m was extrapolated from the measured values using a 5 order polynomial regression $R^2 = 1$. Plots indicated by red ellipses are those included in the current study (ambient O_3: -10 m, high O_3: 1.5 m, low O_3: 10 m).

Soil property	Ambient O_3		Low O_3		High O_3		Treatment effect
	Low N	High N	Low N	High N	Low N	High N	
2008							
pH	5.73± 0.17	5.67± 0.12	5.63± 0.08	5.56± 0.04	5.73± 0.05	5.73± 0.05	ns
EC	27.50± 6.75	18.00± 1.29	15.83± 0.95	17.33± 3.21	22.33± 3.72	22.33± 1.89	ns
Soil moisture %	69.83± 5.58	67.86± 3.93	56.16± 1.89	56.70± 4.30	66.67± 3.12	67.27± 4.20	ns
LOI %	16.45± 1.14	16.37± 0.88	16.30± 1.80	14.61± 1.53	16.75± 0.92	16.22± 1.39	ns
2010							
pH	5.21± 0.03	5.12±0.07	5.22±0.04	5.14±0.07	5.25±0.03	5.29±0.06	ns
Soil moisture %	30.15±1.04	28.45±1.56	24.72±1.07	24.80±0.88	31.39±1.07	31.02±1.81	**
LOI %	13.27±0.65	13.70±1.13	11.68±0.53	11.61±0.75	14.61±1.02	15.22±1.47	ns
NO_3 (mg g^{-1})	5.72E-03± 3.21E-03	1.85E-02± 9.28E-03	6.46E-03± 2.22E-03	6.00E-03± 2.98E-03	1.79E-02± 3.59E-03	1.73E-02± 3.46E-03	ns
NH_4 (mg g^{-1})	2.00E-02± 1.76E-03	2.57E-02± 3.17E-03	1.97E-02± 3.39E-03	1.93E-02± 2.30E-03	1.74E-02± 1.39E-03	1.96E-02± 2.53E-03	ns
Na (meq $100g^{-1}$)	0.18± 0.02	0.15± 0.01	0.16± 0.02	0.16± 0.01	0.18± 0.01	0.18± 0.02	ns
K (meq $100g^{-1}$)	0.24± 0.02	0.28± 0.02	0.25± 0.06	0.22± 0.02	0.22± 0.02	0.22± 0.03	ns
Ca (meq $100g^{-1}$)	33.44± 3.08	30.59± 4.49	35.57± 6.10	30.08± 3.20	40.32± 3.19	40.32± 4.26	ns
Mg (meq $100g^{-1}$)	3.49± 0.21	3.62± 0.44	3.66± 0.56	2.98± 0.24	3.73± 0.19	3.03± 0.22	ns
Kjeldahl N %	0.61± 0.05	0.67± 0.04	0.54± 0.05	0.54± 0.05	0.64± 0.07	0.67± 0.04	**

Table 5.2 Soil properties from Keenley Fell free-air fumigation experimental plots in 2008 and 2010. Values are means ± SE (*n*=6). Effects of ozone denoted as follows: ns $P \geq 0.05$, ** $P \leq 0.01$.

Table 5.2 shows soil properties of the plots measured in 2008 and 2010. No potentially confounding gradients in the measured soil properties were found in 2008, although there was a trend for soil moisture to be lower in the plots 10 m downwind of the O_3 release pipes. Soil moisture was found to be significantly lower in these plots in 2010 ($P \leq 0.01$). Kjeldahl N% was also lower in plots receiving low elevated O_3 in 2010 ($P \leq 0.01$). However, Kjeldahl N was not measured in 2008, and therefore it is not possible to conclude whether this was due to an underlying gradient in the soil properties at the site, or a true effect of the treatments applied to the plots.

Individual plant species cover was heterogeneous between the plots in 2008 (baseline), and varied in relation to their position in the O_3 gradient (see Table 5.3). In both 2008 and 2010, there was the suggestion of a competitive interaction between the dominant grasses *Agrostis capillaris* and *Holcus lanatus* with the percentage cover being lower and higher respectively in the plots receiving high O_3 ($P \leq 0.01$ and $P \leq 0.05$ for both species in 2008 and 2010 respectively). There was consistently higher cover of *Festuca pratensis* in the ambient O_3 plots in 2008 and 2010 ($P \leq 0.05$, 2010), whereas in 2010, *Dactylis glomerata* cover was significantly higher in the low and high O_3 plots ($P \leq 0.05$). *Ranunculus acris* was the most common forb in the sward with a significant negative correlation in terms of percentage cover in plots with high O_3 exposure in both the baseline year and 2010. There were no significant effects of O_3 or N on either the Shannon-Wiener diversity index or the Dominance index in 2008 and 2010 (Table 5.2).

In order to examine the structure of the community as a whole and to take account of other measured factors which may have influenced the species composition of the experimental plots (2008 and 2010 data), as well as the effects of O_3 and N treatment (in 2010 data only), multivariate analysis was performed. Figure 5.3A shows separation of the experimental plots on the PCA ordination by their position in the O_3 gradient in 2008. Plots next to the O_3 release pipes (high O_3 exposure) are positioned to the right of the ordination plot, and those in the ambient and low-O_3 areas of the site are to the left of the ordination plot. As discussed previously, the trend for a soil moisture gradient from wet to dry (ambient O_3 plots> High O_3 plots> low O_3 plots) was evident in 2008. Although soil moisture was not significant when tested using RDA forward selection, the cover weighted Ellenberg F (moisture) explained 39% of the variation in species composition $P \leq 0.001$ (Table 5.4). Figure

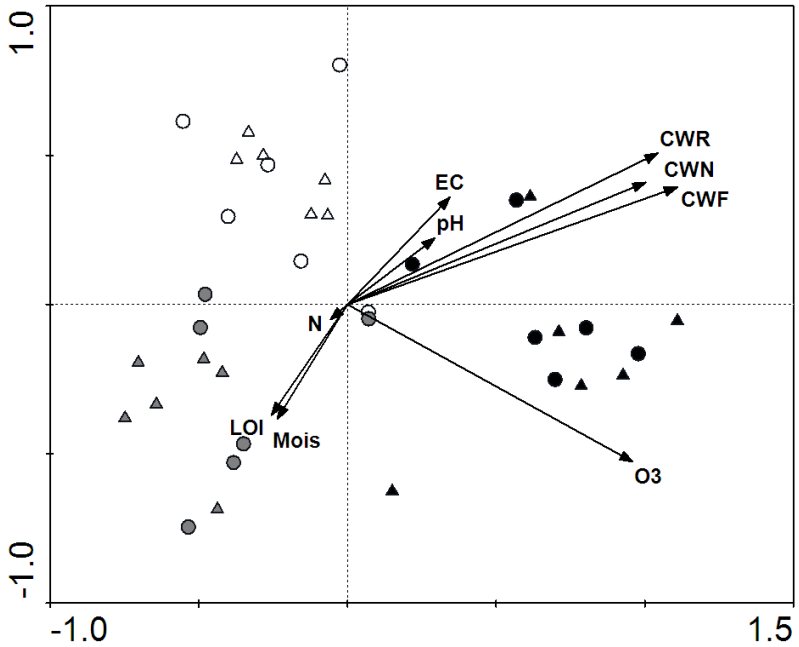
5.3B shows the distribution of the species on the 2008 PCA plot. *Ranunculus repens* and *Holcus lanatus*, which were prevalent in the high O_3 plots, have higher Ellenberg moisture indicator values (seven and six respectively) than *Agrostis capillaris* and *Dactylis glomerata*, prevalent in the low O_3 plots (both have Ellenberg F value of five). Cover weighted Ellenberg Reaction (CWR) explained a further 9 % of the variation in species cover in 2008 (Table 5.4 $P \leq 0.01$). The dominant species in the high O_3 plots had higher average Ellenberg R values, indicating that species which thrive in slightly more basic conditions are prevalent in this area of the experimental site. Cover weighted N (CWN) was not tested for significance due to the possible confounding effect of the added N treatments during the first half of 2008.

The ordination plot of the 2010 species composition RDA is shown in Figure 5.4. As in 2008, the plots next to the O_3 release pipes are separated in the ordination space from those in the ambient and low-O_3 areas of the site, suggesting some differentiation of plot species composition according to situation within the experimental O_3 gradient. However, the species driving the separation along Axis 1, such as *Ranunculus repens* and *Holcus lanatus* (more prevalent in high O_3 plots) and, for example, *Agrostis capillaris* and *Ranunculus acris* (ambient and low O_3 plots) were similarly distributed in the baseline 2008 survey. When analysing the effect of O_3 and N using the Monte Carlo permutation test, neither treatment significantly explained the variation in species composition of the plots when the underlying confounding factors of CWF and CWR were taken into account (Table 5.4). Therefore, even though the percentage contribution of individual species to the sward varied between years, this analysis indicates that the existing species composition structure identified in Figure 5.3B remained relatively constant throughout the experimental period, with no significant shifts in species composition due to exposure to elevated levels of O_3 and/or N.

Species or diversity index		Ambient O_3		Low O_3		High O_3	
	Constancy %	Low N	High N	Low N	High N	Low N	High N
2008							
Agrostis capillaris**	81	14.33± 5.75	18.33± 4.59	43.33± 5.58	51.67± 7.60	0.67±0.42	9.67±8.11
Agrostis sg *	94	43.33± 8.03	49.17± 5.23	6.33± 1.76	7.23± 1.97	6.33± 5.46	25.17± 7.17
Anthoxanthum odoratum*	86	4.50± 1.28	9.67± 2.40	6.37± 2.01	8.83± 2.17	3.00± 1.46	0.33± 0.21
Festuca pratensis	89	5.07±2.48	6.17± 2.87	2.43± 1.58	2.00± 1.10	4.33± 1.36	4.17± 0.40
Holcus lanatus**	100	13.00± 2.58	15.83± 2.39	33.33± 8.13	18.33± 3.07	59.17± 6.38	50.83± 6.64
Ranunculus acris*	78	5.50± 1.45	4.67± 0.84	3.83± 0.91	2.33± 0.33	1.33± 1.15	0.67± 0.49
Rumex acetosa	100	5.00± 2.13	3.03± 1.05	2.33± 0.42	2.83± 0.60	3.17± 0.70	6.67± 3.82
Shannon-Wiener (H')	-	1.75± 0.14	1.70± 0.10	1.69± 0.13	1.74± 0.09	1.22± 0.11	1.31± 0.06
Dominance (D)	-	0.26± 0.04	0.26± 0.03	0.29± 0.04	0.30±0.04	0.43± 0.04	0.37± 0.02
2010							
Agrostis capillaris*	100	38.37± 7.89	38.33± 5.87	38.33± 4.22	37.50± 1.71	26.67± 7.49	12.50± 2.14
Dactylis glomerata*	97	1.53± 0.31	3.00± 1.44	13.67± 2.79	15.83± 3.52	8.50± 2.99	11.67± 3.61
Festuca pratensis*	100	24.17± 7.35	15.83± 5.07	13.33± 4.22	9.00± 2.48	10.37± 2.76	14.17± 2.39
Holcus lanatus*	97	5.33± 1.54	12.17± 4.90	2.50± 0.43	2.83± 0.83	15.50± 4.54	19.17± 4.73
Ranunculus acris*	89	3.03± 1.08	3.20± 0.72	1.23± 0.45	1.57± 0.53	0.40± 0.19	1.43± 1.12
Shannon-Wiener (H')	-	1.82± 0.08	2.03± 0.07	2.12± 0.07	2.17± 0.04	2.14± 0.11	2.12± 0.13
Dominance (D)	-	0.25± 0.02	0.21± 0.02	0.19± 0.02	0.17± 0.01	0.17± 0.03	0.18± 0.04

Table 5.3 Ozone and nitrogen effects on individual species cover and plot diversity by split-plot ANOVA. Only species with ≥80% constancy were included in the cover analysis. *Agrostis sg* is the combined cover of *Agrostis stolonifera*, *Agrostis gigantea* and *Agrostis canina*. Asterisks indicate significant effect of ozone * $P \leq 0.05$, ** $P \leq 0.01$.

A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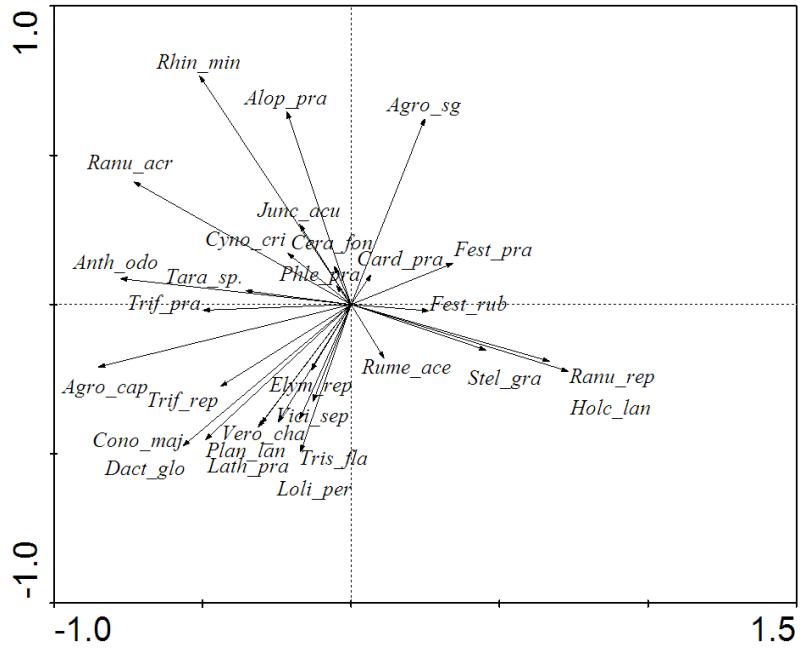
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Holc_lan
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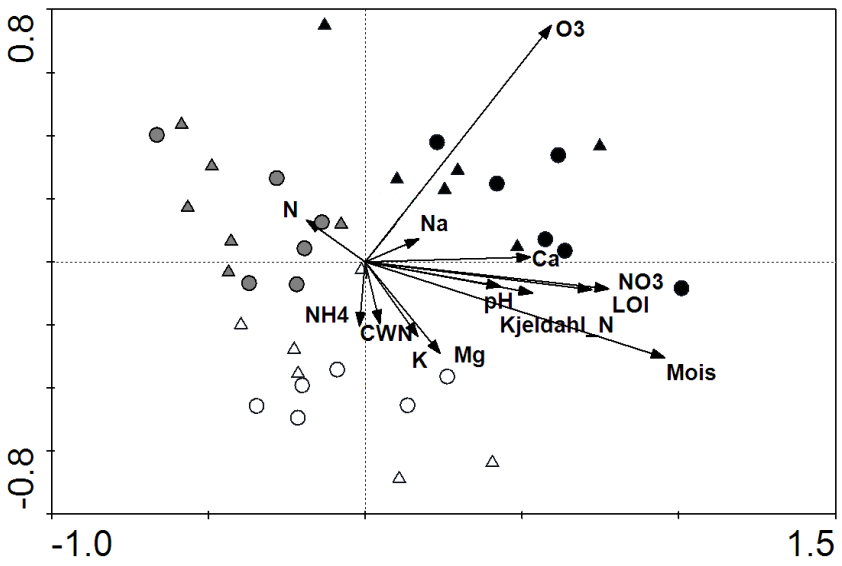
Lath_pra
Loli_per

Figure 5.3 Principal components analysis (PCA) of variation in species cover between the plots in 2008 (at the start of the O_3 fumigation treatment) and supplementary environmental variables. (A): circles = low nitrogen plots, triangles = high nitrogen plots. White symbols = ambient O_3 plots, grey symbols = low O_3 plots and black symbols = high O_3 plots. (B): Variation in plant species cover in the PCA analysis. Environmental variables are: O_3 ozone, N nitrogen, CWR cover weighted Ellenberg acidity, CWF cover weighted Ellenberg moisture and CWN cover weighted Ellenburg nutrients. Soil parameters measured at 0-15 cm (pH, LOI loss on ignition, MOIS soil moisture, EC electrical conductivity,). Species codes: *Agro_cap Agrostis capillaris, Agro_sg Agrostis gigantea Agrostis stolonifera Agrostis canina, Alop_myo Alopecurus myosuroides, Alop_pra Alopecurus pratensis, Anth_odo Anthoxanthum odoratum, Arrh_ela Arrhenatherum elatius, Aven_pub Avenula pubescens, Cyno_cris Cynosurus cristatus, Dact_glo Dactylis glomerata, Elym_rep Elymus repens, Fest_pra Festuca pratensis, Fest_rub Festuca rubra, Holc_lan Holcus lanatus, Junc_acu Juncus acutiflorus, Loli_per Lolium perenne, Phle_pra Phleum pratense, Poa_pra Poa pratense, Poa_tri Poa trivialis, Tris_fla Trisetum flavescens, Card_pra Cardamine pratensis, Cera_fon Cerastium fontanum, Cono_maj Conopodium majus, Hypo_rad Hypochoeris radicata, Myos_sp. Myosotis sp., Plan_lan Plantago lanceolata, Ranu_acr Ranunculus acris, Ranu_rep Ranunculus repens, Rhin_min Rhinanthus minor, Rume_ace Rumex acetosa, Stell_gra Stellaria graminea, Tara_sp. Taraxacum sp., Vero_cha Veronica chamaedrys, Lath_pra Lathyrus pratensis, Trif_pra Trifolium pratense, Trif_rep Trifolium repens, Vici_sep Vicia sepia.*

A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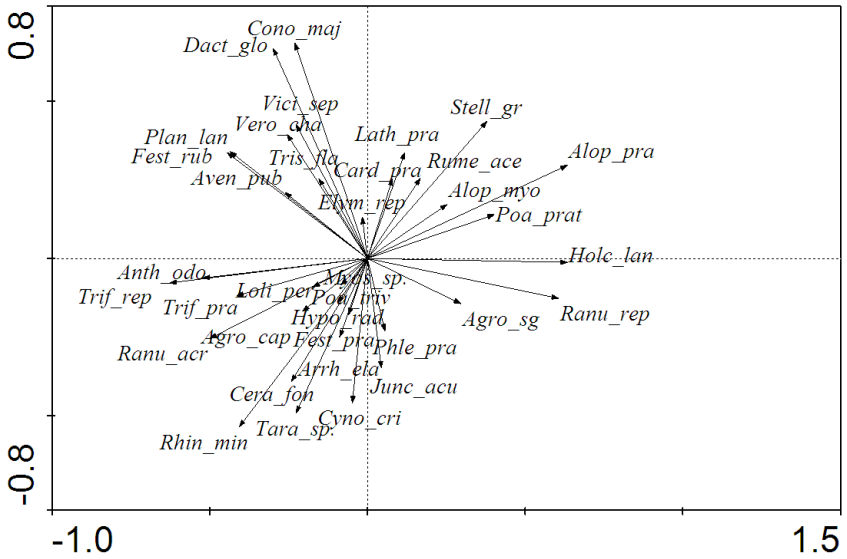
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Fest_rub
Tris_fla
Rume_ace
Alop_pra
Card_pra
Aven_pub
Alop_myo
Elym_rep
Poa_prat
Holc_lan
Anth_odo
Moss_sp.
Loli_per
Trif_rep
Poa_triv
Trif_pra
Agro_sg
Ranu_rep
Hypo_rad
Agro_cap
Fest_pra
Phle_pra
Ranu_acr
Arrh_ela
Junc_acu
Cera_fon
Cyno_cri
Tara_sp.
Rhin_min

Figure 5.4 Redundancy analysis (RDA) of the variation in species cover between the plots in 2010 and supplementary environmental variables, employing cover weighted Ellenberg moisture (CWF) and cover weighted Ellenberg reaction (CWR) as covariates in the analysis. (A): circles = low nitrogen plots, triangles = high nitrogen plots. White symbols = ambient O_3 plots, grey symbols = low O_3 plots and black symbols = high O_3 plots. (B): Variation in plant species cover in the PCA analysis. Environmental variables are: O_3 ozone, N nitrogen, CWR cover weighted Ellenberg acidity, CWF cover weighted Ellenberg moisture and CWN cover weighted Ellenburg nutrients. Soil parameters measured at 0-15 cm (pH, LOI loss on ignition, MOIS soil moisture, Kjeldahl N, K potassium, Mg magnesium, Ca calcium, Na, sodium, NO3 nitrate, NH4 ammonium). Species codes: *Agro_cap Agrostis capillaris, Agro_sg Agrostis gigantea Agrostis stolonifera Agrostis canina, Alop_myo Alopecurus myosuroides, Alop_pra Alopecurus pratensis, Anth_odo Anthoxanthum odoratum, Arrh_ela Arrhenatherum elatius, Aven_pub Avenula pubescens, Cyno_cris Cynosurus cristatus, Dact_glo Dactylis glomerata, Elym_rep Elymus repens, Fest_pra Festuca pratensis, Fest_rub Festuca rubra, Holc_lan Holcus lanatus, Junc_acu Juncus acutiflorus, Loli_per Lolium perenne, Phle_pra Phleum pratense, Poa_pra Poa pratense, Poa_tri Poa trivialis, Tris_fla Trisetum flavescens, Card_pra Cardamine pratensis, Cera_fon Cerastium fontanum, Cono_maj Conopodium majus, Hypo_rad Hypochoeris radicata, Myos_sp. Myosotis sp., Plan_lan Plantago lanceolata, Ranu_acr Ranunculus acris, Ranu_rep Ranunculus repens, Rhin_min Rhinanthus minor, Rume_ace Rumex acetosa, Stell_gra Stellaria graminea, Tara_sp. Taraxacum sp., Vero_cha Veronica chamaedrys, Lath_pra Lathyrus pratensis, Trif_pra Trifolium pratense, Trif_rep Trifolium repens, Vici_sep Vicia sepia.*

Explanatory variable tested for significance	Percentage of total variance	F value	*P* value
2008 species composition RDA			
Cover weighted Ellenberg F (CWF)	38.9	9.524	***
Cover weighted Ellenberg R (CWR)	9.3	2.393	**
2010 species composition RDA			
O_3	10.37	3.700	ns
N	2.3	0.807	ns

Table 5.4 Results from the principle component analysis of the species cover in 2008. Underlying gradients of cover weighted Ellenberg F and R (CWF + CWR) explained 48.2 % of the variation together. Redundancy analysis (RDA) of the 2010 species cover data was re-run and constrained only by ozone (O_3) and nitrogen (N) as variables, i.e. excluding soil variables, and using CWF and CWR as covariables to account for the underlying gradient identified in the baseline year (2008). F statistic significance: *ns* $P \geq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Biomass dry weight (g)	Ambient O_3		Low O_3		High O_3	
	Low N	High N	Low N	High N	Low N	High N
2008						
Grass	473.81± 28.23	481.81± 29.86	599.13± 52.71	566.02± 45.46	530.6± 45.02	604.79± 104.33
Forb *	30.71±7.68	76.27± 32.53	21.91± 3.64	28.36± 7.62	23.06± 4.69	40.77± 14.73
Legume	0.16± 0.16	4.33± 3.11	1.5± 1.03	4.69± 2.13	0.15± 0.15	0.6± 0.35
Total	504.68± 24.55	562.41± 22.36	622.54± 52.57	599.07± 38.96	461.51± 100.85	646.16± 112.36
2009						
Grass	525.73± 53.87	463.35±52.66	622.82± 51.37	544.13± 60.91	513.2± 29.26	571.65± 64.46
Forb	97.01± 16.34	85.51± 18.38	54.39± 9.72	56.33± 11.24	62.65± 20.24	76.98± 20.15
Legume	0.51± 0.51	5.61± 3.65	1.4± 0.69	2.49± 1.28	0.45± 0.39	0.65± 0.65
Total	623.25± 50.65	554.46± 41.84	678.61± 53.81	602.95± 53.57	576.3± 13.41	649.28± 65.31
2010						
Grass	400.13± 16.96	497.54±28.43	490.17± 52.81	486.89± 22.94	452.99± 38.33	485.56± 94.87
Forb	85.82± 23.45	64.6± 11.45	56.23± 11.84	67.08± 14.26	66.38± 17.74	83.22± 25.69
Legume	5.37± 4.61	2.59± 1.68	2.21± 1.81	1.24± 0.42	0.07± 0.07	0.51± 0.49
Total	491.32± 26.29	564.73± 25.29	548.61± 41.98	555.21± 21.8	519.43± 40.38	569.29± 80.09

Table 5.5 Dry weight of above-ground biomass harvest 2008, 2009, 2010 (g dry weight m^{-2}). Values are means ± SE (*n*=6). * indicates increased forb biomass in plots receiving high nitrogen treatment ($P \leq 0.05$).

There was no significant effect of O_3 or N on total above-ground biomass in any year of the experiment (Table 5.5). Alhough there was an increase in forb biomass in plots receiving high nitrogen treatment in 2008 ($P \leq 0.05$) the effect did not persist in the following two years (2009 and 2010). The grass to forb and grass to legume ratios were not affected in any year by O_3 and/or N (data not shown).

There was a significant interaction between O_3 and N on below-ground biomass (Figure 5.5, $P \leq 0.05$), with consistently reduced root biomass in high O_3 plots.

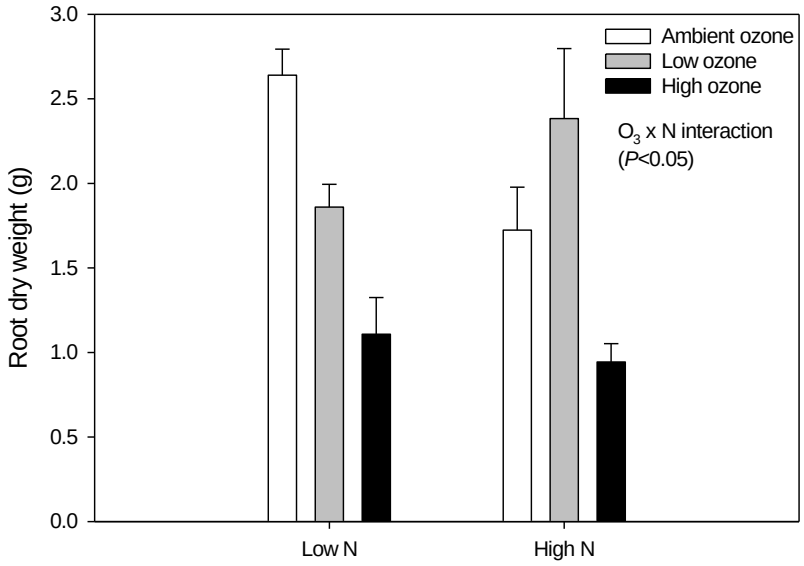

Figure 5.5 Dry root biomass in soil cores (5 cm width, 15 cm depth) taken from each plot in August 2010. Values are means ± SE (*n*=6).

5.4 Discussion

Background O_3 concentrations in the UK are rising at an average rate of 0.2 ppb yr^{-1} (RoTAP, 2011). Therefore mean concentrations during the peak growing season at the Keenley Fell free-air fumigation site reflected the predicted O_3 climate in the years 2030 and 2140 at the 10 m and 1.5 m plots respectively. Due to the design of the O_3 release system, the elevated O_3 concentration was not constant, and the gas was delivered only when wind conditions were appropriate. Therefore effects on the vegetation community are as a result of a series of O_3 'episodes', which may have induced different responses to those from a constant elevated background O_3 dose (Hayes et al., 2010b). Impacts of O_3 on soil chemistry and soil microorganisms are mediated through effects on vegetation (Chen et al., 2009), whereas N impacts are modified by soil processes such as nitrification rates and soil pH (Stevens et al., 2011). Over the three years' of the experiment measured soil properties were not expected to change significantly due to elevated O_3 or N input, but spatial variation in soil properties and cover weighted Ellenberg values were used to aid the interpretation of species cover analysis in 2008 and 2010.

Most previous studies of ozone effects on (semi-)natural grassland communities have been conducted in solardomes or open top chambers (OTCs), and have therefore been constrained to crude species mixtures, mesocosms (Nussbaum et al., 2000; Rämö et al., 2006; Hayes et al., 2010a) or transplanted turfes (Ashmore et al., 1995). Such chamber-based experiments are useful in determining the mechanism of pollution effects on vegetation, and take many edaphic and biotic factors into account. Although it is useful and necessary to build on knowledge gained from OTC and solardome mesocosm experiments with longer-term field based fumigation studies and natural in-situ vegetation communities (Volk et al., 2006), it is important to take in to account the range of factors which can cause variation in the interpretation of such approaches (Stampfli and Fuhrer, 2010). Factors such as pH, soil nutrient status and soil moisture will all influence species composition and productivity (Nussbaum et al., 2000), and gradients within experimental field plots may suppress or enhance responses to pollutant stress. A recent paper by Stampfli and Fuhrer (2010) concluded that changes in productivity and functional group fractions during a 5 year O_3 exposure experiment conducted by Volk and colleagues

(2006) were likely overestimated due to unaccounted-for initial heterogeneity between plots. In the current study, the experimental design of three replicate fumigation transects was constrained by the prevailing wind direction on the site, and did not allow for randomisation of O_3 treatments. Underlying gradients of edaphic and biotic factors became apparent within the experimental plots, and this spatial heterogeneity co-varied with O_3 fumigation. A combination of persistent site-specific environmental characteristics such as soil moisture and plant species competitive interactions appeared stronger drivers of species composition and biomass production than O_3 and/ or N treatment. These factors were previously shown to modify the impact of O_3 on Golden oat (*Trisetum flavescens* L.) in a pot based OTC experiment by Nussbaum and colleagues (2000), and on *Dactylis glomerata* and *Ranunculus acris* in a solardome study by Hayes and colleagues (2011). Although the fumigation system at Keenley Fell was constructed on an area with no severe gradients in slope, minor differences in topography along the O_3 transects may have contributed to the disparity between plots appearing otherwise homogeneous.

Impacts of O_3 and N deposition on mature plant communities are cumulative, and it may take several seasons before measurable changes in community composition or growth is transparent (Thwaites et al., 2006). The results of the current study concur with the premise put forward by Bassin and colleagues (2007a) that established grassland communities show weaker responses to O_3 exposure than early successional communities or pot-based species mixtures, due to large rooting systems, functional redundancy, and high genetic diversity in mature ecosystems. In contrast, in a study on transplanted grassland swards, Ashmore and colleagues (1995) found that the addition of elevated O_3 for 3.5 months caused species composition changes in a community typical of calcareous grasslands, while Wedlich and colleagues (2011) reported reduced forb biomass (specifically for *Ranunculaceae*, and *Rhinanthus minor*), and altered species composition of the forb and legume component of the sward at the Keenley Fell free-air fumigation site between 2007 and 2009. Whilst the current findings do not support these latter observations it is important to emphasize data were generated from experimental plots situated at different locations to those reported by Wedlich and colleagues (2011) at the same site, and the high level of spatial heterogeneity in species

composition and other edaphic factors encountered at the site may have masked effects.

The anticipated antagonistic effects of O_3 and N on above ground productivity were not observed in any year of the experiment. Similarly, Bassin and colleagues (2007b) reported significant impacts of N input (>10 kg ha^{-1} yr^{-1}) on plant biomass and changes to the relative fractions of plant functional groups in a Swiss sub-alpine grassland under long-term O_3 and N exposure. However, no change in species number per plot or species diversity due to O_3 and/or N was observed after 5 years of treatment. This result was attributed in part to the stress-tolerant growth strategy of the sub-alpine vegetation community under exploration. The primary process by which increased N input induces change in vegetation communities is by facilitating a competitive advantage for species with high maximum growth rates over slower-growing species, and this impact should be greatest on vegetation communities adapted to oligotrophic environments (Sala et al., 2000). The lack of significant N effects in the current study may be due to residual fertility pertaining to previous farming practices, and the relatively high background N deposition rate of approximately 22 kg ha^{-1} yr^{-1} compared to the 2009 UK average of 12 kg ha^{-1} yr^{-1} (DEFRA, 2011).

In contrast to the findings from the sub-alpine grassland study by Bassin and colleagues (2007b), Bowman and colleagues (2006) empirically estimated the N critical load for changes in alpine plant community composition over eight years, and showed that N additions of ≥10 kg ha^{-1} yr^{-1} significantly increased species diversity but above-ground biomass changes were transient. Alternative approaches have been employed to investigate the effects of long-term pollution exposure on (semi-)natural habitats, such as the gradient study of N deposition effects on species diversity of acid grasslands by Stevens and colleagues (2004). This wide-ranging study revealed the importance of inorganic N deposition as a driver of species richness loss across calcifuge habitats in the UK. Further exploration of this dataset has led to the identification of tropospheric O_3 as a significant contributor in species composition change, although neither species richness nor diversity was reduced (Payne et al., 2011). The response of an individual species to elevated O_3 is controlled by a number of genetic (Whitfield et al., 1997), climatic (Bassin et al., 2006)

and edaphic factors (Hayes et al., 2011b), and the response of plant communities in the field are determined from the combined primary effects on its component species and their competitive interactions (Bergmann et al., 1999). In the current study, some of the less abundant species may have increased or declined in cover due to O_3 and/or N, without altering the biomass of functional groups or species richness of the vegetation community as a whole. Should an O_3-induced reduction in legume cover or biomass have occurred, this would not lead to strong compositional changes because of their low abundance.

The proportional allocation of biomass between roots and shoots is fundamental to interactions among plants (Callaway et al., 2003). This study is one of very few to consider the combined impacts of multiple pollutants both above and below-ground in a natural plant community. Yet, reductions in carbon allocation to the roots of plants exposed to O_3 and/ or N have been shown in a number of wild species (Sanz et al., 2005; Jones et al., 2010; Hayes et al., 2011). Plots receiving high O_3 and high N treatment had the smallest average root biomass at Keenley Fell, indicating that a change in plant carbon allocation patterns with increasing O_3 and N exposure may have occurred. The interaction between treatment effects was caused by low-O_3 high-N plots having higher mean root biomass than those in the ambient-O_3 high-N plots. Although not explicitly measured in the current study, maintenance of above ground productivity at the expense of below ground biomass could bring about reduced root to shoot ratio of the sward, leading to increased susceptibility to further potential stresses such as drought and grazing (Jones et al., 2010). Should C allocation patterns be altered by the cumulative impacts of elevated O_3 and/or N, there may be secondary effects on plant species which employ stolons for propagation. Wilbourn and colleagues (1995) found a persistent reduction in the density of *Trifolium repens* L. (var, Grasslands Huia) stolons with elevated O_3, due to increased resource allocation to the shoots for defense and repair of the leaves. However, as discussed previously, the co-variance of species composition with O_3 treatment could account for the difference in root biomass between treatments, due to differences in inter-specific root morphology. Moreover, plants are known to allocate proportionally less biomass to roots in nutrient and moisture-rich environments. Further investigation would be needed in order to conclude whether this effect is solely due to the applied treatments.

5.5 Conclusions

Results of this study highlight the potentially problematic nature of investigating tropospheric pollutants on (semi-)natural vegetation by exposure of established grassland communities. Site characterisation and split-plot experimental design with block replication did not account for spatial heterogeneity of plant ecological niches (driven in part by moisture and nutrient availability). Multivariate analysis of species cover provided evidence for the inherent variance in the experimental site characteristics, which co-varied with the O_3 treatments. As pollution effects on perennial vegetation communities are cumulative, ongoing elevated O_3 and N input at Keenley Fell may cause subtle physiological changes with longer term implications on biomass and functional groups. The significant interactive impact of O_3 and N on below-ground biomass of the sward may have been as a result of alteration of C partitioning within the plants. However, it cannot be ruled out that the intrinsic difference in species composition between plots along the O_3 gradient is responsible for this effect. In conclusion, the mesotrophic hay-meadow at Keenley Fell may be less sensitive to ozone and nitrogen than hypothesized.

Chapter 6. General Discussion

6.1 Main findings in relation to the project hypotheses

1. Species of mesotrophic hay-meadows are sensitive to O_3, which can be observed as e.g., increased senescence, reductions in biomass and reduced allocation of carbon to the roots (Chapters 2, 3 and 5).

Both the grass species *Dactylis glomerata* and the forb *Ranunculus acris* were sensitive to elevated O_3 when grown individually, with increased senescence and reductions in root biomass being the primary responses after nine and thirteen weeks exposure respectively. The effect on senescence did not translate into reductions in above-ground biomass. Photosynthesis at the leaf level was not affected by increasing O_3 in *D. glomerata* or *R. acris*. In the short-term mechanistic study (three weeks) on *D. glomerata*, changes in growth and reductions in root biomass in response to elevated O_3 were not yet apparent. Other studies have shown that *Dactylis glomerata* is relatively resistant to the effects of O_3. For example Fuhrer and colleagues (1994) reported an increase in *D. glomerata* yield in managed pastures with elevated O_3. Wedlich and colleagues (2011) and Thwaites and colleagues (2006) have both shown that O_3 stimulates *D. glomerata* production in grassland communities. Hayes and colleagues (2007) conducted a meta-analysis to assess the relative sensitivity of 83 (semi-) natural species to O_3, finding *D. glomerata* above-ground biomass to be stimulated by exposure to O_3. However, in an experiment conducted by Hayes and colleagues (2011), elevating the [O_3] to 60ppb above ambient reduced the root biomass of *D. glomerata* by 30 % compared to plants in ambient O_3. This finding concurs with the results presented in the current investigation (Chapters 2 and 5), and highlights the need to include below-ground assessments in further studies on O_3 impacts on natural vegetation. In contrast to the current findings on O_3 impacts on *Ranunculus acris*, an open top chamber (OTC) study conducted by Rämö and colleagues (2006) reported no significant effect of 40 – 50 ppb O_3 on visible injury or biomass changes in this species. The intact species-

rich vegetation community at Keenley Fell did not exhibit changes in above-ground biomass of plant functional groups with elevated O_3 and/or N, which concurs with findings from chapters 2 and 3, but contrasts with findings from other field-based studies in the literature (Bassin et al., 2007; Wedlich et al., 2011).

2. Plant species vary in their response to O_3 and N. Forb species are more likely to be affected by O_3 than grasses, and grasses will exhibit a greater response to N in terms of increased above ground growth (Chapter 2).

The responses of the grass *Dactylis glomerata* and the forb *Ranunculus acris* to O_3 were broadly similar, with increased senescence, no change in total biomass, and a significant reduction in root biomass with increasing background [O_3]. Neither species exhibited a change in leaf-level photosynthetic capacity (A_{max}) or rate of RubisCO carboxylation ($V_{c,max}$) in response to O_3, N or their combination. Other studies have shown elevated O_3 to reduce photosynthetic capacity in a number of crop and (semi-) natural species (e.g. Fiscus et al., 2005), but these effects were not found at the monitored stage of leaf development in the two selected species. The maintenance of photosynthetic processes in the young leaves of both *D. glomerata* and *R. acris*, in the current study in conjunction with root biomass reductions may be attributable to an increase in repair processes and carbon sink strength in above-ground tissues, and a combination of reduced carbon assimilation, and increased sink strength in older leaves.

The main difference in response to the treatments between the grass and the forb matched the hypothesis for N treatment. Nitrogen induced a significant fertilizing effect on the total biomass of *D. glomerata* plants, and significantly exacerbated O_3-induced reductions in root biomass. *D. glomerata* has previously been shown to increase dry matter partitioning in response to N availability (Harmens et al., 2000) and grasses have been shown to increase in cover in acid grassland throughout the UK in response to N deposition (Stevens et al., 2004).

3. Increased O_3 and N concentrations cause changes in inter-specific competition and consequently in the plant community structure (Chapter 5).

In the free air fumigation experiment, O_3 and N were predicted to have synergistic effects on the species composition of the sward, with an increase in grass species cover to the detriment of forb and legume species. This effect was not manifested after 3 years of exposure to the pollutants, and highlights the caution needed when extrapolating impacts from short-term pot-based studies, to effects on larger ecosystem scales. The findings are in partial agreement with those of Payne and colleagues (2011), who recently showed that O_3 significantly affected the community composition of UK acid grasslands. However, species diversity or richness was not reduced. In contrast to O_3 impacts, Stevens and colleagues (2004) reported significant loss of species diversity with increasing N deposition in the same habitats. Therefore, the combined influence of O_3 and/or N on vegetation community structure and diversity is complex, and is likely to vary with the level of the relative pollution input, and also with habitat type. Further long-term experimentation of intact ecosystems, and also spatial pollutant gradient studies will be essential to further understanding in this research area.

4. N will ameliorate the negative impacts of O_3 on senescence and above ground biomass, but not ameliorate below ground effects (Chapters 2, 3 and 5).

Jones and colleagues (2010) reported that N input equivalent to 100 kg ha^{-1} $year^{-1}$ ameliorated the above ground injury and senescence in *Carex arenaria* induced by increasing [O_3], but did not ameliorate reductions in root biomass. There was little evidence to support the hypothesis that N will ameliorate O_3 damage above-ground in the current project. For example, increased N availability did not protect against senescence in the solardome study (Chapter 2), or promote above ground productivity in an intact species-rich hay-meadow (Chapter 5). However, when mesotrophic grassland species were exposed to the combination of elevated O_3 and N, interactive effects on below-ground pools and/or C fluxes were evident (Chapters 2, 3 and 5). This result is consistent with a study on *Trifolium subteranneum*, reported by Sanz and colleagues (2005). They demonstrated that N additions of 30

kg ha^{-1} yr^{-1} enhanced detrimental effects of elevated O_3 on nutritive quality and on photosynthate allocation to roots. Changes in C partitioning were related to the length of O_3 exposure and reductions in bulk root biomass are likely to be observed on a time-scale of months, and within one growing season for the perennial species *Dactylis glomerata* and *Ranunculus acris*.

5. Exposure to elevated levels of O_3 pollution will not have a significant effect on soil chemical properties, but may reduce the soil microbial biomass and soil C substrate turnover rate *via* limitation of C input to the rhizosphere. High N inputs will increase the labile soil C compound metabolism, but not the metabolism of more recalcitrant litter C, or microbial biomass (Chapter 4).

The response of *D. glomerata* to O_3 and N was dependent both on the [O_3], and the length of exposure to the combined pollutants (Chapters 2 and 3). There was some evidence for secondary impacts of high N treatment on below-ground C pools and fluxes (namely significant reduction in below-ground respiration, and an O_3 x N interaction on soil microbial biomass) in the *D. glomerata* ^{14}C labelling experiment (Chapter 3). In contrast, the study on long-term effects of O_3 and N on *in situ* soil microbial activity from a sub-alpine grassland did not yield results that were consistent with the short-term study, instead showing no effects of O_3 and/or N on the soil microbial biomass.

Findings in the soil of the Swiss subalpine grassland are consistent with the prediction that O_3 will not affect the soil chemical properties directly. Microbial biomass was similarly unaffected by elevated O_3, in the Swiss soil. With regards to reduction in labile C substrate turnover in the soil, this effect of O_3 alone, was only observed for oxalic acid, out of sixteen ^{14}C labelled C compounds investigated. A significant O_3 * N interaction on the metabolism of soil C substrates was evident with an increase in labile C turnover with high-O_3 in ambient-N plots, but a decrease in high-O_3 high-N plots with the addition of sucrose and oxalic acid. The same interaction was observed for the rate of salycilic acid catabolic respiration. McCrady and Andersen (2000) reported that soluble root exudation of recent photosynthate to

the rhizosphere increased in O_3 stressed wheat seedlings compared to controls. Although this could yield a transient increase in sugar availability for the soil microbial community, long-term effects of combined exposure to O_3 and N will likely result in reduced C input from roots to the soil, and a reduction in soil microbial biomass (Treseder, 2008; Chen et al., 2009). Levels of amino acids and reducing-sugar concentration in the root exudates of tomato (*Lycopersicon esculentum*) decreased under elevated O_3 (McCool and Menge, 1983). ^{14}C pulse labelling techniques have been employed to investigate rhizodeposition in *Lolium multiflorum* with N fertilization (Henry et al., 2005). They showed that high N availability increased the root soluble ^{14}C and the ^{14}C recovered in the soil per unit of root biomass, suggesting a stimulation of root exudation. The direction and scale of potential O_3 and N interactive effects on soil microbial communities is uncertain, with few studies to date investigating the combined influence of more than one pollutant. Results from the Swiss alpine site may have been influenced by the timing of soil sample selection (snow melt) as well as the range of other environmental stresses the vegetation and soils are exposed to at this high altitude site.

6. Soil bacterial diversity (analyzed by terminal restriction fragment length polymorphism, T-RFLP) profiles may be altered by N treatment but will be unaffected by O_3 (Chapter 4).

Although studied in detail for the Swiss alpine experiment, no effects on soil bacterial diversity were detected. This result occurred even though there is a trend reported in the literature for the soil bacterial communities to increase relative to the fungal proportion of the microbial community in elevated N environments (Strickland and Rousk, 2010). For example, High N inputs to Beard grass (*Schizachyrium scoparium* (Michx.) Nash) grassland for 18 years increased soil bacterial fatty acid methyl esters (FAMEs), and reduced fungal FAMEs, suggesting the potential for N deposition to disrupt the symbiotic relationships between arbuscular mycorrhizal fungi and plant roots (Bradley et al., 2006). In contrast, eight years of +30 kg N ha^{-1} yr^{-1} in a forest ecosystem in Michigan, revealed that soil microbial biomass was significantly decreased, but the soil community composition was not altered with

increasing NO_3 additions (DeForest et al., 2004). An indirect effect of N deposition changes to the soil rhizosphere and decomposer communities may be an alteration of labile C turnover rates. Currey and co-workers (2010) reported that potential mineralisation of labile C was higher under NH_4 addition than under NO_3 addition in peat soils. In the same study it was also shown that consumption of the amino acid glutamic acid was higher under NH_4 than NO_3.

6.2 The potential for difference in results from experiments on varying spatial and temporal scales.

It is important to be aware of the potential differences in response of plant species to pollutants, such as O_3 and N, when grown individually in pot-based experiments compared to field studies and natural vegetation communities (Ashmore et al., 1995). Furthermore, a species found in multiple habitats may exhibit varying growth rates, and form due to phenotypic plasticity according to resource availability, competition, and other local conditions (Bassin et al., 2006). For example the mechanistic studies of *Dactylis glomerata* physiology and C allocation in response to the pollutants when grown in pots, revealed more severe negative impacts of O_3 and N than seen in other studies on the same species in field experiments (as discussed in the previous section). This could be due to a number of factors, not least the protective effect of the surrounding vegetation canopy itself, in a natural sward. Hayes and colleagues (2011) have reported differences in the response of *Leontodon hispidus* plants to O_3 when grown with either the more dense canopy of *Dactylis glomerata*, or with the more open canopy of *Anthoxanthum odoratum*. This effect is due to a reduction of the O_3 dose within the canopy and towards the soil surface as the gas reacts with vegetation surfaces and/or is removed through gas exchange (Davison et al., 2003). Another factor which greatly influences a species response to O_3 in the field is the effect of relative susceptibility of competing species to the stress when growing within a sward. If competition for light or nutrients is reduced by effects on a sensitive adjacent species, even a species which exhibits some negative impacts of O_3 in pot-based studies may appear to thrive in a vegetation community scenario. Interspecies interactions are dynamic and complex, and the prediction of community composition change and attributing variation to pollutants in the field is challenging. Climatic,

edaphic and other unknown underlying gradients in the system can all confound experimental results (Stampfli and Fuhrer 2010). It was the aim of this project therefore, to design split-plot experiments of varying scales and exposure periods (from pot-based chamber studies of several weeks duration, to long-term field based experiments where impacts of elevated O_3 and N exposures have accumulated over many years). In this way, the potential short-comings of either approach can be recognised, and a comparison of the results aimed to provide a more holistic overview of O_3 and N impacts on C allocation in (semi-) natural vegetation and soil.

6.3 Overall conclusions

The findings from the presented experiments indicate that both O_3 and N will significantly alter C allocation patterns in some grassland plant species. In future research, attention needs to be paid to the combined negative below-ground effects, which although previously recognised as secondary impacts of both O_3 and N pollution separately, have been largely overlooked in the literature thus far. Pot-based studies suggest that the input of N, although permitting increased overall biomass, in fact exacerbates the reduction in C allocation to the roots in the common grass species *Dactylis glomerata*. It could be the case, that in perennial grassland species, the accumulation of this effect over several growing seasons, could lead to significant reductions in the root:shoot ratio, and increased susceptibility to other stresses such as drought. The combined impact of O_3 and N has also been shown to have secondary plant-mediated impacts on soil microbial biomass and below-ground respiration rates. Grasslands store approximately 34 percent of the global stock of carbon in terrestrial ecosystems (Olson, 1994) and are a dynamic source and sink for CO_2. Ozone and N may modify the rate of C turnover and C sequestration in the soil, with long term consequences for these ecosystems. Although no significant shifts in above-ground productivity or species composition of intact grassland was observed with the combined treatment of elevated O_3 and N for 3 years, further long-term experimental fumigation of *in situ* ecosystems are needed in order to characterise vegetation communities at risk from O_3 and N pollution. In conclusion, this work suggests that N deposition modifies vegetation response to O_3 stress and highlights the potentially significant role of rising levels of N deposition and O_3 as drivers of carbon allocation change in the natural environment.

6.4 Further work

There remain many important gaps in current knowledge of the combined impacts of elevated tropospheric O_3 and N deposition on vegetation and associated soils. In particular:

1. Apart from results presented in the current study, four other (semi-) natural grassland species have been investigated for the combined effects of O_3 and N. These are; *Trifolium subterraneum* (Sanz et al., 2005)*, Trifolium striatum* (Sanz et al., 2007*), Carex arenaria* (Jones et al., 2010) and *Briza maxima* (Sanz et al., 2011). It would be of value, therefore, to experimentally test a wider range of species for susceptibility to combined O_3 and N in order to better predict effects on vegetation communities as a whole. In particular, investigation of forb and legume species as they are predicted to be negatively affected by combined exposure to O_3 and N in natural ecosystems (Wedlich et al., 2011 and Stevens et al., 2006 respectively).

2. Although not explicitly measured in the current study, findings presented here suggest that O_3 and/or N will alter root enzyme activity and/or rhizodeposition. This could be further investigated utilizing ^{14}C pulse labelling techniques in combination with hydroponic plant culture, and/or axenic sand media. In this way, temporal (and spatial) samples of root tissue and exudation could be taken in order to explore the mechanism for secondary O_3 and/or N effects on the soil microbial community.

3. The increased susceptibility of legumes to O_3 and N compared to other plant functional groups is not clearly understood. It would be of interest to conduct a ^{14}C pulse-labelling experiment in legume species under combined O_3 and N stress, as below-ground effects may contribute to the loss of legume species reported in the literature (Volk et al., 2006; Gimeno et al., 2004). If alteration of root morphology, or changes in rhizodeposition occur, it is possible that the rhizobia/legume relationship could be disrupted by the combined impacts of O_3 and N.

4. The soil fungal community, and in particular plant- mycorrhizal associations are extremely important for ecosystem functioning and plant community health (e.g. Mack and Rudgers, 2008; Jones et al., 2004; Landeweert et al., 2001). Further examination of the soil microbial community at long-term free-air fumigation experiments (such as Alp Flix), using phospholipid fatty-acid (PLFA) analysis could reveal secondary impacts of O_3 and N on the bacterial:fungal ratio. It would be expected, as shown by Bradley and colleagues (2006) and Porras-Alfaro and colleagues (2007) under high N deposition and by Andrew and Lilleskov (2009) and Chen and colleagues (2010) under elevated O_3, for the fungal: bacteria ratio to decrease under the combined exposure.

5. In parallel with further exploration of the microbial community *in situ*; C allocation changes below-ground in natural communities could be investigated using carbon isotope pulse labeling of vegetation swards under elevated O_3 and N. This approach could provide an insight into the fate and dynamics of C turnover in a field situation as employed by Hill and colleagues (2007).

6. The short-term chamber study on C partitioning in *Dactylis glomerata* showed initial differences in below-ground C pools after a 3-week exposure. A combination of shorter and longer O_3 and N exposures may help separate short term versus long-term effects. Furthermore the C isotope pulse-labelling technique could be employed to explore differences in response of *D. glomerata* grown singly versus in competition or in natural swards.

Appendix A Peer reviewed paper: Hayes, F., Mills, G., Harmens, H. and Wyness, K. (2011)

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