Impacts of Tropospheric Ozone on Semi-natural Ecosystems

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Diploma (MSc) in Agricultural Biology

October 2009

A thesis submitted for the degree of
Doctor of Philosophy of the University of York

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Abstract

Experimental work on the effects of air pollutants such as ozone on semi-natural ecosystems is somewhat limited, and not much of the knowledge of such systems derives from community level studies. The overall aim of this study was to better understand the impacts of elevated ozone on semi-natural ecosystems.

Effects of ozone were studied on a semi- natural grassland at High Keenley Fell in the Northern Pennines. The grassland of the study had been fumigated with elevated ozone concentrations in a free air fumigation gradient system for two years. Elevated ozone reduced the above- ground biomass of forbs and certain forb species such as *Ranunculacae* and *Rhinanthus minor*. A multivariate analysis showed that next to ozone other environmental factors such as nitrate, calcium and soil water content were also accountable for the species variation. Furthermore, a reduction in ozone concentrations was found within the canopy, and the elevated ozone concentrations led to an increased stomatal closure in *Briza media* and *Trifolium repens*.

Effects of ozone on a mire ecosystem were studied at the University of Newcastle field station Close House, Northumberland. Lowland mire community mesocosms had been exposed to elevated ozone concentrations in open-top chambers for three years. The biomass of the key species *Eriophorum vaginatum* declined and methane emissions were reduced by ozone, and so was the available ammonium in the soil water. In a separate litter experiment (litter of *E. vaginatum*), in which the litter was soaked with ozone-treated soil water, the microbial respiration was also negatively affected.

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Acknowledgement

This study was carried-out under the tenure of a Department for the Environment, Food and Rural Affairs (DEFRA) Studentship.

I would like to thank my supervisors, Professor Mike Ashmore and Dr. Sylvia Toet for their advice and guidance throughout the study.

Furthermore I want also to thank Dr. Lisa Emberson and Dr. Patrick Bücker for their helpful input to the current study.

Many thanks to Rebecca Sutton and Dr. Leon van den Berg who helped me much with laboratory work.

Special thanks to Professor Jeremy Barnes and Dr. Simon Peacock for letting me use the mire and the grassland mesocosms in the open top chambers for this study and for providing instruments and help during the intensive measuring campaign.

To all members of the 'Ozone Umbrella Group' over the last three years, who put much work into this project and have made this study possible. I would like especially to thank Professor Neil Cape, Dr. Mhairi Coyle, and Dr. Simon Peacock for keeping the ozone system running at High Keenley Fell.

And last but not least I am grateful for all the help and support from my friends and my family throughout the last three years.

Declaration

The work in this thesis was developed by the author between July 2006 and October 2009. I declare that all the work contained within this thesis, apart from work whose authors are clearly acknowledged, is the result of my very own and original work.

1. Introduction

1.1 Ozone – characteristics, formation and atmospheric concentrations

Ozone is a pale blue gas with a sharp odour. It is a molecule consisting of three oxygen atoms (O_3), with a molecular weight of 48 g mol^{-1} and a density of 2.144 g l^{-1} (Daintith, 2004). Ozone is a very reactive, oxidant gas, which can react with unsaturated organic chemical bonds, forming free radical ozonides. The free radicals can destroy the unsaturated bonds which also occur for example in fatty acids (Kley, 1999).

Its character in the earth's atmosphere is quite contradictory. In the stratosphere, ozone protects humans from the ultra-violet radiation from the sun, whereas in the lower troposphere it is harmful for humans at high concentrations causing health problems such as inflammation of the lungs and bronchia (Royal Society, 2008). In addition to effects on human health, it has impacts on the environment, including causing severe damage to plants. Acute plant responses of ozone have been recognized as foliar injury, while chronic responses include reductions in plant growth and productivity. Tropospheric ozone contributes to global warming because it acts as a strong greenhouse gas (Kley, 1999).

1.2 Ozone formation and exposure

Ozone is a secondary pollutant that is formed from precursors in a series of photochemical reactions. Ultraviolet light (UV) drives the dissociation of oxygen (photolysis), producing ozone.

$O_2 + h\nu \rightarrow O + O$ where $h\nu$ = 280-290 nm

$O + O_2 \rightarrow O_3$

This UV-driven reaction is responsible for most atmospheric O_3 production, most of the ozone is found in the stratosphere, creating the stratospheric ozone layer. Some of the stratospheric ozone is transferred to the troposphere, which occurs during the tropopause folding (a mechanism whereby stratospheric air is irreversibly transporteddirectly along isentropic surfaces into the troposphere; Price & Vaughan, 1993), and cut-off lows (isolated cyclonic vortices in the upper level flow (around 300 mb) which form as a result of meridional excursion of jet streams and are capable of transferring stratospheric air into the troposphere; Price & Vaughan, 1993) (Kentarchos *et al.*, 1999, Beekman *et al.*, 1997). Other tropospheric ozone concentrations derive from a series of reactions from precursor pollutants such as nitrogen oxides (NO_x), carbon monoxide (CO) and volatile organic compounds (VOCs). The main reactions which govern the concentration of ozone are as follows:

$NO + O_3 \rightarrow NO_2 + O_2$

$NO + h\nu \rightarrow NO + O$ where $h\nu$ = 280-290 nm

$O + O_2 (+M) \rightarrow O_3 (+M)$ M = any molecule

However, these reactions mainly occur under typical daytime conditions when the atmosphere is well mixed, and then ozone concentrations stay fairly low. Additional ozone is produced in very complex processes which involve several hundreds of radicals, NO_x and VOCs (NEGTAP, 2001).

These reactions can be summarised as follows (PORG, 1998):

$OH + RH \rightarrow R + H_2O$

$R + O_2 (+M) \rightarrow RO_2 (+M)$ M= any molecule

$RO_2 + NO \rightarrow RO + NO_2$

$RO \rightarrow$ carbonyl products + HO_2

$HO_2 + NO \rightarrow OH + NO_2$

Where OH = hydroxyl radical

RH = saturated hydrocarbon

R = alkyl radical

RO_2 = alkyl peroxy radical

HO_2 = hydroxyperoxy radical

RO = alkoxy radical

As OH is generated from HO_2 and NO in the last reaction, the process forms a catalytic cycle and several molecules of ozone can be produced from the oxidation of a single hydrocarbon compound (Figure 1). The oxidation of carbon monoxide (CO) also involves hydroxperoxy and alkyl peroxy radicals. These processes can also disturb the photostationary state and generate ozone (PORG, 1998).

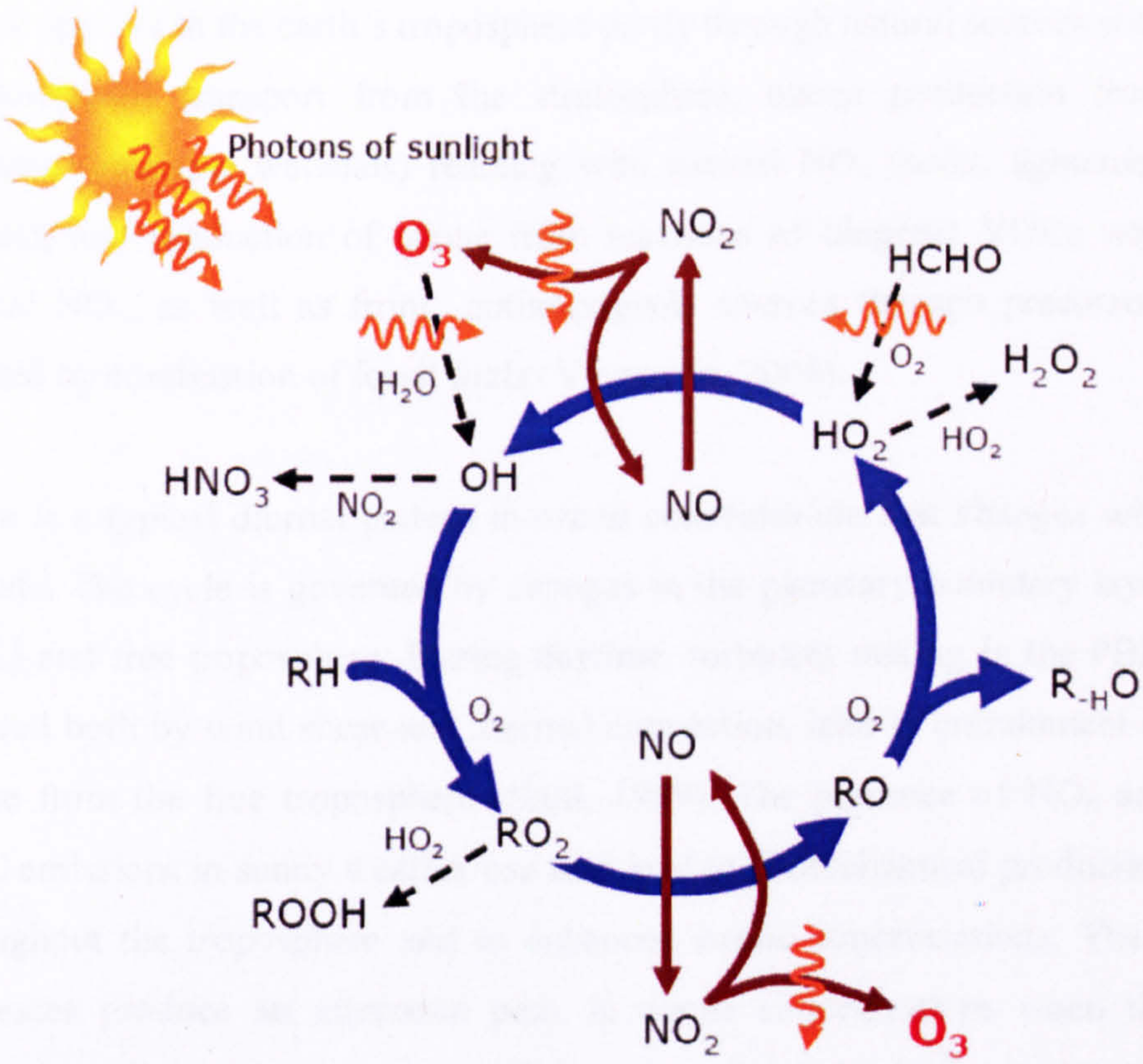


Figure 1: Schematic representation of ozone chemistry showing the free-radical (OH) catalysed oxidation of a generic saturated hydrocarbon, RH (after PORG, 1998).

Although the major oxidation processes occur during the daytime, as ozone formation is triggered by sunlight, there are potentially significant processes at night which lead to O_3 removal (PORG, 1998).

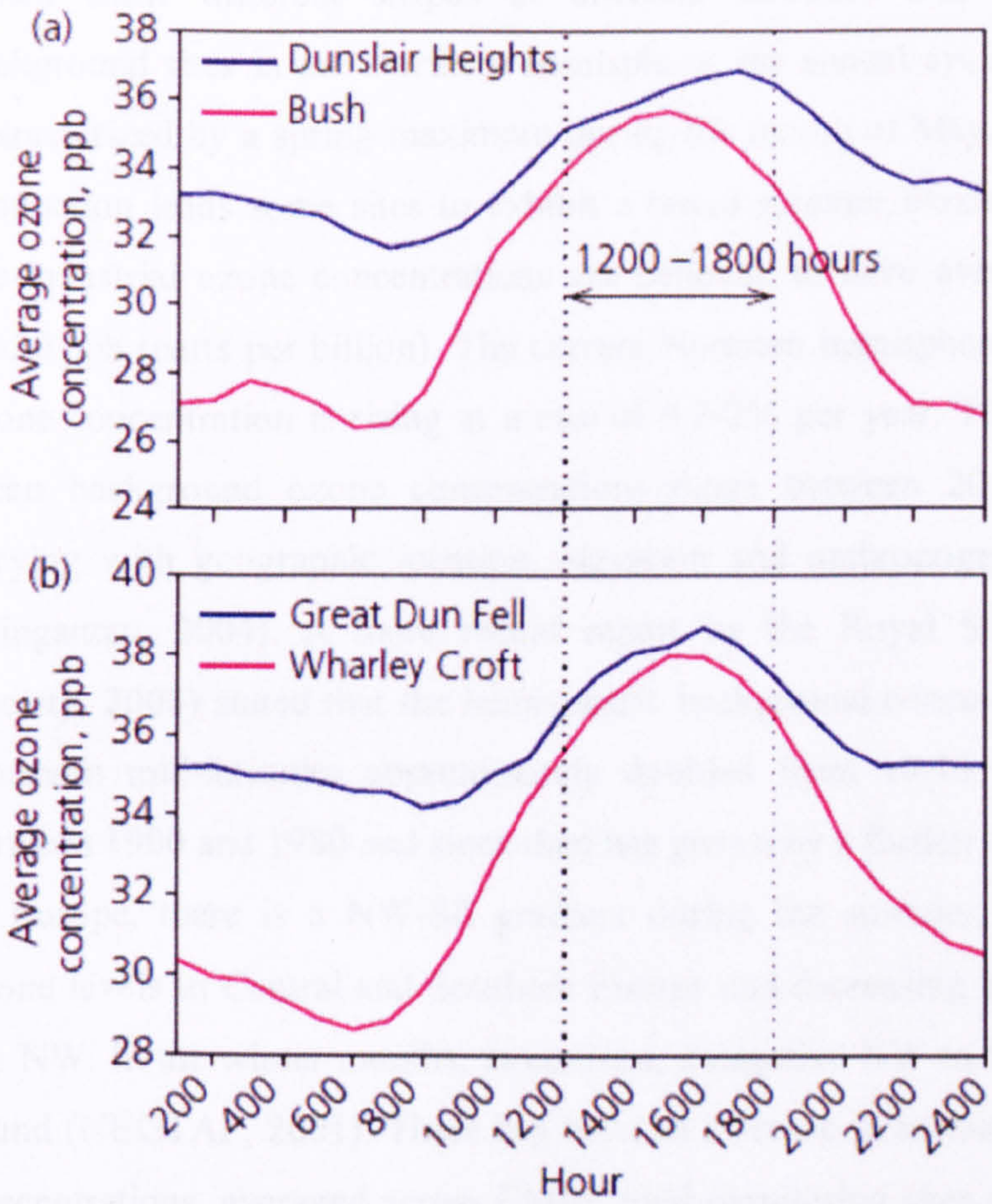
Once sunlight strikes, NO_3 is photolysed and NO_2 and O_3 are regenerated.

$NO_2 + O_3$	$\rightarrow$	$NO_3 + O_2$	
$NO_3 + h\nu$	$\rightarrow$	$NO_2 + O$	where $h\nu$ = 280-290nm
$O + O_2$ (+M)	$\rightarrow$	O_3 (+M)	M = any molecule

Ozone appears in the earth's troposphere partly through natural sources such as downward transport from the stratosphere, ozone production from methane (swamps, wetlands) reacting with natural NO_x (soils, lightening strikes), and production of ozone from reactions of biogenic VOCs with natural NO_x, as well as from anthropogenic sources through precursors emitted by combustion of fossil fuels (Vingarzan, 2004).

There is a typical diurnal pattern in ozone concentration that changes with altitude. The cycle is governed by changes in the planetary boundary layer (PBL) and free troposphere. During daytime, turbulent mixing in the PBL, induced both by wind shear and thermal convection, lead to entrainment of ozone from the free troposphere (Stull, 1989). The presence of NO_x and VOC emissions in sunny weather can also lead to photochemical production throughout the troposphere and to enhanced ozone concentrations. These processes produce an afternoon peak in ozone concentrations when the atmosphere is most turbulent and UV levels are at their maximum (Coyle, 2006). Ozone declines during the night in stable layers at low elevation sites. Provided that thermal stratification of the atmosphere leads to stable nocturnal layers, with virtually no ozone exchange between them, ozone concentration can decrease to zero. In urban areas, ozone depletes because of the reaction with nitric oxide (produced by cars and other combustion processes), whereas in most rural areas the nitric oxide concentrations are too low to effectively reduce ozone concentrations and dry deposition dominates the process of ozone depletion (Figure 2). The minimum concentration is usually reached between midnight and dawn. At higher elevations the diurnal variation is suppressed, because of the lack of nocturnal stable layers, and ozone levels remain high at night (WHO, 2000).

Figure 2: The diurnal cycle in ground-level ozone at rural sites in the UK illustrating the much larger range in the diurnal cycle at (a) low altitude and valley sites (Bush and Wharley Croft) relative to (b) hill top locations (Dunslair and Great Dun Fell) (after the Royal Society 2008).



1.3 Concentration patterns and trends

Ground-level ozone concentrations fluctuate in space and time due to the variability of sources and sinks. There are pronounced seasonal cycles which show different shapes at different latitudes and altitudes. At background sites in the Northern hemisphere, the annual cycle of ozone is characterized by a spring maximum during the month of May. Local ozone production leads some sites to exhibit a broad summer maximum as well. Pre-industrial ozone concentrations are believed to have averaged around 10-20 ppb (parts per billion). The current Northern hemisphere background ozone concentration is rising at a rate of 0.5-2% per year. Today's annual mean background ozone concentrations range between 20 and 45 ppb, varying with geographic location, elevation and anthropogenic influence (Vingarzan, 2004). A more recent report by the Royal Society (Royal Society, 2008) stated that the hemispheric background concentration in the Northern mid-latitudes approximately doubled from 10-15 to 20-30 ppb between 1900 and 1980 and since then has grown by a further 5 ppb.

In Europe, there is a NW-SE gradient during the summer, with highest ozone levels in Central and Southern Europe and decreasing levels towards the NW. In the winter months, in contrast, a negative NW to SE gradient is found (NEGTAP, 2001). There has been an increase in annual mean ozone concentrations, averaged across EMEP rural monitoring sites across Europe that are situated below 500 m of 0.3 $\mu g\ m^{-3}\ yr^{-1}$ with 17 showing a downward trend (2 of which were statistically significant) and 29 showing an upward trend (11 of which were statistically significant) from 1990 to 2002 (AQEG, 2009).

Globally, ozone concentrations are higher over industrialised latitudes of the Northern hemisphere and lower in the Southern hemisphere, although there are enhanced concentration levels over parts of South America and Africa. As precursor emissions increase in Eastern Europe, Asia and some regions of the Southern Hemisphere, global background ozone concentrations are predicted to rise in the future. In contrast, in North America and Europe, peak ozone concentrations are anticipated to fall due to emission control

strategies (Collins *et al.*, 2000a & b; Lelieveld & Dentener, 2000). Modelling studies argue that increments of global NO_x emissions since the 1970s account for 10-20% of increasing background ozone concentrations, whereas methane levels have an influence about 3-4%. In contrast, increases in hydrocarbons have been modest and only affected regional high ozone episodes (Fusco & Logan, 2003). The average global surface ozone concentrations based on different IPCC emission scenarios is expected to be in the range of 35-48 ppb by 2040, 38-71 ppb by 2060, 41-87 ppb by 2080 and 42-84 ppb by 2100 (Figure 3; Vingarzan, 2004).

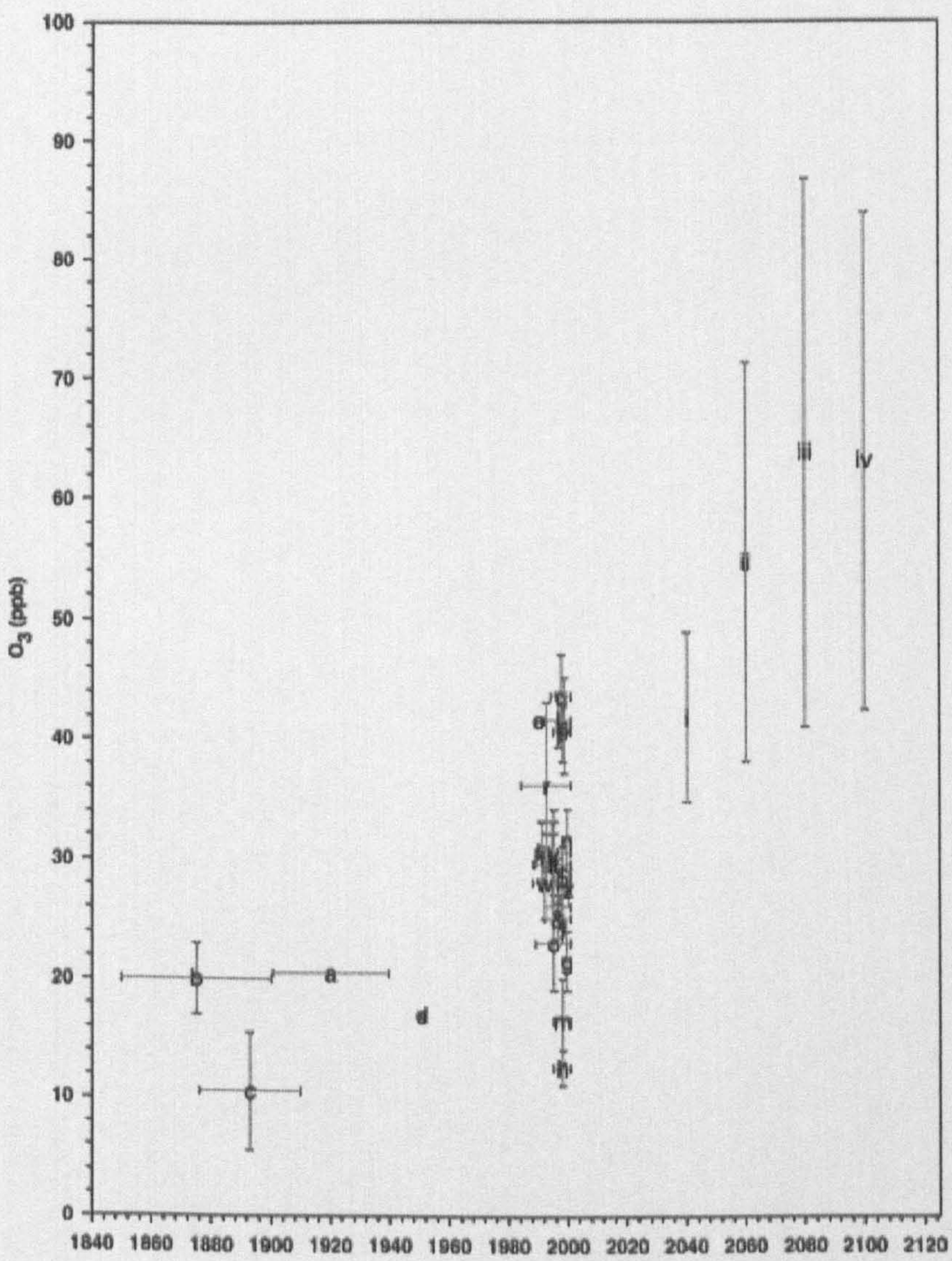


Figure 3: Historical, current and projected background surface ozone annual concentrations.

a—Athens, Greece (Varotsos and Cartalis, 1991). b—Europe (Bojkov, 1986)–avg of daily maxima. c—Montsouris, France (Volz and Kley, 1988). d—Arosa, Switzerland, (Staehelin et al., 1994). e—Arosa, Switzerland, (Staehelin *et al.*, 1994). f—Pt. Barrow, Alaska (CMDL, 2004). g—Virgin Islands National Park, US Virgin Islands (CASTNet, 2004). h—American Samoa (CMDL, 2004). i—South Pole, Antarctica (CMDL, 2004). j—Arrival Heights, Antarctica (CMDL, 2004). k—Ny Alesund, Svalbard, Norway (CMDL, 2004). l—Mauna Loa, Hawaii (CMDL, 2004). m—Mount Rainier National Park, Washington (CASTNet, 2004). n—Denali National Park, Alaska (CASTNet, 2004). o—Glacier National Park, Montana (CASTNet, 2004). p—Lassen National Park, California (CASTNet, 2004). q—Rocky Mountain National Park, Colorado (CASTNet, 2004). r—Theodore Roosevelt National Park, North Dakota (CASTNet, 2004). s—Yellowstone National Park, Wyoming (CASTNet, 2004). t—Kejimkujik, Nova Scotia (CAPMoN, 2003). u—Montmorency, Quebec (CAPMoN, 2003). v—Algoma, Ontario (CAPMoN, 2003). w—Chalk River, Ontario, (CAPMoN, 2003). x—Egbert, Ontario (CAPMoN, 2003). y—Experimental Lakes Area (ELA), Ontario, (CAPMoN, 2003). z—Bratt's Lake, Saskatchewan (CAPMoN, 2003). $—Esther, Alberta (CAPMoN, 2003). &—Saturna, British Columbia (CAPMoN, 2003). i—Range of surface O_3 projections for the 2040 (IPCC-DDC, 2004). ii—Range of surface O_3 projections for the 2060 (IPCC-DDC, 2004). iii—Range of surface O_3 projections for the 2080 (IPCC-DDC, 2004). iv—Range of surface O_3 projections for 2100 (IPCC-DDC, 2004).

1.4 UK ozone levels

In the UK, the annual mean ozone concentration varies from values of around 10 ppb in urban areas to 25 ppb in rural areas and 35 ppb in upland regions. There is a summer-time spatial pattern in mean concentrations that shows a regional gradient from low concentrations in the NW to higher concentrations in the SE towards mainland Europe. The opposite pattern is found in winter, with the highest mean concentrations in the NW and the lowest in the SE. Also, a seasonal cycle is found with peaks in both spring

and summer. The spring peak is due to seasonal variation in hemispheric background concentrations and is most pronounced at less polluted northern sites. Several peaks typically occur during summertime, which are spread over a period from April to September, and are more pronounced at southern sites due to the effect of local and regional scale photochemical ozone production (NEGTAP, 2001). On average there has been an increase in mean annual ozone at rural and remote sites (18 locations throughout UK) of 0.4 μg m^{-3} yr^{-1}, with 6 of 18 sites showing statistically significant increases from 1990 to 2005 (AQEG, 2009).

According to previous analysis of trends in UK annual mean and maximum ozone concentrations (PORG, 1998; Coyle *et al.*, 2000), background annual mean ozone concentrations at rural sites have increased and peak concentrations during photochemical episodes have decreased. From the 1970s to the 1990s, a large decline in the monthly maximum concentration was found with a decrease in peak values in the order of 30 ppb. This change was also reflected in the maximum 8 hour running mean concentrations (highest 8h mean in a day), and AOT40 (an accumulated ozone exposure over the threshold of 40 ppb, i.e. the differences between an hourly mean in excess of 40 ppb and 40 ppb are summed over a specific period of time for each vegetation type) trends. For the UK, surface ozone concentrations are expected to rise steadily within this century (NEGTAP, 2001), primarily due to increases in Northern hemisphere background concentrations.

1.5 Effects of ozone on plants

Ozone is one of the most phytotoxic air pollutants, and causes considerable damage to vegetation throughout the world (Karnosky *et al.*, 2007). Ozone sensitivity varies between species as well as between cultivars. Thomas (1951) first observed visible symptoms of oxidant injury of crops, ornamentals and wild species, in California. In North America, ozone is still the most important air pollutant for crops, and foliar symptoms have been reported in the field for potato, dry bean, soybean, tobacco and several other

species (Pearson & Percy, 1997). Other vegetation types such as trees have also been studied in detail. Miller *et al.* (1982) reported O_3 induced foliar injury and early needle loss, accompanied by decreased nutrient availability and reduced carbohydrate production, in the San Bernadino Forest, in Southern California. In Europe, the first injuries to crops were noticed in the 1970s. Ashmore *et al.* (1980) reported visible injury on radish and peas in the UK after a period of high O_3 concentrations and Velissariou (1999) noticed severe reddening and necrosis on chicory near Athens. Such visible damage has now been reported in the field in Europe for bean, clover, maize, potato, soybean and many other crop species (Ashmore, 2003). Visible damage to trees has been detected in Switzerland and Spain for a large number of shrubs and deciduous tree species (Skelly *et al.*, 1999). Baumgarten *et al.* (2000) found visible injury caused by ozone on young seedlings and mature beech trees in Germany, and showed that the association betwee injury and ozone exposure was improved when the cumulative O_3 uptake was used.

Ozone enters the leaf mainly via stomata. Once it has passed the stomata it can react with organic molecules to be found on the cell walls of the sub-stomatal cavity, as well as in the intercellular air space. This reaction results in the formation of highly reactive radical species that react with component of the cell membrane. In the cell radical scavengers such as superoxide dismutase, catalase, glutathione peroxidise and ascorbate usually prevent this reaction, scavenging harmful oxygen radicals and inhibiting lipid peroxides for instance. A complex system of protection from oxidative damage operates in the extracellular and intracellular space (Tausz *et al.*, 2007; Heath, 2008; Matyssek *et al.*, 2008; Sandermann, 2008). Ozone dissolves in the apoplastic fluid where it decomposes to hydrogen peroxide, singlet oxygen and hydroxyl radicals (Sandermann, 2008). Reactions of ascorbate with ozone proceed via ozonolysis with the production of zwitter ions and of singlet oxygen, which is toxic and needs to react again with ascorbate. End products of the electron transfer reactions are mono-dehydroascorbate and dehydroascorbate. After transport through the

plasmalemma, these compounds are reduced, and ascorbate is re-translocated to the extracellular space (Sanmartin *et al.*, 2003).

At high ozone levels, the protection mechanisms can fail, with a subsequent collapse of targeted cells, leading to a local visible tissue destruction which can often, but not necessarily, be observed as visible foliar injury (chlorosis, bleaching, bronzing, flecking, stippling, uni- and bifacial necrosis). Further physiological changes, such as alterations in the membranes and their functions, stomatal functioning and a decline in photosynthetic capacity may occur also (WHO, 2000). A decline in photosynthetic capacity could also derive from an up-regulation of defence mechanisms, leading to chronic ozone effects. Such chronic effects can have serious consequences for the fitness of the plant, determined as reductions in growth rate, and diminution in resource allocation and reproductive performance (Kley, 1999; Ramö, 2006). Ozone stress has been found to reduce nutrient-use efficiency (NUE) in wheat, causing a decline in the efficiency of N uptake and N assimilation with increasing O_3 concentrations (Fuhrer, 1997). Effects on water use efficiency (WUE) are another important matter because high ozone levels are associated with warm weather and reduced soil moisture. Decreased leaf WUE has, for example, been detected in wheat (Nussbaum *et al.*, 1995) and soybean (Vozzo *et al.*, 1995).

1.6 Ecological effects

While there has been great progress in understanding the effects of ozone on agricultural crops and individual forest species, there is relatively little knowledge about potential impacts of ozone on natural terrestrial ecosystems (i.e., grasslands, wetlands, alpine or arctic tundra, unmanaged forest stands and deserts; Laurence & Andersen, 2003). A challenge is to identify key species in natural systems that might affect the overall functioning of the ecosystem. Organisms in ecosystems process energy at different rates, respond to disturbance differently, and are often ecosystem specific (Moore, 1994). One of the best examples of ecosystem change in

response to ozone is a native ponderosa pine ecosystem of Southern California. Miller and colleagues (Miller, 1973, 1984; Miller *et al.*, 1982, 1989) showed in their studies how ozone may alter the structure and the function of ponderosa pine ecosystems, and might reduce the root biomass, although there is still an uncertainty about the extent of ozone's role since other pollutants such as nitrogen deposition also occurred in association with ozone (Fenn & Bytnerowicz, 1993; Grulke *et al.*, 1998). However, concentrations ranging from 50-60 ppb ozone induced foliar injury and early needle loss, decreased nutrient availability in stressed trees, reduced carbohydrate production with lessened tree vigour which resulted in decreased height/diameter growth and increased susceptibility to bark beetles (Miller *et al.*, 1982). With diminishing ozone stress since 1974, Miller *et al.* (1989) have reported improvements in the foliar injury index except at the most exposed plots.

As ozone has an impact on the growth and productivity of individual plants, it certainly has the potential to affect the plant community as well. These effects can be defined as shifts in species composition, loss of biodiversity and changes in genetic composition (WHO, 2000). A shift in the relative proportions of the component species in response to ozone has been demonstrated by several studies of simple mixtures of herbaceous species, particularly where species are actively competing (Fuhrer *et al.*, 1994; Montes *et al.*, 1982). This is because neighbouring individuals compete for resources above- and belowground, and changes in resource availability, resulting from effects of ozone on one of the competing species, lead to adaptions of the other competing species (Callaway *et al.*, 2003). For instance competition for light in dense stands can cause changes in root growth and architecture (Turkington *et al.*, 1994). Differing ozone sensitivity of grasses and legumes has been found, causing a shift in the grass/ legume ratio in favour of the grass fraction, and therefore altering the protein concentration and other qualitative traits in the plant community (Fuhrer, 1997).

The impact of ozone on the community may be related to the sensitivity of the component species of that community, which is primarily determined by stomatal conductance (g_s), specific leaf area (SLA) and defence capacity (Bassin *et al.*, 2006). Mills *et al.* (2006) identified 83 species from existing publications for which it was possible to define a sensitivity index based on responses of above-ground biomass. They identified physiological and ecological characteristics associated with species sensitivity. The therophyte life-form was particularly sensitive, and plants with higher leaf N concentration were more sensitive than those with lower N concentration. Weak relationships were found with Ellenberg habitat requirements for light and salinity. However, no relationship with ozone sensitivity could be found for leaf phosphorus (P) concentration, Grime's CSR strategy, leaf longevity, flowering season, stomatal density and maximum altitude.

1.7 Grasslands

Grasslands are a major component of European landscapes. Grassland is the most widespread land cover in the UK, covering 13 million ha, or 54% of the total area (Wilkins, 2005). Many of these perennial-dominated systems are exposed to ozone over long growing seasons and for many years. There are many issues related to long-term effects of ozone that are relevant to the use of grasslands for grazing animals, including changes in forage quality (due to altered leaf chemistry and/ or shifts in species composition), increased levels of phenolic acids and flavonoids, increased senescing tissue, increased lignification, and decreased leaf/stem ratio which may negatively affect ruminant micro-organisms and enzyme systems (Fuhrer & Booker 2003).

Most studies of ozone effects on grassland communities have been done in open-top chambers (OTCs), and have focused on artificial communities (e.g. Ashmore & Ainsworth, 1995; Bungener *et al.*, 1999; Nussbaum *et al.*, 2000; Kanerva *et al.*, 2006; Rämö *et al.*, 2006). Bungener *et al.* (1999) found that depending on species, injury symptoms were expressed as flecking, leaf yellowing or anthocyanin formation. *Carum carvi,*

Onobrychis sativa, Trifolium repens and *Trifolium pratense* were found to be most responsive species to ozone. Nussbaum *et al.* (2000) used an experiment with binary mixtures showing that competition changed the species reaction to ozone. *Trisetum flavescens*, planted with either *Trifolium repens* or *Centaurea jacea* in different mixing ratios was affected by ozone when grown with *T. repens* but not with the other species. Rämö *et al.* (2006) reported a reduction of the total above-ground biomass of a mixture of seven grassland species in response to elevated ozone concentrations with *Campanula rotundifolia, Fragraria vesca* and *Vicia cracca* being most strongly affected. In the same community Kanerva *et al.* (2006) found reduced soil N_2O, and CO_2 emissions and reduced CH_4 fluxes in elevated ozone.

One of the few OTC studies that used non-artificial communities was carried out by Thwaites et al. (2006). They fumigated intact transplanted turfs of calcareous grasslands with three different ozone regimes (40-50, 60-70, 80-90 ppb and ambient, filtered air) over three summers. The high ozone treatment caused a decline in cover of *Festuca rubra* and *Campanula rotundifolia* was lost from all ozone treatments, whereas the frequency of *Galium verum* and *Plantago lanceolata* was increased. Although cumulative ozone exposure was a significant factor, it only explained 4.6% of the compositional change over the three year study because of other changes in time induced by the transfer to artificial chamber environments. In the study of Evans & Ashmore (1992), which compared charcoal-filtered with ambient air-treated grassland plots, a reduction in the biomass of the forb fractions and an increase of the legume fraction was detected in the highest ozone treatment.

Although OTC studies take many biotic and edaphic factors into account, they still differ from field conditions. Nussbaum & Fuhrer (2000) found a higher atmospheric and boundary layer conductance in OTCs, which led to increased pollutant fluxes in the chambers when compared with field conditions. Other disadvantageous features are reduced photosynthetically radiation, higher temperature and associated decreases in water vapour pressure deficit. Water vapour pressure deficit (VPD) modifies the relative

sensitivity of species because of the differential response to stomata to VPD. Protection from ozone is species specific, and depends on a reduction in stomatal conductance (g_s) due to decreased soil water potential while protection under increased VPD depends upon the relationship between VPD and g_s (Bungener *et al.*, 1999, Nussbaum *et al.*, 2000).

Using a free-air exposure system could overcome these disadvantages as it provides close-to natural conditions and allows for sufficient plot size and space to study the interactions with additional factors such as water and nutrition (Bassin *et al.*, 2006; Volk *et al.*, 2006). There are currently four studies on long-term free air ozone fumigation of grasslands that are completed: Wilbourn *et al.* (1995); Bassin *et al.* (2005); Erbs & Fangmeier (2005) and Volk *et al.* (2006). Wilbourn *et al.* (1995) used an unenclosed field fumigation system for 22 days with ozone treatment concentrations varying from 50-70 ppb compared to ambient concentrations of 10-28 ppb. They found significant effects on yield and herbage composition of a sown grass-clover mixture, with the highest yield loss in ozone for *Trifolium repens.* They also noted a persistent effect of ozone via impacts on stolon density. Briede & Fangmeier (pers. Com.) detected a decline in the cover of *Chrysanthemum leucanthemum* and *Dianthus cartusianorum,* and an increase in the cover of *Centaurea jacea,* when exposing a sown chalk grassland to ozone using a ring system (Ambient, 66 ppb and 100 ppb treatment). They also reported an increase in biomass for *Centaurea jacea,* but a decrease in *Salvia pratensis.* Volk *et al.* (2006) found, in their fully established grassland FACE study, that increasing ambient ozone levels by 50% over 5 years caused reduced productivity (23% yield loss) accompanied by a change in the composition of different functional groups, with a strong negative response of legumes to ozone. Bassin *et al.* (2007) fumigated an alpine pasture for three years with mean concentrations of 20 and 40 ppb of ozone above ambient, detecting negative effects on the ecosystem respiration and canopy greenness (reduced positive effects of N on greeness of the canopy trhough accelereated leaf senescence) but not on productivity.

Besides the impact of ozone on these communities, cutting and grazing is also of great importance. Intensive plant growth and leaf development are distributed over much of the growing season in a managed temperate grassland, which increases the probability of co-occurrence of ozone-sensitive developmental phases and ozone peaks. After cutting, the microclimatic conditions change and low-growing species which have been previously protected by the canopy can now be exposed to higher ozone levels, and to more light, during the initial phase of re-growth (Bassin *et al.*, 2006). Jaeggi *et al.* (2006) found a two-layered structure in their grass canopy, in which species occupying the lower part of the canopy were less exposed to ozone than taller species. In one of the few studies looking at the effects of cutting and ozone in combination on species composition, Ashmore & Ainsworth (1995) found decreased biomass in *Trifolium repens,* but increased biomass of *Festuca rubra,* in the high O_3 treatment (70 ppb 8h mean) when it was uncut. In the cut treatment, the biomass of *T. repens* declined again, but this time *Agrostis capillaris* increased in the high ozone treatment. Overall the response of forbs to ozone was greater in the cut pots. Wilbourn *et al.* (1995) observed not only effects of ozone on the yield and species composition of dominant grasses, but also on the capacity of *T. repens* to recover between ozone episodes or between cuts; the species replaced ozone-damaged leaves quite quickly but with a loss of stolon density. Accoring to Ryle *et al.*, 1981, a very high proportion of the carbon fixed in the leaf is partioned into the stolon, hence they eplained the decrease of stolon density with the loss of resources in maintaining the canopy. Nussbaum *et al.* (1995) and Fuhrer *et al.* (1994) found similar results for *T. repens.* The continued growth and survival of *T. repens* depends on stolon development and replacement, (Frame & Newbould, 1986) and its vigour in spring is related to the amount of stolon over-wintering (Harris *et al.*, 1983), so effects of ozone, especially ozone episodes in summer, may have lasting effects on the survival of that specie.

1.8 Peatlands

Peatlands can act both as sources and sinks for atmospheric carbon. Especially important is their dual role as a sink for CO_2 and as a source of CH_4. The C reservoir of Northern latitude peatlands is estimated to be 270-370 x 10^{15} g, which is more than 20% of the total global soil C pool (Post *et al.*, 1982; Turunen *et al.*, 2002). The strength of the C sink of a peatland is determined by the balance between CO_2 fixation (photosynthesis) and decomposition. The decomposition process is slowed down under the waterlogged, acid and cool conditions at Northern latitudes, leading to increased accumulation of the plant material which forms the peat. Depending on environmental conditions, such as drought, a peatland may change from a sink into a source of CO_2. In the anoxic peat horizon, methanogenic bacteria produce CH_4. Plant roots provide the substrate (e.g. root exudates and/or decay) for CH_4 production that penetrates this anoxic zone. The CH_4 reaches the atmosphere primarily via three main pathways, namely diffusion, ebullition and plant-mediated transport through the aerenchyma (Siegel *et al.*, 1995; Rinnan *et al.*, 2003). A large amount of CH_4 is generated in the anaerobic peat layers, so there is typically a CH_4 gradient from peat to the atmosphere that drives molecular diffusion. Overall, diffusive flux is slow compared to the other two transport mechanims but it is important biogeochemically by facilitating the contact of CH_4 with methanotrophic community in the upper aerobic layer (Whalen, 2005). Ebullition is a process that releases CH_4 into the atmosphere in the form of gas bubbles. Owing to very high rates of methanogenesis, supersaturation of CH_4 can be found in the porewater of deep anaerobic peat layers. When the partial pressure of all disolved gases in solution is greater than the hydrostatic pressure in peat, gas bubbles are formed (Chanton & Whiting, 1995). Some vascular plants in peatlands develop an internal gas-space venitlation system (aerenchyma) to provide aeration for submerged organs in anoxic peat under the water table (Jabsson *et al.*, 1999). These aerenchymatous tissues can serve as gas conduits for the CH_4 transport from roots in the anaerobic zone to the atmosphere, bypassing the aerobic,

methane-oxidizing peat layers (Whalen, 2005). Two major mechanisms are involved: molecular diffusion (upward diffusion gradient through respiratory uptake of oxygen by plants) and bulk flow (driven by pressure differences such as temperature or water-vapor pressure between the internal air spaces in plants and the surrounding atmosphere generate a pressure gradient) (Joabsson *et al.*, 1999). CH_4 emissions are thereby affected by organic substrate supply, water table depth and the peat acidity (Lai, 2009).

Although it is assumed that vascular plants of peatlands may have a high ozone uptake, as their stomata are less likely to close in dry summers due to their moist habitats, peatlands have to date received little attention in research on ozone impacts (Franzaring *et al.*, 2000; Power & Ashmore, 2002). There are hardly any studies on vascular mire plants. Mörsky *et al.* (2008) studied the shoot length, and the structure of *E. vaginatum,* leaves which were exposed in an open-air ozone exposure system to elevated ozone for about 4 years. They found that neither the shoot length nor the structure of the sedge was affected by ozone, but the total number of leaves increased towards the end of the experiment.

Rinnan & Holopainen (2004) exposed three vascular plant species (*Eriophorum vaginatum, Vaccinium oxycoccus* and *Andromeda polifolia*) and three moss species (*Sphagnum angustifolium, S. magellanicum* and *S. papillosum*) to a concentration of 0, 50, 100 and 150 ppb of ozone in growth chambers for 4 or 5 weeks. For the vascular plants, they found that O_3 exposure reduced chloroplast size in *E. vaginatum* and also reduced the relative area of starch but not in number and total area of plastoglobuli. *V. oxycoccus* and *A. polfolia* showed an ozone induced thickening of the cell wall and an increased size of plastoglobuli under summer conditions, whereas under autumn conditions there was a transient increase in chloroplast and starch areas, and in the number and size of plastoglobuli. *Sphagnum* moss leaves from the capitulum showed a decrease in chloroplast area and in granum thickness plus various changes in plastoglobuli and cell

wall thickness. However, the significance of these anatomical changes is uncertain.

There have been few other studies on responses of peat mosses to ozone fumigation (Gagnon & Karnosky, 1992; Potter *et al.*, 1996 a,b; Niemi *et al.*, 2002; Rinan *et al.*, 2003). Gagnon & Karnosky (1992) found reduced growth, photosynthesis and chlorophyll content in *Sphagnum magellanicum*, *S. flexosum* and *S. rubellum* after exposing them to 80 ppb ozone for 10 weeks. Potter *et al.* (1996 a, b) examined the susceptibility of four *Sphagnum* species to 150 ppb for 6 hours in controlled cabinets. They detected lowered net CO_2 assimilation and quantum efficiency of photo-system II (F_v/ F_m), and higher membrane leakage of potassium, only in *S. recurvum,* in this short -term and also in a longer term study (70-80 ppb for 6-9 weeks) in controlled environment cabinets and open-top chambers. Niemi *et al.* (2002) fumigated fen communities with four different concentrations (0, 50, 100, 150 ppb) in autumn and summer for 4-6 weeks. In autumn, they detected a significantly higher conductivity and magnesium leakage in *Spagnum angustifolium.*

Questions remain whether tropospheric ozone may upset the balance of the carbon cycle in peatlands via its influence on peatland vegetation, and there is limited information available on long-term effects of ozone on methane dynamics in peatlands. Niemi *et al.* (2002) found, during their summer experiment, no effect on gross photosynthesis but an increase in dark respiration of the ecosystem and more than doubled methane emissions following O_3 exposure of 100 ppb for 6 weeks. Similar substantial changes in carbon cycling were also found by Rinan *et al.* (2003). Elevated ozone concentrations (100, 200 ppb for 7 weeks) increased the dark respiration of the ecosystem and reduced the net CO_2 exchange over time in microcosms dominated by *Eriophorum vaginatum* L. and *Sphagnum spp.* Mörsky *et al.* (2008) studied methane dynamics of boreal peatland microcosms in response to elevated ozone (2 x ambient) in open-field conditions. They observed only slight increases in methane fluxes in the elevated ozone

treatment towards the end of the experiment. They also found that microbial biomass was higher in elevated ozone and they also observed more organic acids in elevated ozone. Jones *et al.* (2009), in a study of impacts of elevated ozone on peatland below-ground dissolved organic carbon (DOC) characteristics, reported changes in DOC only for fens but not for bogs. They reported a decline of 55% of DOC in the fen cores exposed to elevated ozone, and rising values of pore water specific UV absorbance (SUVA).

1.9 Ozone flux

As current concentrations of ozone across Europe are very likely to cause damage to many types of vegetation, robust indicators of risk are needed for policy assessment. In 1994, the United Nations Economic Commission for Europe (UNECE) and the Convention on long-range Transboundary Air Pollution (CLRTAP) first set critical levels (the atmospheric concentrations of pollutants in the atmosphere above which adverse effects on receptors, such as human beings, plants, ecosystems or materials may occur according to present knowledge (LRTAP Convention, 2007b). These critical levels were based on atmospheric concentrations of ozone at the top of the plant canopy. Based on the results of many OTC-experiments, a cut-off concentration of 40 ppb for the protection of plants against ozone was set and the ozone exposure was characterised as the accumulated ozone exposure over this threshold of 40 ppb (AOT40). Fuhrer *et al.* (1997) reviewed critical levels for ozone effects on vegetation in Europe. There are two major approaches in defining critical levels. The Level 1 critical level approach provides an environmental standard/threshold to minimise the effects of ozone on sensitive receptors, but dos not seek to quantify the impacts of exceeding the critical level under field conditions: Use of the AOT40 concept gave a good linear fit to experimental data from open-top chambers for arable crops but not for trees and not for semi-natural communities. The available data allowed the threshold for defined adverse effect (e.g. 5% loss of yield) to be defined. However, major uncertainties exist in relation to the choice of response parameters and species, absence of

data for many receptors, and extrapolation of data to field conditions from relatively short term OTC experiments. TheLevel 2 approach is to estimate the impacts of ozone in the field and defines a range of critical levels depending on vegetation type, soil conditions, climate and other information, which is still poorly developed to date. In practice, the ozone critical levles have employed a Level 1 approach (Fuhrer *et al.*, 1997). There is limited data on the responses of semi-natural vegetation to ozone, but despite these limitations critical levels are available. For communities dominated by annuals an AOT40 of 3 ppm h accumulated over three months and for communities dominated by perennials, an AOT40 of 5 ppm h accumulated over six months have been accepted by the LRTAP Convention (Hayes *et al.*, 2007).

However, the AOT40 index does not take into account any environmental (e.g. climatic, phenological etc.) factors defining the aperture of the stomata and hence the flux of ozone into the leaf. At two international ozone workshops, the AOT40-based critical levels have been reviewed and there has been a general agreement that a flux-based methodology provides a biologically stronger metric for the determination of ozone critical levels and risk assessment. Stomatal flux describes the movement of ozone from the outside of a leaf, through the stomatal pore and into the air spaces inside. It is modelled by predicting the transport of ozone through the stomatal pores per unit leaf area at any moment in time (Hayes *et al.*, 2007).

Ozone flux is partitioned to stomata and to external surfaces; hence it is also influenced by surface destruction of ozone, which depends on leaf surface characteristics and surface conditions such as wetness (Zhang *et al.*, 2006) and temperature (Fowler *et al.*, 2001). Highest ozone concentrations tend to occur under meteorological conditions which limit the flux of ozone to the plant, both because of high resistance to ozone flux across the atmospheric boundary layer to the vegetation, and because such concentrations tend to occur with high vapour pressure deficits, which lead to low values of stomatal conductance (Grünhage *et al.*, 1997). Grünhage *et al.* (1997) developed a soil-vegetation-atmosphere transfer model for ozone and used it to calculate an effective AOT40 in the canopy, taking into account the effect

of atmospheric boundary layer resistances, stomatal resistance, and the phenological stages at which ozone uptake was limited by leaf senescence.
New critical levels have now been proposed, which are based on the modelled accumulated flux (AFst), which provides an estimate of the critical amount of ozone entering the plant through the stomata and reaching the sites of potential damage inside the plant. Stomatal flux-based critical levels take into consideration the varying influences of temperature, water pressure deficit (VPD), radiation (PAR), soil water potential (SWP), ozone concentration and plant development on stomatal conductance (Coyle, 2006).

The exceedance of critical levels or fluxes is difficult to calculate on a regional or national level because the evidence from field-based or chamber studies has to be scaled up to a larger scale using modelling techniques. One approach to do this is the EMEP model, which implements a deposition module (the DO3SE model) (Emberson *et al.*, 2000; Simpson *et al.*, 2003; Tuovinen *et al.*, 2004; Ashmore *et al.*, 2007). The EMEP model can map AFst across Europe and show how this will be altered by different policy initiatives (Coyle, 2006; Simpson *et al.*, 2007).

This flux-based approach has been mainly applied to major forest and crop species, for which the parameterisation is well-developed (Mapping Manual 2008). This enables realistic ozone flux calculations to crops and forests to be made on a regional scale. However, the modelling of ozone fluxes to grasslands is still difficult and prone to errors. The application of the DO_3SE model to grasslands is so far limited in many ways: one problem is the large variety of different species and/ or functional groups in grassland communities, each of them being affected by ozone in a very dissimilar way, and with each of them having different stomatal responses The different sensitivity to ozone stress leads to important shifts in the community, favouring grasses over legumes for instance (Fuhrer *et al.*, 2003; Jäggi *et al.*, 2006). Another problem is the position of a species inside the plant canopy. Individual species inside a grassland canopy occupy layers at different heights, where they are exposed to different ozone-levels. Additionally, the ozone concentration profile inside a canopy is controlled

by the strength and distribution of sinks such as uptake via stomata (which is modified by changes in stomatal conductance (g_s) due to changing VPD and PAR within the canopy), destruction at surfaces or gas-phase reactions, and by the turbulent mixing of air into the canopy. In comparison with other ecosystems, such as those of forests and crops, grasslands reach similar leaf area indices (LAI) of 4-7 m^2 m^{-2}, but their leaf density is much higher. The few studies on in-canopy ozone concentration profiles have been focused on forests, tall herbaceous plants and agricultural crops. The major difference in comparison to grasslands is that most of the forest and crop canopies have their maximum of active leaf area in the upper part of the canopy (Jäggi *et al.*, 2006). Davison *et al.* (2003) and Jäggi *et al.* (2006) measured ozone profiles in herbaceous stands. Davison *et al.* (2003) examined the canopy of *Rudbeckia laciniata* and found a greater reduction in PAR than in ozone along vertical gradients. The ozone concentration varied from 15% to 90% of ambient levels at 50 cm above ground and PAR showed a consistent value of below 10% of ambient. However, Jäggi *et al.* (2006) observed a moderate reduction throughout the canopy in a two-layered grass canopy, with less than 20% of the LAI in the upper half. They found that leaves of *Alopecurus pratensis* at 0.5 m were exposed to 92% of the ozone levels above the canopy whereas leaves of *Trifolium repens* at 0.25 m had only an exposure of 64% of the level above the canopy.

1.10 Impact of ozone on below ground processes and nutrient cycling

Beside effects of ozone on above-ground sources (e.g. physiological changes in the leaf), ozone also alters the source-sink balance and carbon allocation below ground (Andersen, 2003). In theory, carbon allocation to various sinks is controlled both by sink demand (activity and size) and source control of photosynthate production. Carbohydrate depletion up-regulates genes which are responsible for photosynthesis, mobilization and export, while carbohydrate abundance up-regulates genes responsible for storage and use (Koch, 1996; Farrar & Jones, 2000).

Ozone may alter soil carbon pools. Elevated ozone may increase rhizodeposition by accelerating root turnover, increasing exudation, or increasing litter inputs shifting microbial communities from older to newer, more labile forms of carbon (Islam *et al.*, 2000). Decreased carbon allocation to roots would lead to smaller root systems, smaller plants and less carbon flux to soils. Long-term ozone exposure leads to shrinking labile pools, an increased decomposition of organic matter and a decreasing sequestration of carbon in soils, with important consequences for soil micro-organisms which are usually carbon limited (Zak *et al.*, 1994).

Ozone may change nutrient cycling in soils by altering litter quality and quantity (root and leaf), and by changing rates of energy flow through soil food webs. Alteration in the quantity or quality of soil carbon may affect nutrient retention in soils. As plant response to ozone is affected by nutrient availability, any change in nutrient cycling and availability, may in turn have a negative feedback in terms of plant sensitivity to ozone (Whitfield *et al.*, 1998).

Through structural or functional changes in components of the soil food web, ozone can alter the plant species' diversity indirectly as feedbacks between roots and soil microbial population have an effect on plant species' diversity and community structure (Mills & Bever, 1998). Shifts in mycorrhizal species' occurrence in response to ozone have been observed which might reflect structural changes in soil fungal diversity (Andersen, 2003). As most land plants form symbiosis with mycorrhizal fungi, their role in mediating plant responses to atmospheric change may play an important part in predicting ozone impacts on managed or natural ecosystems (Shafer & Schoeneberger, 1991). Miller *et al.* (1997) found significant interactions between ozone and symbiont type and showed that the presence of an arbuscular mycorrhizal fungus suppressed growth of host *T. subterraneum* in ozone.

1.11 Aim of thesis

Experimental work on the effects of air pollutants such as ozone on semi-natural ecosystems, as described above, is somewhat limited, and not much of the knowledge of such systems derives from community level studies. Furthermore, very few experiments have followed the changes in a semi-natural community over long periods in time, in order to establish the dynamics involved as a result of exposure to ozone. The overall aim of this study was to better understand the impacts of elevated ozone on semi-natural ecosystems. The main objectives of the study were to determine the responses of species composition, growth and ecosystem processes to ozone concentrations and flux. The work described here addressed the following specific objectives:

- to monitor the impacts of ozone on plant growth, productivity and species composition of a semi-natural grassland;
- to assess canopy dynamics, stomatal conductance and ozone concentrations within the canopy;
- to provide basis for modelling ozone flux and effects in upland grassland communities;
- to determine the effects of ozone on species composition, productivity and methane production of peat mesocosms;
- to assess the impacts of ozone on litter decomposition and to evaluate the effects of ozone on carbon and nutrient dynamics.

Two semi-natural ecosystems were investigated, a semi-natural grassland and a lowland mire. The first study was a grassland in the uplands in the Northern Pennines, which was fumigated with elevated ozone concentrations in a free air fumigation gradient system for about two years. It was suggested, that long-term exposure of ozone affects the balance of functional groups and has an effect on the frequency of individual species (Objective 1, Chapter 2).

Another component of the grassland study was the effect of ozone on the stomatal conductance of different functional groups. Next to the grassland free-air fumigation study also the long-term grassland mesocosm experiment run by Newcastle University at the Close House site was included. It was proposed that the ozone concentration within the canopy differs from the ozone concentration above the canopy, showing a vertical gradient, and that the stomatal conductance of certain species is affected by ozone (Objectives 2 and 3, Chapter 3).

The second study consisted of mire mesocosms which had been exposed to elevated ozone concentrations in open-top chamber for three years. This part of the study aimed at the effects of ozone on the C-cycle (Objectives 4 and 5, Chapter 4). The suggestions were that long-term effects of ozone alter the C-cycle: 1. through effects on dark ecosystem respiration and methane emissions, 2. through effects on the biomass of key species, 3. through effects on soil chemistry (such as changes in the N cycle) and litter decomposition (such as litter quality and microbial activity).

2. Effects of ozone on an upland grassland

2.1 Introduction

Although there have been many studies on ozone and its effects on vegetation, the main focus has been on forests and crops and relatively few studies have dealt with semi-natural vegetation. Hayes *et al.* (2006) have analyzed the available literature on the responses of semi-natural vegetation to ozone and have estimated the relative sensitivity of over 80 individual European species based on above-ground growth. Species of the *Fabaceae* family were found to be particularly sensitive. Based on the sensitivity of individual component species, Mills *et al.* (2006) suggested that calcicolous and mesotrophic grasslands are likely to be the most ozone-sensitive ecosystems, whereas mires, heathlands, swamps and tall herb fens were likely to be less susceptible to ozone-induced damage. Jones *et al.* (2006) applied a regression-based model for predicting changes in biomass of individual species exposed to ozone, based on their Ellenberg indicator values. They tested the model on the grassland 'Le Mouret' experiment in Switzerland (Volk *et al.*, 2006) and showed a model prediction of 27% decrease in above-ground biomass which was similar to the observed 23% biomass decline. Additionally, they compared 48 grassland and montane communities under the UK classification system, and found that one community of calcareous grasslands was predicted to be least sensitive to ozone while another community of calcareous grasslands was predicted to be most sensitive. They concluded that ozone sensitivity is probably driven by component species rather than by broad community types. Nevertheless, effects on individual species in isolation may not be good predictors of effects in whole communities, and more field experiments are needed on grassland communities.

In order to assess the impacts of ozone on semi-natural vegetation, several potential sites were investigated in 2006-2007 to locate a site with non-acidic semi-natural species-rich grassland suitable for a field fumigation experiment. One, close to West Linton, South of Edinburgh was spatially very limited and too close to a major trunk road; another, on disturbed ground close to Dunbar cement works, was at risk of development for a new road. Hence, attention turned to two sites close to Hexham, Northumberland. Both were on land that was managed for conservation, but one appeared to have been better managed, and was close to electrical power. The site finally chosen was a mesotrophic grassland in the uplands of Northumberland.

This field site is situated in High Keenley Fell and is under the Environmental Stewardship management. A new free air ozone fumigation system using a fumigation gradient was set up in April 2007 and started to fumigate in July 2007. To my knowledge, only two free air ozone fumigation systems targeting fully established grassland communities have been set up so far, both in Switzerland (Volk *et al.*, 2006; Bassin *et al.*, 2007), with this being the first free air ozone fumigation system applied to grassland in the UK.

Exisitng urban models runs for the UK only consider the 2020 time horizon (e.g. AQEG, 2008), and so far only modelling work for London projected into the future to 2100 has been completed. This modeling work has been based on a Pollution Climate Model which is an empirically-based model which uses an oxidant partitioning methodology, in conjunction with NO_x emissions maps and projections, which takes into account changes in the background concentrations of O_3 and the local NO_x titration effect over the century. One scenario representing gradually increasing background O_3 by 0.3% per year, showed a substantially increase from 12 ppb in 2000 to 30 ppb in 2100. In a second scenario, using a decreasing background O_3 by 0.2%, the increase in O_3 was effectively halted by 2050 and remained close to 20 ppb for the remainder of the century (The Royal Society, 2008). As the project aimed at assessing the effects of the increased ozone background concentrations that have been predicted for the years 2050-2080, this study focused on the measurement of

plant responses to target ozone concentrations of +10 ppb and +25 ppb as 24h mean concentrations. During the vegetation period of 2007, the study concentrated on establishing a good baseline, assessing the plant cover and determining the main soil constituents of the study plots. A first harvest was conducted at the end of the vegetation period and a second harvest in 2008.

The work reported in this chapter aimed to test the following hypotheses:

i. Long-term ozone exposure affects the frequency of individual species
ii. Long-term ozone exposure has an impact on the balance of functional groups (e.g. grass/forb ratio)
iii. The response of a plant community to ozone can be predicted from the response of individual species to ozone.

2.2 Methods

2.2.1 Field site

The field site, a mesotrophic grassland, is located in the uplands of Northumberland at High Keenley Fell, Allendale, about 18 km from Hexham (NY 7922 5586). It lies at an altitude of 360 m above sea level. Its N-deposition rate[1] is estimated to be 22 kg ha^{-1} a^{-1}. The field is managed under the Higher Level Stewardship Scheme (HLS), for creation, maintenance and restoration of species-rich, semi-natural grassland. The management plan is described as follows. It is grazed by sheep (Scottish Blackface, a large breed of >70 kg) in spring until eight weeks before the cut, in order to open-up the sward, and grazed post-cut in September in order to achieve a sward height of between 2 cm and 10 cm in October/November. For haymaking the meadow is cut, and the hay is dried on site and removed once each year. Under the HLS there is only one manure treatment per year (well-rotted farmyard manure at 12.5 t ha^{-1} maximum) and no other application of nutrients such as fertilizers or other organic manures are allowed, although soil pH may be raised to 6.0 with lime on neutral grassland. Ploughing, sub-surface cultivation and reseeding are not permitted. This management plan is supposed to increase the species diversity within the field[2].

2.2.2 Ozone fumigation system

The experimental area is on level ground at the foot of a slope which rises to the SW, providing an uninterrupted air flow of around 200 m upwind (Figure 4 and Figure 5). The experimental area is situated 10 m to the SE of a cabin

[1] www.apis.ac.uk
[2] http://www.defra.gov.uk/erdp/schemes/hls/default.htm

containing monitoring and control equipment (see Figure 6), and comprises three replicate treatments.

Each treatment comprises a 6 m long pipe through which ozone is released at a controlled rate, so as to maintain an air concentration at the vegetation surface at 10 m downwind (NE) of the release pipe of 20 ppb above ambient (this was adjusted to 30 ppb in 2008). This is projected to produce a long-time average enhancement of the ozone concentration of 10 ppb, assuming that fumigation will occur over 50% of time (adjusted to 15 ppb in 2008), ozone is generated and released only when the wind is blowing from the SW (between 245° and 295°, later adjusted to 180°-270° in 2008), and when wind speeds are sufficient to ensure dispersion of the gas. Each replicate has an independent ozone generator (ozone is generated by electrical discharge from pure oxygen, which is generated on site), which is controlled real time using monitoring of ozone concentrations in ambient air, at 10 m downwind from the release pipes. All three replicate systems are under computer control, which also checks for malfunctions that could produce abnormally high concentrations. In such an event, ozone generation is terminated at source.

Figure 4: View to NE from site of the monitoring cabin, looking across the experimental area before equipment was installed.

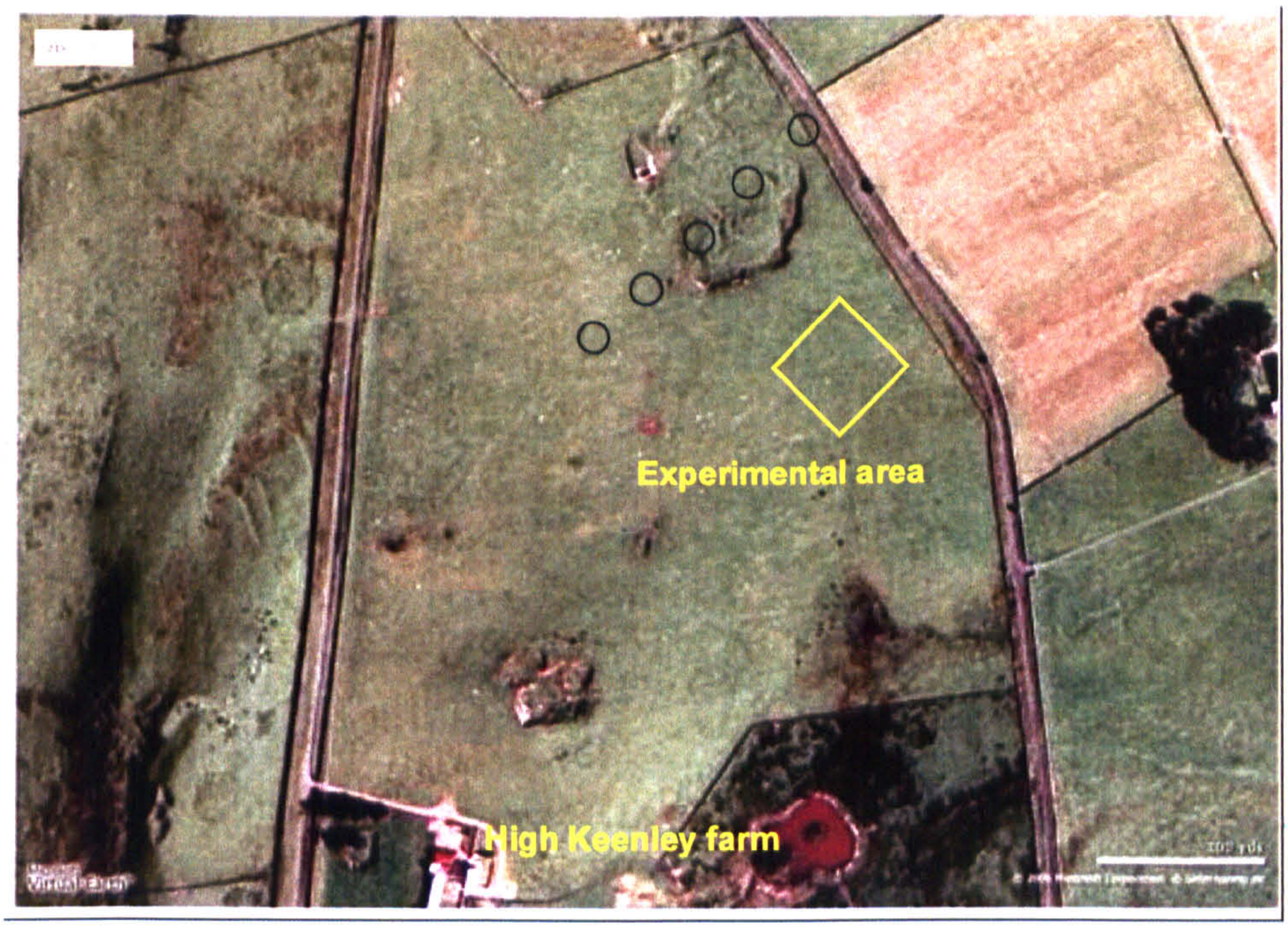


Figure 5: Aerial view of High Keenley farm showing experimental area[3] .

[3] picture copyright Microsoft Corporation, https://local.live.com/

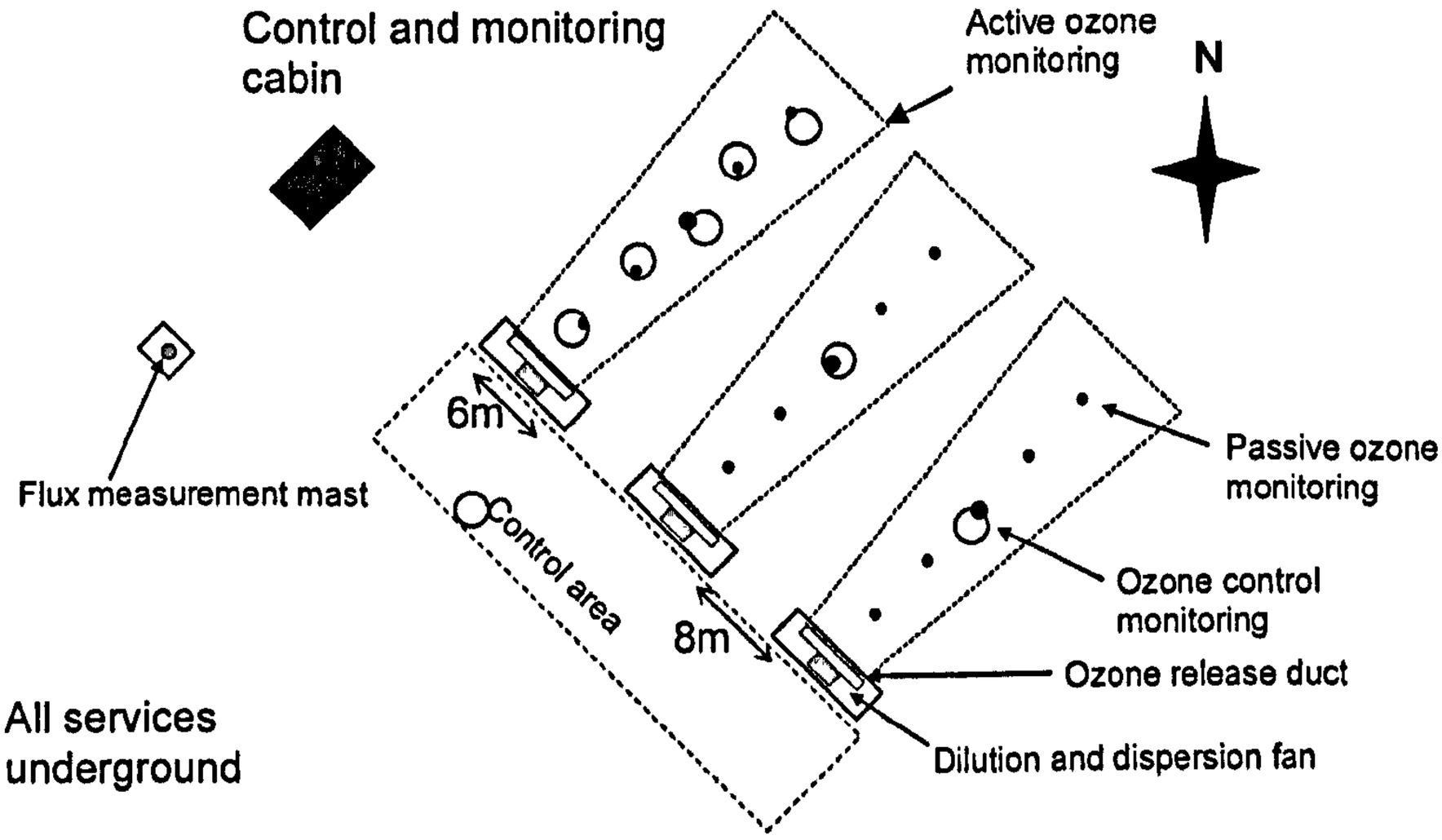


Figure 6: Schematic diagram of experimental design, showing relative location of the 3 replicate areas, the cabin and the flux mast.

The layout of the experimental area (Figure 6) shows a region 10 m wide upwind of the release pipes, to be used as a 'control' area exposed only to ambient ozone. Sampling sub-plots were laid out 2.5 m downwind of the release pipes, where target concentrations were 75 ppb above ambient, 5m downwind, where target concentrations were 25 ppb above ambient, and at 10m downwind, where target concentrations were around 10 ppb above ambient and in the control area.

Ozone exposure data for 2007 and 2008 in the three transects at 10 m downwind of the release pipes is listed in Table 1 and Table 2. In 2007 the three fumigation units released about 40% less ozone than in 2008. The mean ozone concentrations released from the fumigation units were about 23 ppb in 2007, whereas in 2008 the ozone concentrations were about 40 ppb. The mean achieved ozone concentrations at 10 m downwind of the release line were about 33.6 ppb and threshold level (AOT40) was at 4397 in 2008. The O_3 concentrations measured at different distances along Transect A in 2007 and

2008 are presented in Figure 7, Figure 8 and Figure 9. The projected additional ozone concentrations of ambient plus 10ppb at 10 m downwind were not achieved in 2007. In 2008, the mean ozone concentrations were higher than in 2007 and were closer to a target of 20 ppb at 10 m downwind from the release line A. In Figure 9 the average O_3 concentrations along each transect in 2008 measured with passive samplers (Gradko tubes) is shown. The ozone gradient in Transect B was overall lower than in the Transects A and C. The difference is largest at 2.5 m and 5 m downwind, while at 10 m downwind the ozone concentrations varied less between the transects.

A measurement mast was installed at 10 m to the S of the cabin, and fitted with a rapid-response ozone analyzer and sonic anemometer, and an inlet to measure CO_2 and water vapour concentrations by infrared photometry. The sonic anemometer also provides wind speed and wind direction data to the ozone release program. Gas fluxes were measured using eddy covariance, and in SW flow provide data on the fluxes of ozone, CO_2 and H_2O vapour to/from the grassland canopy upwind of the experimental area.

The Centre for Ecology and Hydrology (CEH) Edinburgh built the fumigation system in collaboration with the University of Newcastle (Professor Jeremy Barnes and Dr Simon Peacock). Most of the CEH effort was from Professor Neil Cape (experimental design and project management) and Dr Mhairi Coyle (computer programming and flux measurement installation). Dr Simon Peacock was mainly in charge of managing the site and took the lead on practical aspects associated with the day-to-day operation of the experimental site.

Table 1: Percentage of time (June 2007-August 2008) the system was in operation, showing the % possible ozone fumigation, the ozone concentrations released from the three fumigation units (Transect 1, 2 and 3), and the ozone concentrations from the ambient control unit.

	% possible	*Ambient [ppb]*	*Transect 1 [ppb]*	*Transect 2 [ppb]*	*Transect 3 [ppb]*
Jun-07	3%	17.2	19.1	17.3	17.6
Jul-07	54%	18.5	19.4	18.6	18.4
Aug-07	21%	19.8	23.1	20.7	20.6
Sep-07	14%	27.2	27.1	27.0	26.9
Oct-07	24%	24.9	28.0	26.4	25.6
Nov-07	36%	22.4	24.5	22.9	22.5
Dec-07	9%	33.0	32.7	31.4	32.0
Mean	**23%**	**23**	**25**	**23**	**23**
Jan-08	0				
Feb-08	5%	35.2	43.7	47.9	52.0
Mar-08	49%	48.9	49.6	48.3	49.4
Apr-08	78%	47.6	48.9	48.3	48.9
May-08	79%	42.5	43.4	43.6	43.8
Jun-08	76%	31.0	36.3	35.7	35.0
Jul-08	77%	26.4	29.8	29.6	29.6
Aug-08	76%	26.4	29.8	29.6	29.6
Mean	**63%**	**37**	**40**	**40**	**41**

Table 2: Ozone exposure data for 2008 at 10m downwind of the release pipes. 8 h mean, and the accumulated exposures over a threshold of 40 (AOT 40) of ambient and elevated treatments of the fumigation units A (Transect 1), B (Transect 2), and C (Transect 3). The AOT40 values are based on 12 minute means.

	Ambient	*Transect 1*	*Transect 2*	*Transect 3*
mean O_3 [ppb]				
May-08	43.0	43.7	42.5	44.1
Jun-08	31.0	34.9	33.5	33.9
Jul-08	26.4	29.2	29.0	29.0
Aug-08	23.0	28.4	27.0	28.7
average	**30.8**	**34.0**	**33.0**	**33.9**
AOT40				
May-08	1785	2181	1659	2289
Jun-08	133	1273	1002	784
Jul-08	205	530	449	590
Aug-08	10	870	672	890
sum	**2133**	**4855**	**3782**	**4554**

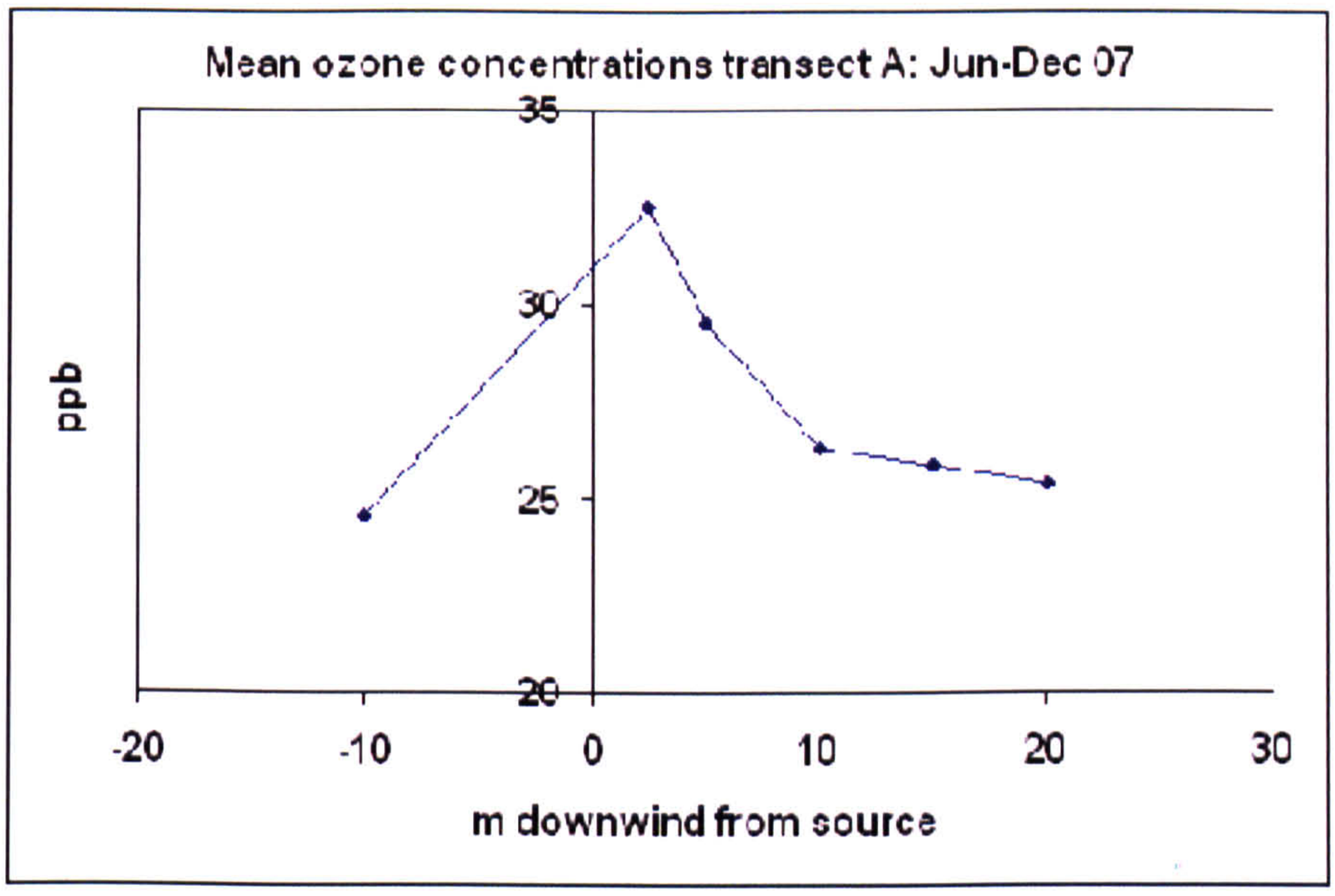


Figure 7:The average ozone concentration measured with a Dasibi analyzer along Transect A (Transect 1) for the period Jun-Dec 2007.

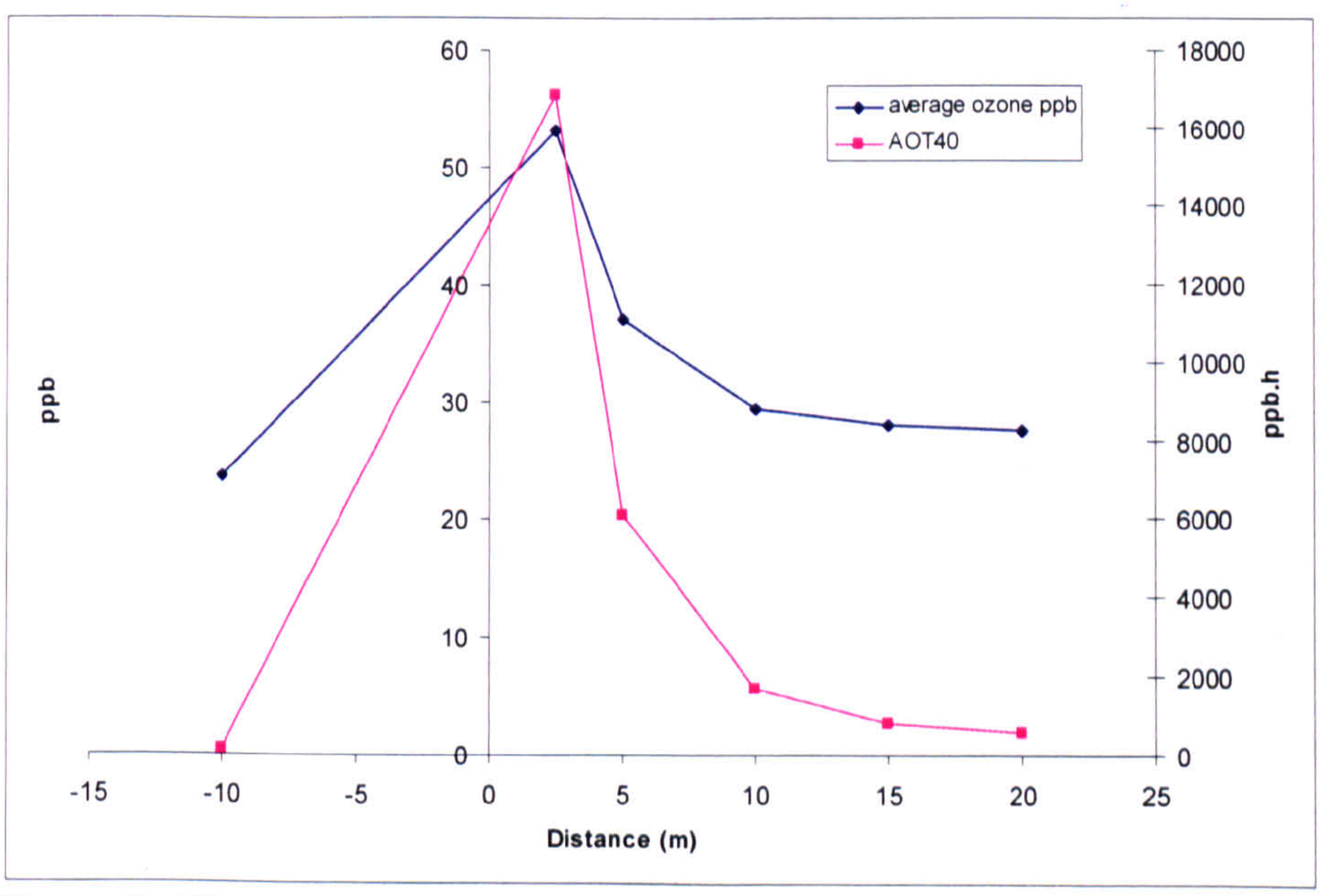


Figure 8: The average ozone concentration measured with a Dasibi analyzer along Transect A (Transect 1) for the period Jun-Aug 2008.

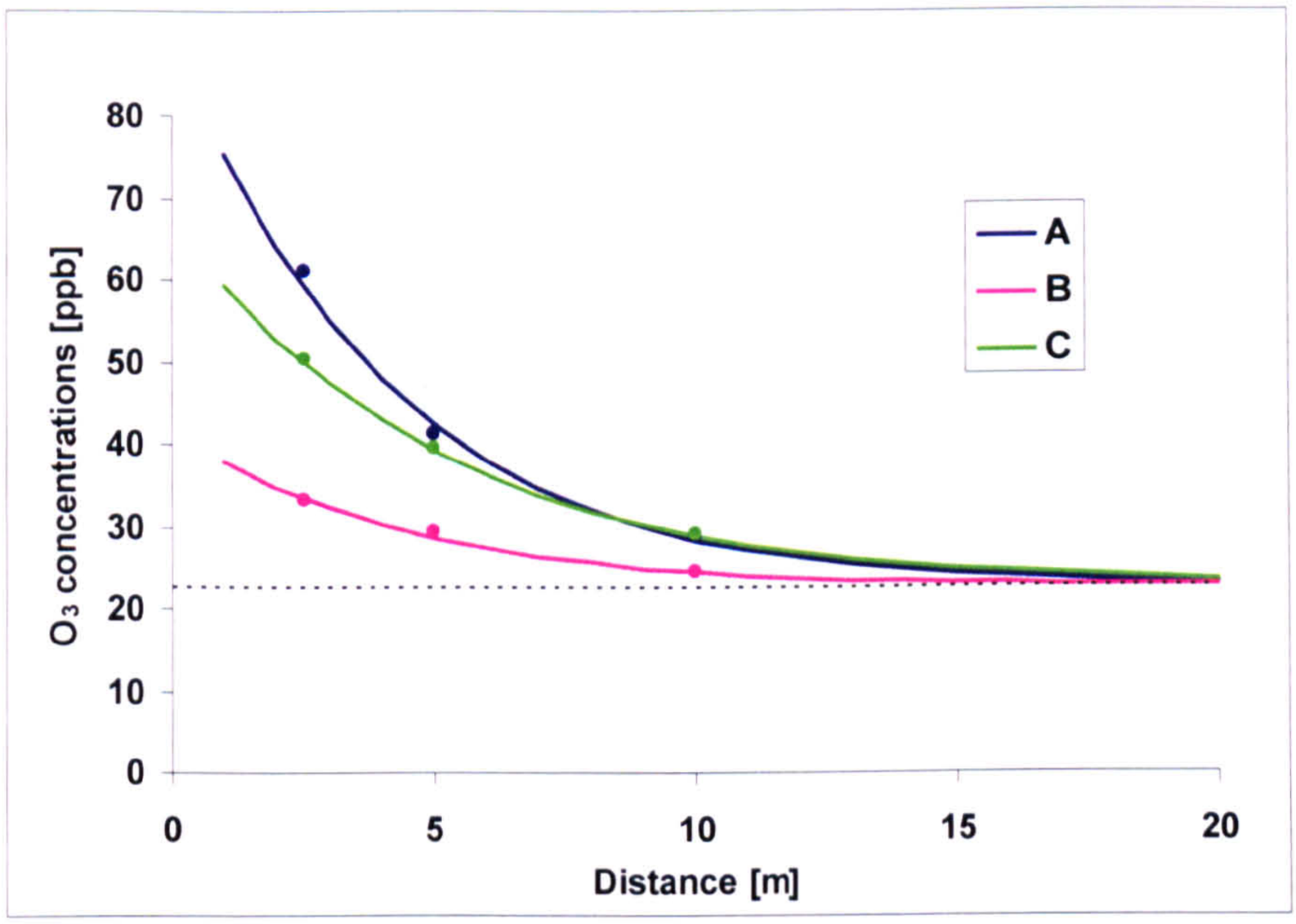


Figure 9: Average O_3 concentrations along each transect in 2008. Modelled decrease in ozone based on excess ozone = C0.exp(-kx), or ln(excess) = ln(C0)-k.x (corrected Gradko diffusion tube data up to September 2008, using correction factor of 1.52).

2.2.3 Weather conditions at the field site

Climatic data collected from the measurement mast during the vegetation period in 2007, are listed in Table 2.3. The highest monthly mean temperatures were recorded in July with 21.4°C. Whereas the monthly mean temperatures ranged from 9.5-21.4°C, the humidity levels were high and stayed at 80-90%. Monthly rainfall was low during the whole vegetation period with 221 mm; the highest rainfall was recorded in July. Photosynthetically active radiation (PAR) reached its highest mean in July at 344 μmol m^{-2} s^{-1}. The highest mean ambient O_3 concentrations were measured in July with 26.6 ppb; the monthly mean O_3 concentrations ranged from 22.7 -26.6 ppb (Table 3).

Table 3: The monthly means of photosynthetically radiation (PAR), temperature (T), humidity (RH) and ambient O_3 concentration and the total monthly rainfall during the main vegetation period in 2007.

Month	*PAR [µmol m^{-2} s^{-1}]*	*T [oC]*	*RH [%]*	*O_3 [ppb]*	*Rainfall [mm]*
June	235.4	20.9	91.9	22.7	0.0
July	344.0	21.4	90.3	26.6	84.2
August	307.4	12.4	84.7	26.1	61.8
September	230.0	10.9	86.3	23.5	38
October	148.6	9.5	87.6	26.4	37
Total Vegetation period	**253.1**	**15.0**	**88.2**	**25.1**	**221**

In 2008, the weather was much colder. The highest monthly mean temperatures, recorded in July was 13.3°C. Overall the monthly temperatures were relatively low, with monthly mean temperatures ranging from 6.7-13.3°C, while the humidity levels were high and stayed at 80-90%. Monthly rainfall was much higher than in 2007; during the whole vegetation period rainfall was 606 mm. The highest rainfall was recorded in July. Photosynthetically active radiation (PAR) reached its highest mean in June at 375.8 µmol m^{-2} s^{-1}. The highest mean ambient O_3 concentrations were measured in May with 43.4 ppb; the monthly mean O_3 concentrations were around 10-20% lower from June until October (Table 4).

Table 4: The monthly means of photosynthetically radiation (PAR), temperature (T), humidity (RH) and ambient O_3 concentration and the total monthly rainfall during the main vegetation period in 2008.

Month	*PAR* *[µmol m^{-2} s^{-1}]*	*T* *[°C]*	*RH* *[%]*	*O_3* *[ppb]*	*Rainfall* *[mm]*
May	359.8	9.8	82.0	43.4	18.6
June	375.8	11.0	82.2	33.2	77.8
July	334.7	13.3	86.5	29.3	101.4
August	240.9	13.1	88.9	25.5	122.6
September	168.9	10.6	89.3	26.7	154.6
October	121.9	6.7	88.8	26.3	131.4
Total Vegetation period	**267.0**	**10.8**	**86.3**	**30.7**	**606.4**

2.2.4 Characterization of the field site – preliminary surveys (2006-2007)

The potential site at High Keenley Fell was first selected in early September 2006. An overall species list was established, and the part of the field site suitable for the ozone fumigation was given special attention (Table 5). The species composition of the field site was assessed using the Domin scale (A system devised by K. Domin for describing the cover of a species in a vegetation community. The scale ranges from simple presence through 10 grades of linked cover-abundance measures. The scheme is based on the original (1927) five-point cover scale of Braun-Blanquet, but the finer subdivisions allow more detailed interpretation (sensu Dahl & Hadac, 1941)) in a preliminary survey which, according to a key of upland types[4], revealed the vegetation to be a *Festuca rubra – Holcus lanantus – Anthoxanthum odoratum* grassland. This type of grassland is not described in the NVC classification, but has been characterised by Rodwell *et al.* (1998). Species identification and nomenclature in all surveys followed Hubbard (1984) and Stace (1997).

[4] www.jncc.gov.uk

Table 5: List of the grass and forb species found at High Keenley Fell in September 2006.

Forbs	*Grasses*
Achillea atrata	*Agrostis capillaris*
Achillea millefolium	*Alopecurus pratensis*
Bellis perrenis	*Arrhenatherum elatius*
Cardamine pratensis	*Anthoxanthum odoratum*
Centaurea nigra	*Bromus hordaceus*
Cerastium fontanum	*Cynosurus cristatus*
Cirsum arvense	*Dactylis glomerata*
Conopodium majus	*Festuca rubra*
Euphrasia officinalis	*Holcus lanatus*
Heracleum sphondylium	*Lolium perenne*
Hypochaeris radicata	*Poa trivialis*
Lathyrus pratensis	*Trisetum flavescens*
Leontodon hispidus	
Leontodon acris	
Lotus corniculatus	
Mentha arvensis	
Plantago lanceolata	
Prunella vulgaris	
Ranunculcus acris	
Ranunculcus repens	
Rumex acetosa	
Taraxacum spec.	
Trifolium pratense	
Trifolium repens	
Veronica chamaedrys	
Vicia sativa	

Subsequent to this, a more detailed survey was conducted in mid-September 2006, which included two transects along a line downwind from the provisional ozone release points. Transects were 30 m in width and 90 m in length, aligned Southwest/Northeast. The aim was to assess whether there was a natural gradient in community composition which might confound the interpretation of the experimental ozone gradient. The cover was recorded in 12 2x2 m quadrats across the area. The cover of dominant grass species did not differ greatly between quadrats and this was also the case for some of the more important forb species (e.g. *Ranunculus acris* and *Rumex acetosa*). However, for other forb species (e.g. *Trifolium repens*, *Trifolium pratense*, *Lathyrus pratensis* and *Leontodon hispidus*) there was much greater variation between quadrats. This reflected a patchy distribution across the site that was also apparent on visual inspection (Table 6 and Table 7). Nonetheless, no systematic gradient in the cover of species was found along the two transects.

Table 6: Main forb species found at High Keenley Fell along SW-NE transects (Transect A and Transect B) across the site. The cover of the species was assessed in 2 m x 2 m quadrats via the Domin scale in September 2006.

Forb species						
Distance (m)	**15**	**30**	**45**	**60**	**75**	**90**
Transect A						
Leontodon hispidus	3	3	4	5	0	0
Taraxacum spec.	3	4	4	5	0	0
Cerastium fontanum	6	6	0	4	4	4
Cardamine pratensis	4	5	5	0	0	0
Ranunculus repens	7	6	5	6	6	5
Ranunculus acris	7	6	5	6	6	5
Prunella vulagaris	3	2	3	3	2	3
Veronica chamaedrys	0	0	0	0	0	0
Trifolium repens	0	0	6	5	7	0
Trifolium pratense	0	0	7	0	6	6
Plantago lanceolata	0	0	4	0	0	0
Lathyrus pratensis	0	0	6	0	5	6
Rumex acetosa	5	7	7	6	6	5
Transect B						
Leontodon hispidus	3	4	0	4	0	4
Taraxacum spec.	3	4	0	4	0	4
Cerastium fontanum	8	6	5	4	4	4
Cardamine pratensis	4	0	4	4	4	0
Ranunculus repens	7	6	6	6	6	5
Ranunculus acris	7	6	6	6	6	5
Prunella vulagaris	3	3	2	2	3	2
Veronica chamaedrys	0	0	6	0	0	0
Trifolium repens	0	0	6	5	0	5
Trifolium pratense	0	0	5	5	5	0
Plantago lanceolata	0	4	0	0	0	0
Lathyrus pratensis	0	0	0	5	5	6
Rumex acetosa	5	7	7	6	6	6

Table 7: Main grass species found at High Keenley Fell along SW-NE transects (Transect A and Transect B) across the site. The cover of the species was assessed in 2 m x2 m quadrats via the Domin scale in September 2006.

Grass species						
Distance (m)	**15**	**30**	**45**	**60**	**75**	**90**
Transect A						
Dactylis glomerata	10	10	10	10	10	10
Agrostis capillaris	9	9	9	9	8	9
Cynosurus cristatus	8	7	7	8	8	8
Anthoxanthum odoratum	10	10	10	10	10	10
Festuca rubra	9	9	9	9	9	9
Holcus lanatus	10	10	10	10	10	10
Poa trivialis	9	9	9	9	9	8
Trisetum flavescens	8	7	8	8	8	8
Lolium perenne	8	8	8	8	8	7
Transect B						
Dactylis glomerata	10	10	10	10	10	10
Agrostis capillaris	9	9	8	9	9	9
Cynosurus cristatus	8	8	8	8	8	8
Anthoxanthum odoratum	10	10	10	10	10	10
Festuca rubra	9	9	9	9	8	8
Holcus lanatus	10	10	10	10	10	10
Poa trivialis	9	9	9	9	9	9
Trisetum flavescens	8	8	8	8	7	7
Lolium perenne	8	8	8	8	8	7

The September field survey was followed by a more detailed survey of the area identified for ozone exposure, which was carried out in March 2007. This time the survey focused on variation in conditions across the field site. A survey area 75 m (width) by 50 m (length) was established and species composition and soil conditions were assessed every 15 m by 10 m. Overall 24 quadrats (2m x 2m) were surveyed and 12 soil samples (5 pooled sub-samples from each quadrat) were analysed for pH, extractable nitrate (NO_3^- [μmol g^{-1} DW]) and ammonium (NH_4^+ [μmol g^{-1} DW]), and organic matter ([%]). The analytical methods used are described in Section 2.2.6.2.

The plant survey revealed no major differences compared to the first survey in September 2006. Nine grass species and ten forb species were identified. Most of the grass species had a relatively uniform cover, whereas most of the forb species, except *Rumex* and *Ranunculus,* showed very patchy distribution (Table 8).

Table 8 : Mean Domin values of the main grass and forb species found at High Keenley Fell. The cover of the species was assessed via the Domin scale. At each distance along a SW-NE transect of 75 m, four 2 m x2 m quadrats were assessed across a width of 50 m.

Distance (m)	*0*	*15*	*30*	*45*	*60*	*75*
Species (domin scale)						
Forbs						
Leontodon hispidus	1	1	1	1	1	0
Cerastium fontanum	4	4	4	5	5	4
Cardamine pratensis	4	2	3	3	4	1
Veronica chamaedrys	0	0	0	4	0	0
Trifolium repens	5	3	0	1	5	5
Trifolium pratense	0	5	0	0	5	0
Rumex acetosa	5	5	5	5	5	5
Ranunculus spec.	5	5	5	5	5	5
Conopodium majus	5	1	2	2	0	0
Vicia sativa	5	5	5	3	2	4
Grasses						
Dactylis glomerata	8	8	8	8	8	8
Agrostis capillaris	7	7	7	7	7	7
Cynosurus cristatus	7	7	7	7	7	7
Anthoxanthum odoratum	8	8	8	8	8	8
Festuca rubra	7	7	7	7	7	7
Holcus lanatus	8	8	8	8	8	8
Poa trivialis	7	7	7	7	7	7
Trisetum flavescens	7	7	7	7	7	7
Lolium perenne	7	7	7	7	7	7

The results of the soil assessment showed no obvious gradients in pH, NH_4^+/ NO_3^- concentrations, and organic matter across the potential study area. Mean values of soil parameters are summarised in Table 9. Silty clay soils were predominant across the area. Similar soils were described by Jarvis *et al.*

(1984), who described soil associations near Alston, Cumbria (18 km from High Keenley Fell), which were waterlogged for long winter periods, and showed slowly permeable loam/clay subsurface horizons. The fertility of the site was moderate, according to the ammonium and nitrate concentrations, and the organic matter content (Charman, 2007; Mausbach, 1998).

Table 9: Mean and standard error of values of soil parameters measured in March 2007 in the study area at a depth of 0-10 cm, based on 12 samples.

Soil parameters	*Mean ± SE (n=12)*
pH	5.43 ± 0.04
Extractable NH_4^+-N concentration [mg kg^{-1} DW]	11.55 ± 1.35
Extractable NO_3^--N concentration [mg kg^{-1} DW]	4.83 ± 1.08
Organic matter content (%)	16.1 ± 0.92

This survey did, however, identify a change to soil characteristics, with increased clay content (assessed via by unconfined compression test (Terzaghi, 1996)) towards the Southern edge of the proposed experimental area; this area also appeared to be wetter and have a greater frequency of sedge species. For this reason, this zone of the survey area was avoided when the long-term plots were finally marked up, partly by reducing the width of the three experimental transects.

2.2.5 Experimental design

Having identified an appropriate study area, the next step was to define the experimental design.

Filion *et al.* (2000) published a paper on experimental designs of FACE studies. They focused on "two-way nested designs" (treatments are randomly applied to distinct rings), "split-plot designs" (test of interactions between 2 treatment factors) and on "two-way factorial design with repetitions in time or space" (treatment factor is fixed and time or site is random). They also analyzed the statistical power and weaknesses of these designs. According to their findings, the most suitable study design for this project was a two-way nested design with two treatments (ambient and +10 ppb) and three replicates (critical F-value: 7.71), or three treatments (ambient, +10 ppb and maybe +20-40 ppb; and three replicates (c. F-value: 5.14). Considering the lower critical F-value and therefore the higher statistical power of the latter experimental design, a nested design with three treatments and three replicates was chosen.

Based on these surveys, and further site assessment, the experimental area was laid out in early April, resulting in a total of 36 experimental plots. These were divided between three experimental treatments (ambient, +10, +25). Within each treatment, four plots were established in each of the three replicate ozone transects. Each plot was 1m by 1m; within this area, a permanent 0.5 m by 0.5 m quadrat was initially reserved for recording of above-ground vegetation responses, with the remaining area used for other samples and measurements. In addition, a fourth treatment (+75 ppb) with two subplots (1 m by 2 m) per transect was used for measurements in 2008. Usually two subplots were chosen along each side of the transect mid-line. Due to the variability of forbs and to the activity of moles on the site, the alignment of the subplots had to be adjusted in some cases. In particular, the subplots in Transect B at 25 ppb were placed in a line (Figure 10 and Figure 11).

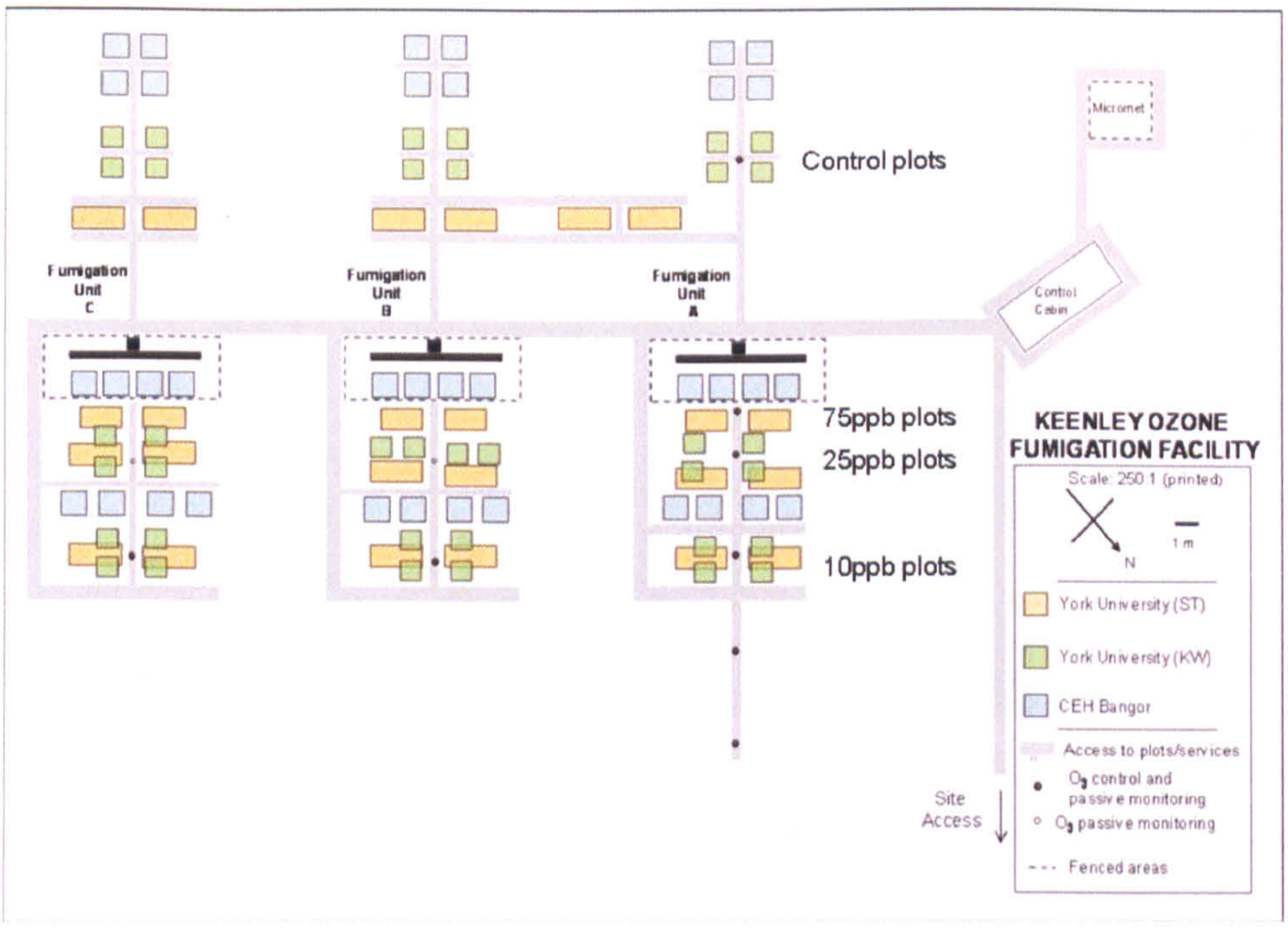


Figure 10: Keenley fumigation plot map showing different plots in use by different research groups, the three fumigation units A, B and C, the control cabin, the micromet tower and the location of passive and active O_3 samplers. The plots used in this study are the ones from York University (KW) and partly from York University (ST). Control, 10ppb and 25ppb plots are in green (■), and the 75ppb plots are in orange (▬).

Figure 11: Picture of experimental layout showing the different plots of the research groups and the three fumigations unit A, B and C (from the front to the back).

2.2.6 Survey methods

This section describes in detail the methods to assess responses to ozone within the 1 m x 1 m quadrat shown in Figure 10.

2.2.6.1 Initial plant survey

Measurements of species composition were made in April 2007, prior to the start of the ozone fumigation, in each of the 36 0.5m plots. In order to estimate the vegetation cover before the start of any ozone fumigation the plant survey was carried out with the point intercept method. The point intercept method is designed to sample within-plot variation and to quantity changes in plant species cover, height and ground cover over time. A special quadrat (consisting of a permanent 0.6 m x 0.6 m grid (horizontal and vertical) with a measurement device which can be adjusted to various distances) was set up in each subplot,

always facing the same direction (Figure 12). Within the 0.6 m grid, a sampling pole was systematically lowered every 10 cm. Only single points (contact with plant species or ground cover class) were counted. Overall 36 hits were recorded per subplot. The slope of each subplot was noted and a complete photo record of the site was taken (general and close-up view).

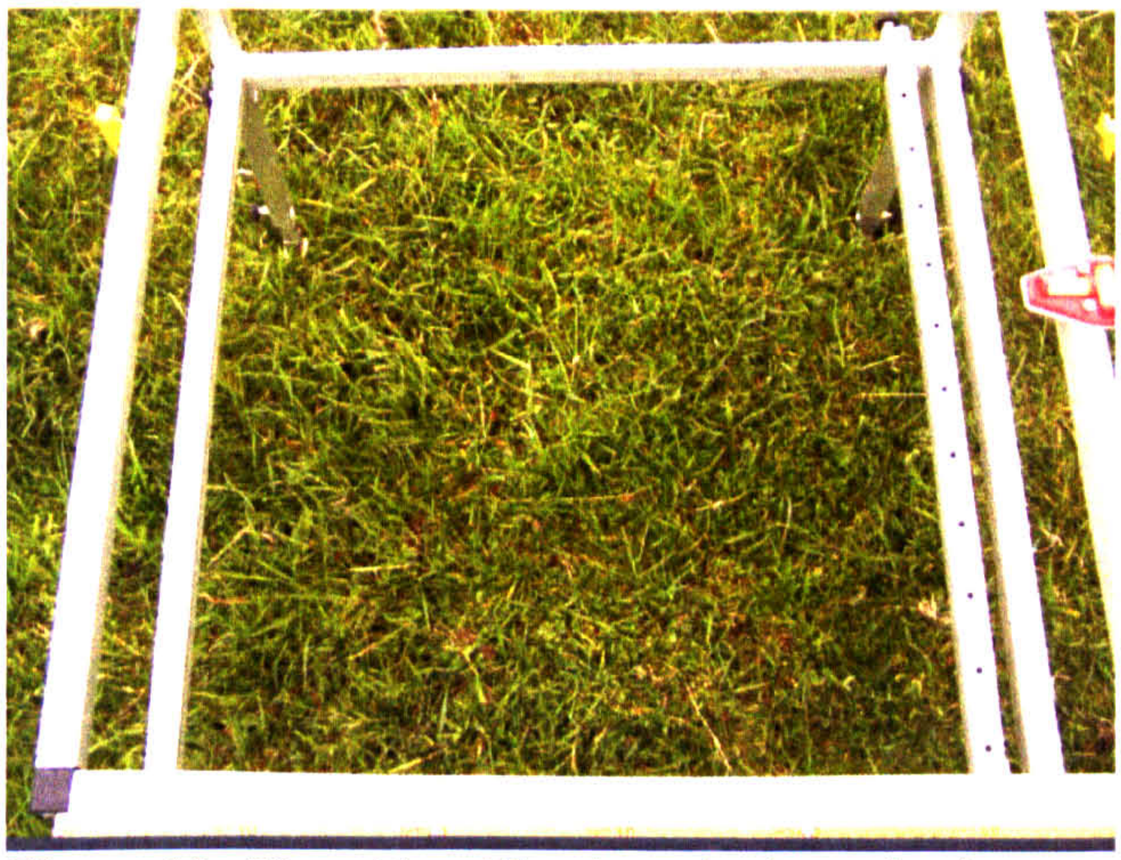


Figure 12: Plant identification via the point intercept method.

2.2.6.2 Soil survey

The remaining area of the $1m^2$ of each subplot was used for soil analysis. In April 2007 the rooting depth was checked with an auger, which showed a rooting depth of 0-20 cm. As the rhizosphere determines nutrient content and exchange, it was decided to take soil samples from two depths. Five subsamples were taken with a soil corer at five sampling points from two different depths at each subplot; 0-10 cm and 10-20 cm. Additionally an extra soil sample at each depth was taken for determination of bulk density.

Soil samples were placed in plastic bags and placed in a fridge until their extraction. The fresh soil samples were cleared of roots and stones. One part was spiked with water for pH determination and one part with 0.5 M sodium chloride (NaCl) for ammonium and nitrate-extraction (50 ml of NaCl solution or water added to 17.5 g of fresh soil; Rowell, 1994). They were all shaken for 1 hour at 120 rpm. The pH of the water sample extracts was measured using a

pH meter (Thermo Orion, model 420) and recorded directly afterwards. The NaCl soil solutions were extracted overnight using rhizon samplers which were connected to 60 ml syringes. The extra soil sample (10 ± 0.01 g) from each subplot was weighed into a crucible and placed in an oven at 105°C overnight in order to determine the soil water content. Next day, after cooling down, it was put in a desiccator and reweighed. The samples were crushed with a mortar and pestle after drying and, in order to determine the loss on ignition (LOI), were then placed in a furnace (carbolite furnace, CWF 1200) at 550°C for about 5 hours. Thereafter, samples were let cool in a desiccator and reweighed.

All NaCl extracts were placed in a freezer at -20°C. Later they were analysed for ammonium and nitrate concentrations using a continuous flow Autoanalyser (Autoanalyzer 3, Bran + Luebbe), and for potassium and calcium using an Atomic Absorption Spectrometer (AA-6300, Shimadzu). Samples for potassium analysis were diluted 1:1 with 10,000 ppm Caesium Chloride (CsCl); for calcium analysis 10,000 ppm lanthanum added in a 1:1 ratio. CsCl works as an ionization buffer (for better results in the calibration curve) and lanthanum as a releasing agent (because of interference from silicon, phosphorus and aluminium; Rowell, 1994; Smith & Cresser, 2004).

The plant available phosphorus (PO_4^{3-}) concentrations were determined using the Ohlsen method (Rowell, 1994). 5 g (±0.05) of <2 mm homogenated air-dry soil were added to 100 ml of sodium bicarbonate solution (0.5 M $NaHCO_3$) and shaken for 30 min at 120 rpm. The suspensions were extracted via rhizon samplers and syringes overnight. Extracts were stored in a freezer and then analysed using the phosphomolybdate method. Absorbance of the soil extracts were measured at 880 nm using a UV/Vis Spectrometer (Lambda 25, Perkin Elmer).

The extractable ammonium, nitrate, potassium, calcium and phosphorus concentrations were expressed in terms of soil dry weight.

In order to determine the bulk density, soil samples were taken from each depth (0-10 cm, 10-20 cm) with a corer (radius = 1.25 cm) and placed into bags. Their volume was measured by displacement (Rowell, 1994). The bulk density was expressed as g dry soil per litre.

2.2.6.3 Harvest – plants

All 36 plots were cut from an area of 0.36 m^2 (the same are used for point intercept measurements) at a height of 5 cm in early August 2007. This height was similar to the height used by the farmer in the late summer cut. The material was brought back to York where it was stored in a fridge. The material, which had been thoroughly mixed before, was sorted into individual species and weighed. Because of the large bulk of material, 200 g fresh weight of each sample was sorted into species; for the remainder only the total dry weight was recorded. Because of the mass of leaf fragments, some of the 200 g of grasses remained unidentified, whereas for forb species all the material was identified. The species abundance was later calculated as a percentage of the dry weight of the sample that was sorted into species.

In 2008, the 36 plots were cut at the same height as in 2007 but, instead of the 0.36 m^2 area the full 1 m^2 of each plot was used. The harvest took place in early August 2008. The material was brought back to York where each subplot was separated into 4 parts which were mixed thoroughly before it was sorted into species. 50 g was taken from each part, making up 200 g in total. The sorting of the species was then processed as in 2007.

2.2.6.4 Data analysis

The data summary was carried out using Microsoft Excel (2003) and the statistic analysis was carried out using SPSS 15.0. Each dataset was first explored via histograms, q-q-plots and boxplots. Examining the datasets for

skewness, kurtosis and normality using the Shapiro-Wilk-test, showed that most of the data needed to be transformed. The data was log_{10} or square-root transformed in order to gain normality. Some of the data did not reach normality despite transformation; these datasets were analysed using non-parametric tests. This applied especially to data on forbs, which were absent from a significant number of plots. For these data, both treatment and transect effects were tested using the Kruskal-Wallis test, and in order to test the significance of specific contrasts the Mann-Whitney-test was applied. To correct for Type 1 errors, and to determine significant differences, a Bonferoni adjustment of $P< 0.016$ was used.

The transformed data were tested with parametric tests. One-way-ANOVA was used to compare the means and to determine significant differences between the three transects and the three treatments. Homogeneity of variance was assessed via the Levene-test, and the significance of contrasts was tested with the Tukey-test. Differences were tested for significance at $P< 0.05$, unless the outcome of the Levene-test was inhomogeneous, in which case the P-level was set to $P< 0.01$. In order to estimate if there was an interaction between treatments and transects, a univariate general linear model was applied, with the significance tested in the same way as for the ANOVA. In order to test for significant differences between the years a simple t-test was used. The significance was tested in the same way as described above.

Multivariate analysis was carried out using CANOCO ver. 4.5 (a tool for constrained and unconstrained ordination in ecological applications; ter Braak and Šmilauer, 2002). An initial Detrended Correspondence Analysis (DCA) showed that the gradients within the soil data set (length of gradients < 1.0) and within the initial plant cover data (length of gradients < 2.1) were short. For the harvest data in 2007 and 2008, the gradients within the grass species data were short (length of gradients < 1.6 (2007), length of gradients < 1.16 (2008)), whereas the gradients within the forb species data were long (length of gradients < 4.0 (2007), length of gradients < 3.7 (2008)).

In order to interpret patterns extracted from all variations of the soil, and the initial plant cover data, an unconstrained analysis was executed using principal component analysis (PCA; an orthogonal linear transformation that transforms the data to a new coordinate system such that the greatest variance by any projection of the data comes to lie on the first coordinate (called the first principal component), the second greatest variance on the second coordinate, and so on). For the harvest data, a constrained analysis was carried out in order to extract patterns from the variation only, using redundancy analysis (RDA; an extension of multiple regression when there is more than one response (species) variable). For the grasses and the forbs this was performed using cumulative O_3 exposure as an environmental variable. Although the length of the gradients, with values of 4.0 and 3.7 within the forb species data pointed to a canonical analysis being more suitable, the outcome of this approach did not improve compared to a redundancy analysis. Therefore, the same analyses were performed for all groups of plant species. The significance of the ordination was assessed via Monte Carlo permutation. In order to get a better understanding of the significant axes of the RDA, a stepwise canonical analysis (CCA; extension of multiple regression analysis from one criterion variable to a set of criterion variables) was performed in that special case. The four most significant variables, which explained most of the species variance, were chosen for this analysis.

2.3 Results

2.3.1 Field characterisation

2.3.1.1 Plant survey – early season cover

The most dominant grass species in the early season pre-fumigation cover assessment in April 2007 were, in descending order, *Holcus lanatus, Agrostis tenuis, Poa pratensis, Anthoxanthum odoratum, Phleum bertolonii, Dactylis glomerata, Festuca pratensis, Alopecurus pratensis, Lolium perenne,* and *Trisetum flavescens,* while the most dominant forb species, in descending order of the cover, *Rumex acetosa, Cerastium fontanum, Conopodium majus, Veronica chamaedrys, Ranunculus acris, Ranunculus repens, Stellaria graminea, Trifolium repens, Lathyrus pratensis* and *Cardamine pratensis* (Figure 13 and Figure 14). Whereas the grass species had a quite uniform cover across the plots, the forb and legume species showed a very patchy distribution except for the species *Rumex acetosa.* This was consistent with the findings of the preliminary surveys in September 2006 and March 2007. The control plot in Transect 2 had a particularly low cover of forb species.

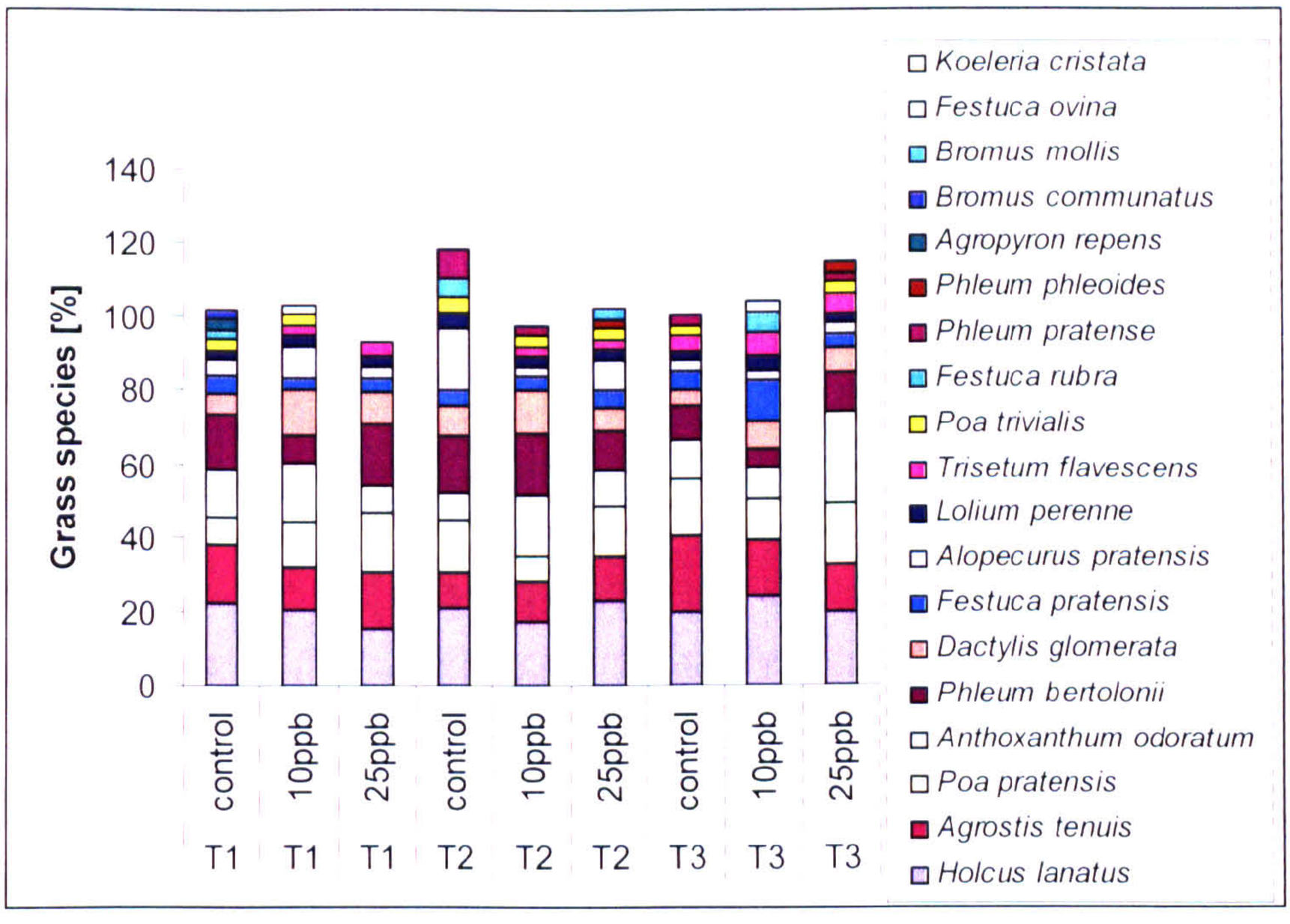


Figure 13: The mean frequency of each grass species in all nine combinations of transect (T1, T2 and T3) and ozone treatment (Control, 10 ppb and 25 ppb). Data are the mean of values for four subplots measured using the point intercept method in April 2007. Unidentified grass species are not shown.

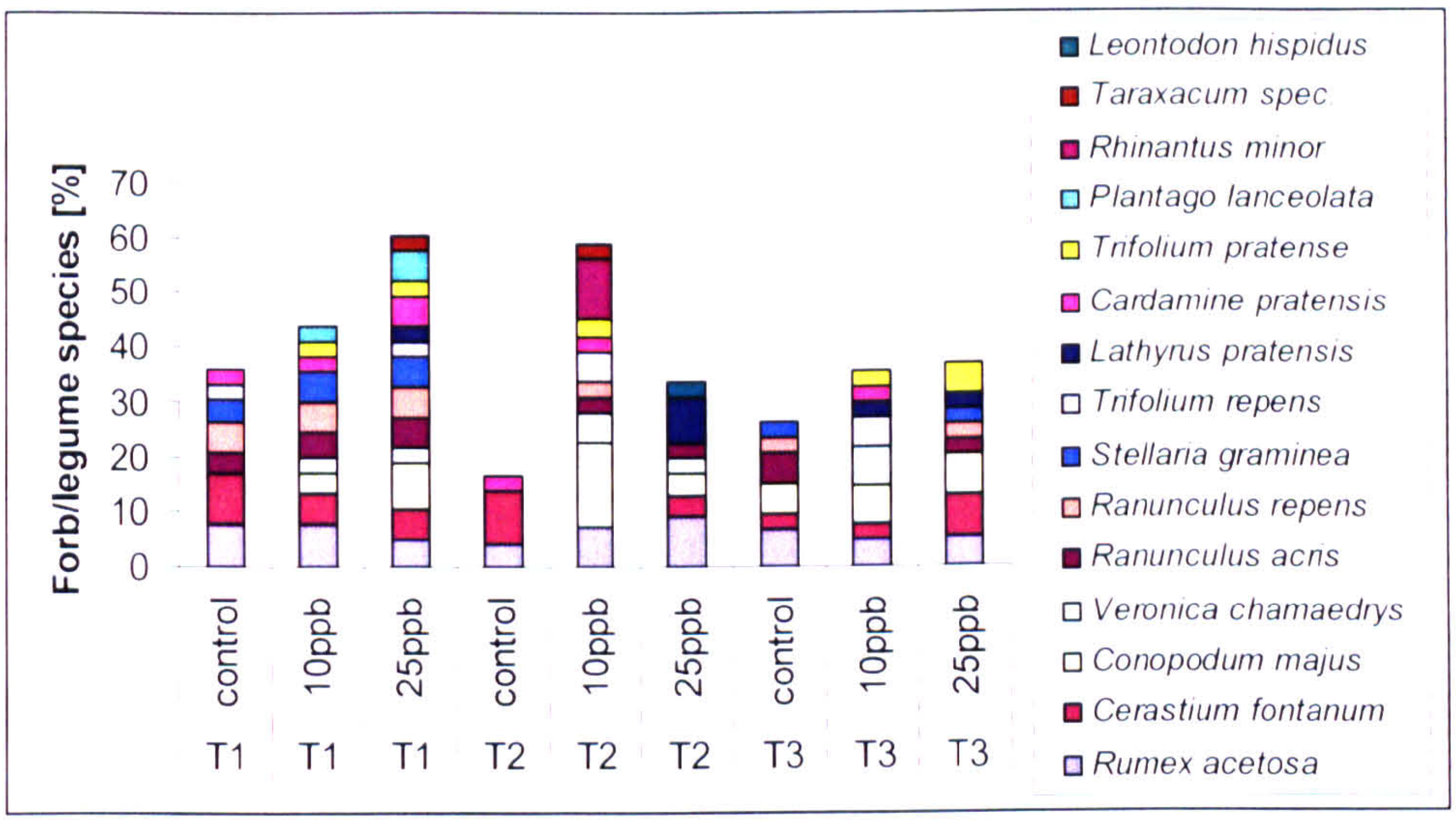


Figure 14: The mean frequency of each forb species in all nine combinations of transect (T1, T2 and T3) and ozone treatment (Control, 10 ppb and 25 ppb). Data are the mean of values for four subplots measured using the point intercept method in April 2007. Unidentified forb species are not shown.

2.3.1.2 Soil survey – soil chemistry

The soil characteristics, averaged over all the study plots, are summarised in Table 10. The results of the soil assessment showed, that overall, nutrient concentrations were higher at 0-10 cm than at 10-20 cm. The pH was around 5.5 at 0-10 cm and around 6 at 10-20 cm, being slightly acidic. The NH_4^+ concentrations were moderate; 7 mg kg^{-1} DW at 0-10 cm and 3 mg kg^{-1} DW at 10-20 cm. NO_3^- concentrations were 17 mg kg^{-1} DW at 0-10 cm and 8 mg kg^{-1} DW at 10-20 cm, pointing to a high availability of that nutrient. High organic matter contents and high soil water content were found at both depths. Organic matter was about 18.5% at 0-10 cm and 13.4% at 10-20 cm. The soil water content showed a high percentage with 58.8% at 0-10 cm and with 41% at 10-20 cm. Available phosphorus (PO_4^{3-}) concentrations pointed towards a limitation in this nutrient, with P concentrations of 6.7 µg kg^{-1} DW at 0-10 cm

and 2.6 μg kg^{-1} DW at 10-20 cm. Potassium (K^+) concentration were moderate; 16.5 mg kg^{-1} DW at 0-10 cm and with 5.3 mg kg^{-1} DW at 10-20 cm, and there was not much difference in the calcium (Ca^{2+}) concentration between depths (10.4 mg kg^{-1} DW at 0-10 cm and with 8.6 mg kg^{-1} DW at 10-20 cm). The bulk density also did not show much difference between the both depths, with a value of 0.12-0.13 g L^{-1} (Charman, 2007; Mausbach, 1998)

Table 10: Mean and standard error values (se) of soil parameters in the study area, based on 36 samples.

Soil parameters	*Mean ± SE (n=36) 0-10cm*	*Mean ± SE (n=36) 10-20cm*
pH	5.51 ± 0.05	6.01 ± 0.07
NH_4^+-N concentration [mg kg^{-1} DW]	7.19 ± 0.77	2.98 ± 0.22
NO_3^--N concentration [mg kg^{-1} DW]	16.89 ± 1.31	8.46 ± 0.39
PO_4^{3-} [μg kg^{-1} DW]	6.65 ± 1.27	2.55 ± 0.72
K^+ [mg kg^{-1} DW]	16.47 ± 2.2	5.33 ± 0.48
Ca^{2+} [mg kg^{-1} DW]	10.39 ± 0.3	8.6 ± 0.4
Organic matter content (%)	18.58 ± 0.94	13.4 ± 0.61
Soil water content (%)	58.79 ± 3.29	40.97 ± 1.85
Bulk density (g L^{-1})	0.13 ± 0.01	0.12 ± 0.01

The data obtained in April 2007 showed no significant differences between ozone treatments and transects in terms of pH and bulk density. There were significant differences for NH_4^+, K^+ and Ca^{2+} 0-10 cm, and for soil water content, organic matter content (LOI), NO_3^-, available P (PO_4^{3-}), and Ca^{2+} at 10-

20 cm (Table 11 and Table 12), between the transects. Usually Transect 3 showed higher concentrations than the other two transects.

Furthermore some soil parameters differed significantly between O_3 treatments At 0-10 cm significant differences were found for SWC, LOI, NO_3^- and Ca^{2+}, and at 10-20 cm significant differences were found for SWC and for pH. In general higher concentrations were found in the control treatment. More detail on the soil analysis is shown in the Appendix 7.1.

Table 11: The significance of effects of transect and ozone on soil parameters measured at 0-10 cm. Post-hoc differences are significant at $P < 0.05$.

Soil parameter	*Effect of Ozone*	*Post-hoc*	*Effect of Transect*	*Post-hoc*
0-10 cm				
pH	0.216	ns	0.201	ns
NO_3^--N concentrations	**0.003**	Control > 10ppb	0.794	ns
NH_4^+-N concentrations	0.839	ns	**0.000**	T1, T3 > T2
P	0.202	ns	0.153	ns
K^+	0.472	ns	**0.000**	T1> T2, T3; T3> T2
Ca^{2+}	**0.005**	Control > 10ppb, 25 ppb	**0.001**	T3 > T2, T1
Organic matter content	**0.028**	Control > 10ppb, 25 ppb	0.15	ns
Soil water content	**0.000**	Control > 10ppb, 25 ppb	0.118	ns
Bulk density	0.431	ns	0.226	ns

Table 12: The significance of effects of transect and ozone on soil parameters measured at 10-20 cm. Post-hoc differences are significant at P< 0.05.

Soil parameter	*Effects of Ozone*	*Post-hoc*	*Effects of Transect*	*Post-hoc*
10-20 cm				
pH	**0.011**	25 ppb > Control, 10 ppb	0.241	ns
NO_3^--N concentrations	0.186	ns	**0.003**	T1 < T3
NH_4^+-N concentrations	0.365	ns	0.096	ns
P	0.792	ns	**0.001**	T3 > T2, T1
K^+	0.194	ns	0.122	ns
Ca^{2+}	0.236	ns	**0.000**	T3 > T2, T1
Organic matter content	0.304	ns	**0.003**	T3 > T2, T1
Soil water content	**0.044**	Control > 10 ppb	**0.006**	T3> T2
Bulk density	0.559	ns	0.479	ns

Overall, Transect 3 was wetter, and had a higher organic matter content, than the other two transects. Nitrate, phosphorus and calcium concentrations were also greater in the Transect 3. For ammonium and potassium concentrations elevated concentrations were found in Transect 3, but higher concentrations were also found in Transect 1 than in Transect 2.

The organic matter content, soil water content, and calcium, potassium and nitrate concentrations were all higher in the control plots than in the 10 ppb and 25 ppb plots. In contrast the pH was higher overall in the 25 ppb plots.

Hence, the soil analysis revealed differences in nutrients between transects and plots, which is probably responsible for the plant distribution. Likewise a large variation of the species distribution and the species frequency is expected (more detail on the soil analysis is shown in the Appendix 7.1).

2.3.2 August 2007 harvest

Total above-ground biomass

The mean total above-ground biomass in 2007 showed no significant effect of ozone treatment (Table 13), but did show a significant gradient across the site, with biomass in each transect being different from the other two (P=0). This is presented in Figure 15. The biomass of legumes (P= 0.029) and grasses (P= 0) showed significantly greater values in Transect 3 than Transect 1. The greater total biomass and the greater biomass of grasses, and legumes in T3 were consistent with the soil fertility data presented in the previous section, which suggested that fertility overall was greater in T3. There was no significant transect effect for the forb biomass nor for the grass/ forb ratio (Table 13, Figure 17 and Figure 19).

Figure 16 and Figure 18 demonstrate the large transect effect on the grasses and legumes. The transect effect was strong for legumes but the overall biomass was only a very small fraction of that of grasses. In Figure 19 the grass/ forb ratio (including legumes) is presented. In this case a significant O_3 effect rather than a transect effect was observed. Significantly lower values were found in the 25 ppb treatment than in the control treatment (P= 0.01).

Table 13: Results of the ANOVA of effects of ozone and transect on total above ground biomass, and the biomass of grasses, forbs and legumes in August 2007. Ratio refers to the grass/ forb ratio. Post-hoc differences are significant at $P < 0.05$ (ns = non significant).

	Effects of ozone	***Post-hoc***	***Effects of transect***	***Post-hoc***
total	0.873	ns	**0**	All
grasses	0.814	ns	**0**	T1 < T3
ratio	**0.01**	Control > 25ppb	0.751	ns
forbs	0.122	ns	0.756	ns
legumes	0.231	ns	**0.029**	T1 < T3

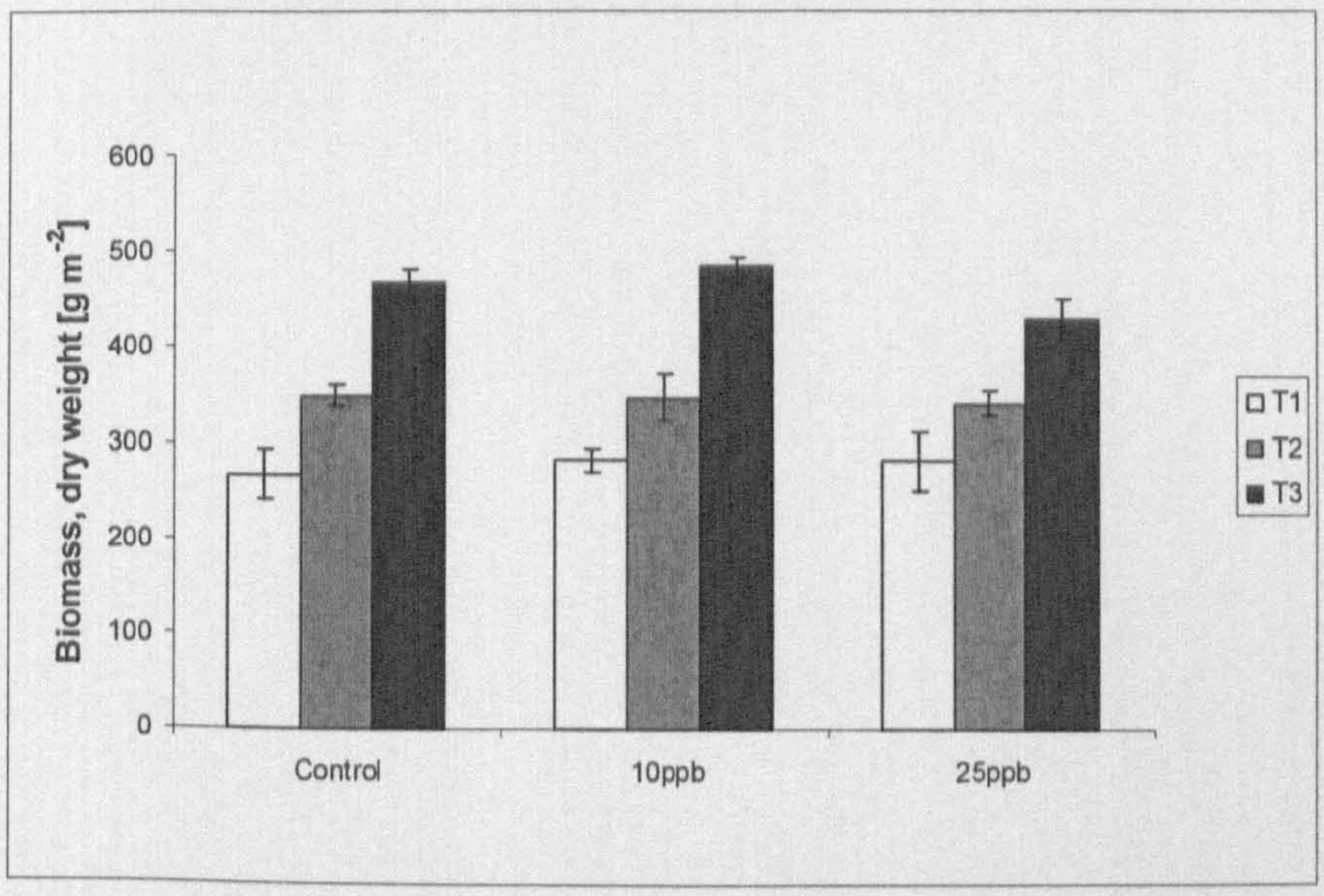


Figure 15: Mean total above ground biomass (g dry weight m^{-2}) in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2007. Error bars represent the standard error between replicate sub-plots.

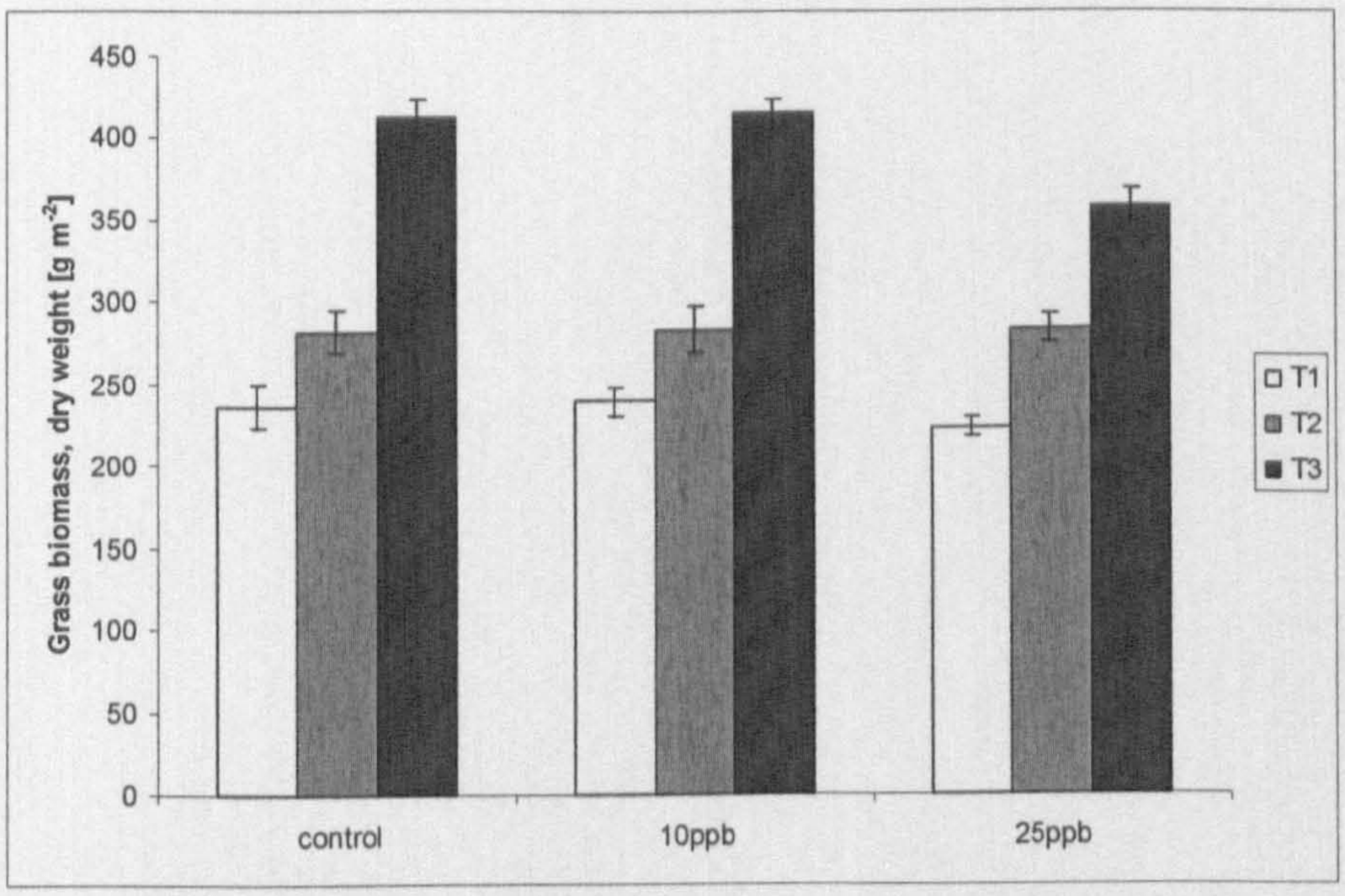


Figure 16: Mean grass biomass (g dry weight m^{-2}) in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2007. Error bars represent the standard error between replicate sub-plots.

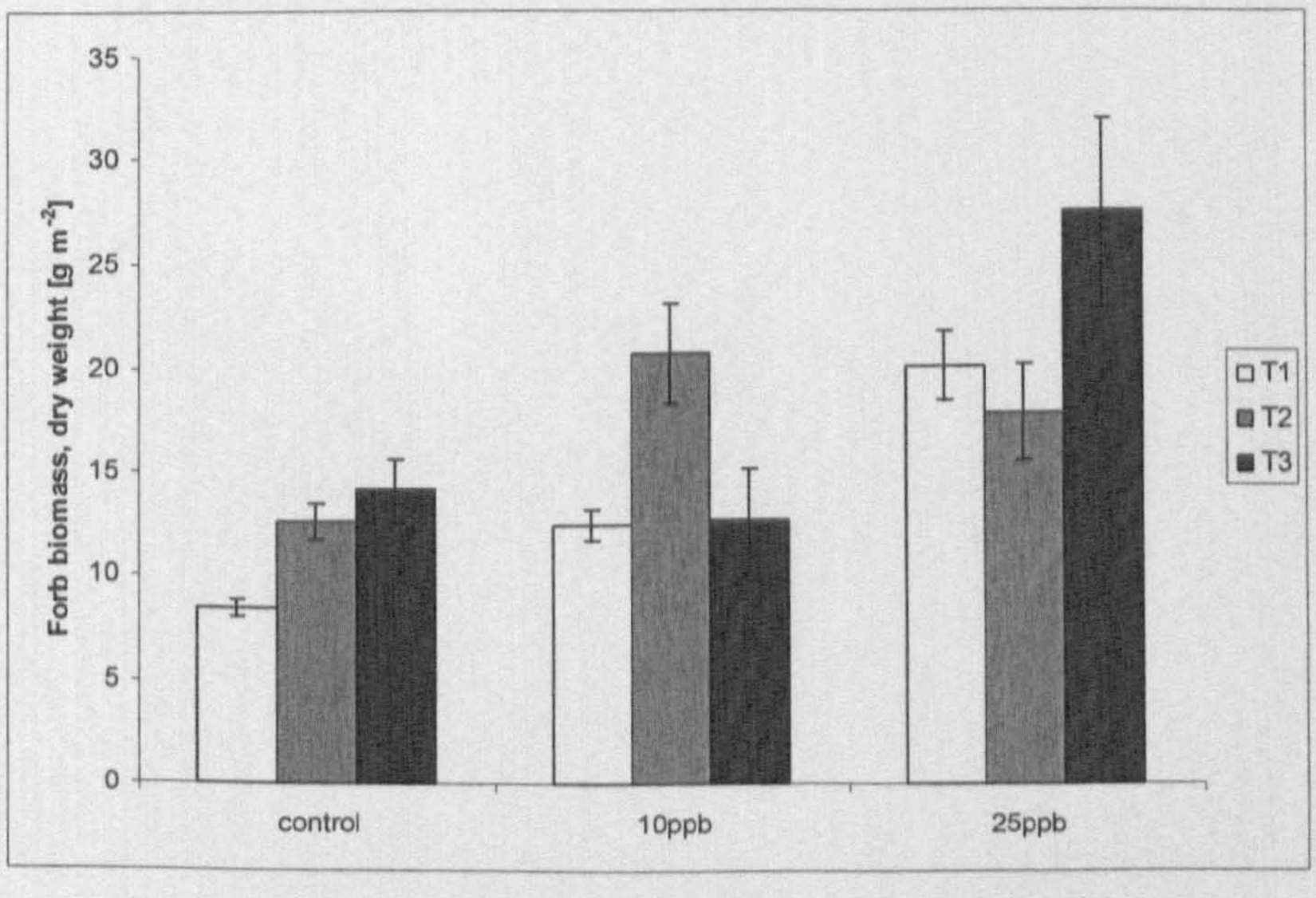


Figure 17: Mean forb biomass (g dry weight m^{-2}) in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2007. Error bars represent the standard error between replicate sub-plots.

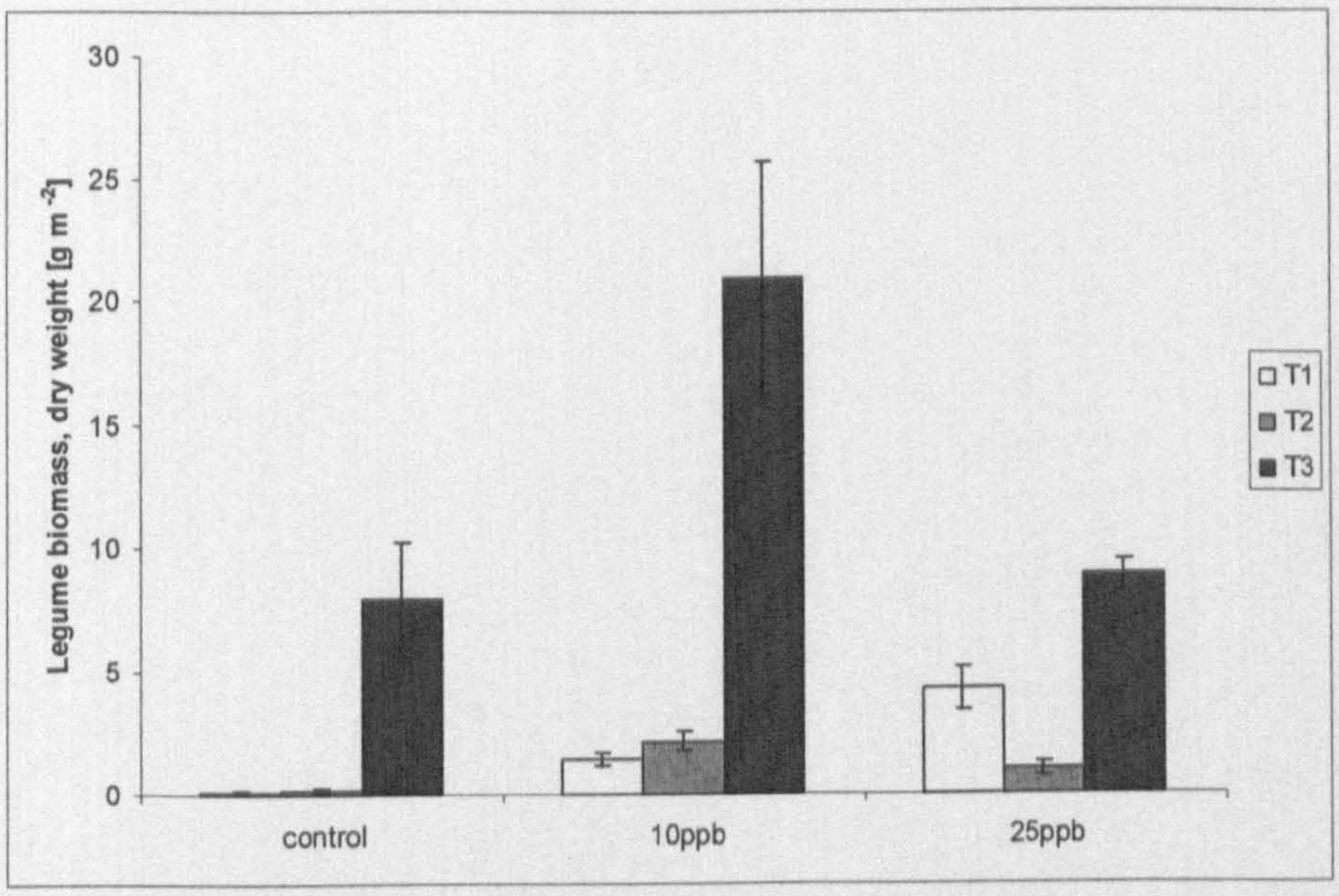


Figure 18: Mean legume biomass (g dry weight m^{-2}) in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2007. Error bars represent the standard error between replicate sub-plots.

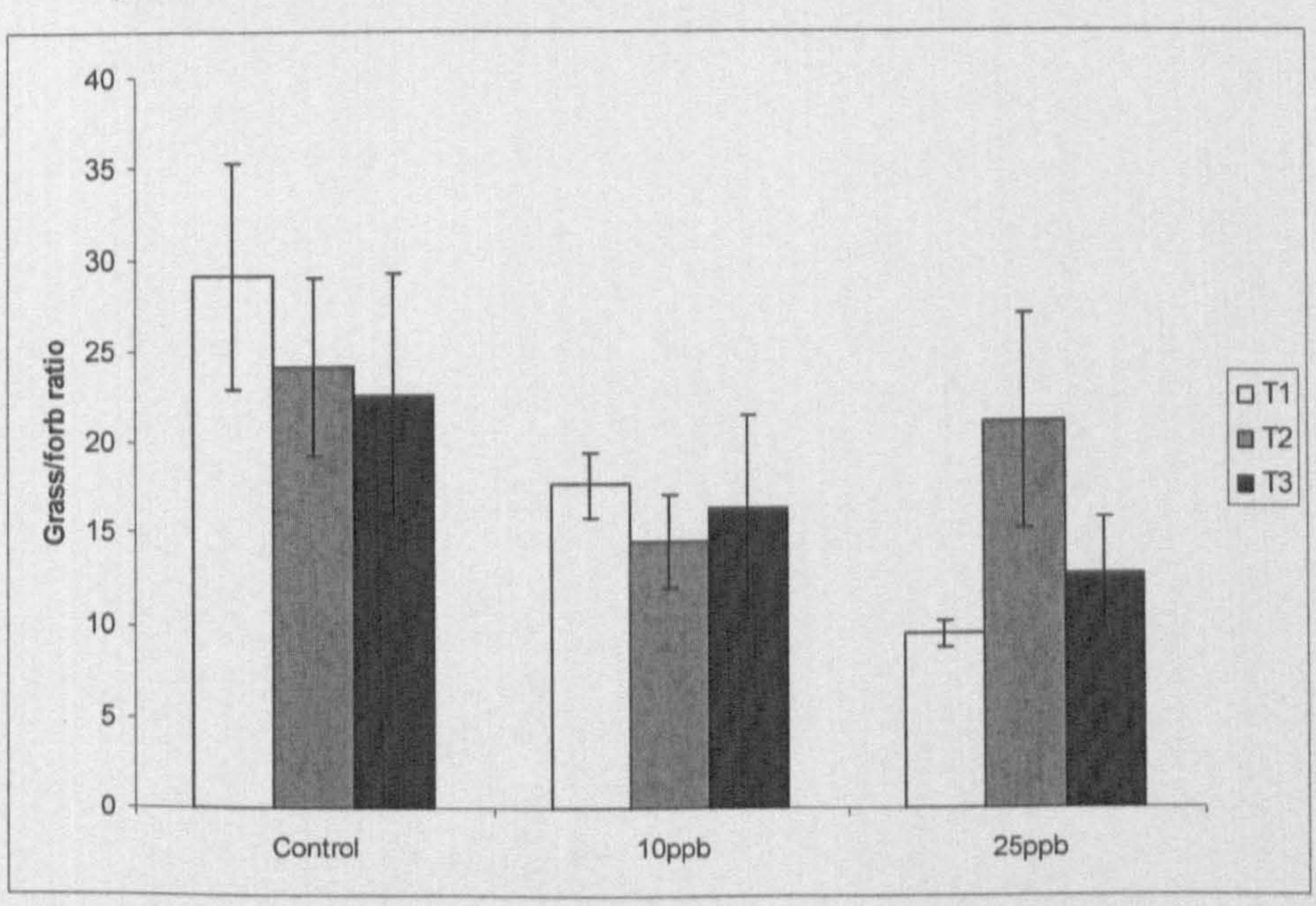


Figure 19: Grass/forb ratio in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2007. Error bars represent the standard error between replicate sub-plots.

Species biomass

Figure 20 shows the percentage of the major grass species in the same treatments; in contrast to total production, there were no clear gradients in grass species composition across the site, although there was substantial variation between replicate plots. This finding is consistent with the initial cover estimates. For the biomass of *Lolium perenne* and *Poa pratensis,* significant differences were found between transects (Table 14). *L. perenne* biomass was significantly greater in the 3rd transect than in the 2nd and 1st transect (P= 0.016), while. *P. pratensis* biomass was significantly greater in the 3rd transect than in the 2nd Transect (P= 0.033). The higher biomass of these two grass species in T3 is consistent with the fertility gradient. Significant O_3 effects were found for *Festuca pratensis* (P= 0.009) and *Phleum bertolonii* (P= 0.015), with biomass being significantly lower in the 10 ppb plots than in control plots (Table 14).

Data for the main forb species are shown in Figure 21. These show a great variation between the nine plots, but Table 15 suggests that none of this is systematically related to ozone or treatment. The dominant forb species was *Rumex acetosa,* the only species which was present in every subplot. One of the other more dominant forbs, *Rhinanthus minor* had a significantly greater biomass in the 10 ppb treatment than in the control (P= 0.02) (Table 15). No significant interactions were found between transects and treatments. The effects of O_3 on the species for which significant effects were found are presented in Figure 22.

Table 14: ANOVA of effects of ozone and transect on aboveground biomass of grass species in 2007. Post-hoc differences are significant at P< 0.05 (ns= non significant).

	Effect of ozone	*Post-hoc*	*Effect of transect*	*Post-hoc*
Agrostis tenuis	.418	ns	**.041**[5]	ns
Agrostis gigantea	.544	ns	.056	ns
Alopecurus pratensis	.168	ns	.075	ns
Anthoxanthum odoratum	.492	ns	.507	ns
Bromus ramosus	.391	ns	.391	ns
Dactylis glomerata	**.048**[6]	ns	.426	ns
Festuca pratensis	**.009**	Control > 10 ppb	.920	ns
Festuca rubra/ovina	.911	ns	.091	ns
Holcus lanatus	.776	ns	.522	ns
Lolium perenne	.737	ns	**.016**	T1,T2< T3
Phleum bertolonii	**.015**	Control > 10 ppb	.150	ns
Poa pratensis	.615	ns	**.033**	T2< T3
Poa spec	.367	ns	.257	ns

[5] Inhomogenous; values significant at P<0.01
[6] Inhomogenous; values significant at P<0.01

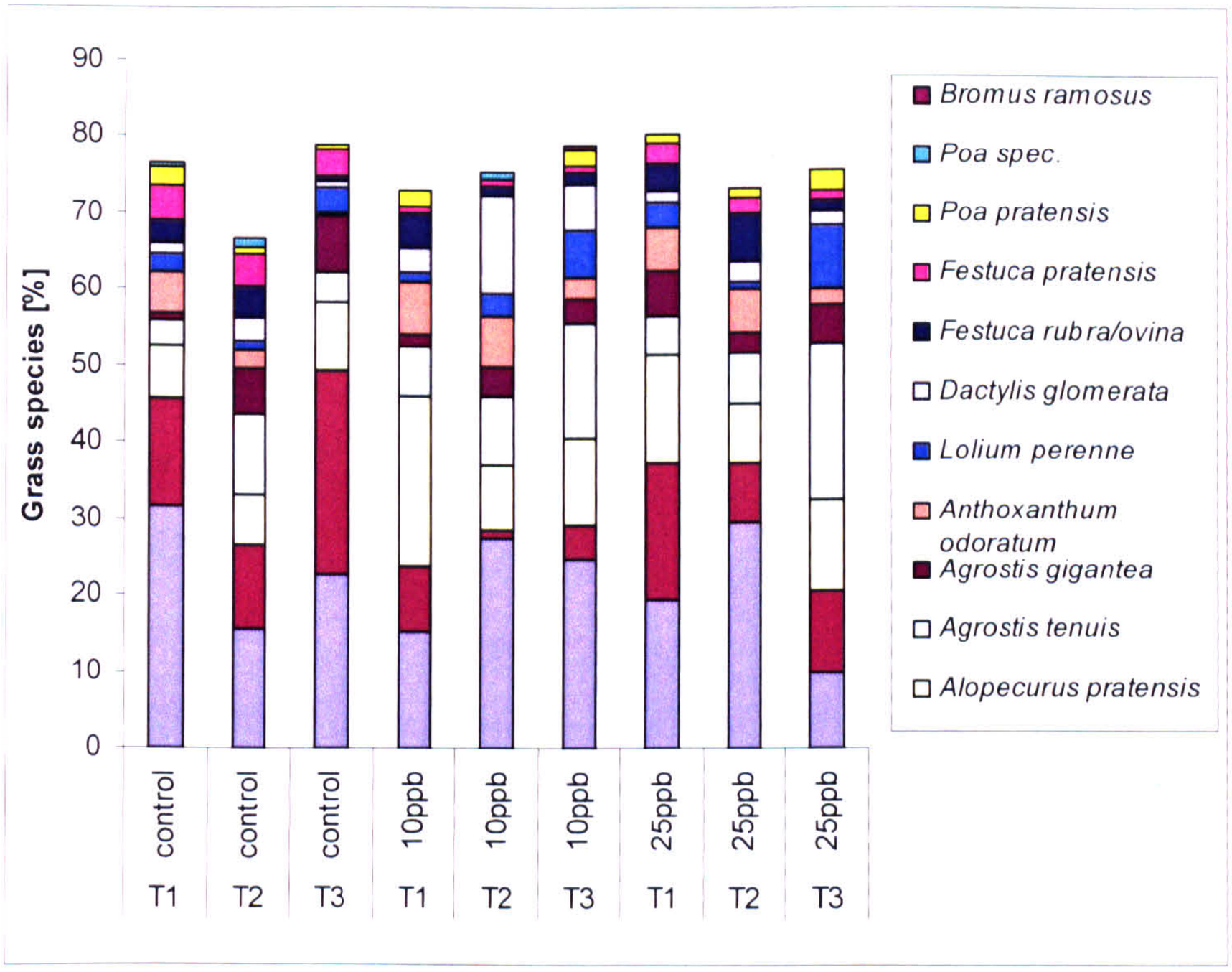


Figure 20: The percentage of the total aboveground biomass of major grass species in all treatments in August 2007. Unidentified grass species are not shown.

Table 15: ANOVA of effects of ozone and transect on aboveground biomass of forb species in 2007. Post-hoc differences are significant at P< 0.05 (ns= non significant).

	Effect of ozone	*Post-hoc*	*Effect of transect*	*Post-hoc*
Cardamine pratensis	0.352	ns	0.175	ns
Cerastium fontanum	0.277	ns	0.108	ns
Conopodium major	0.317	ns	0.317	ns
Lathyrus pratensis	0.143	ns	0.117	ns
Plantago lanceolata	0.121	ns	0.180	ns
Ranunculus acris	0.732	ns	0.683	ns
Ranunculus repens	0.256	ns	0.88	ns
Ranunculus spec.	0.551	ns	0.143	ns
Rhinanthus minor	**0.020**	Control < 10ppb	0.180	ns
Rumex acetosa	0.294	ns	0.335	ns
Taraxacum spec.	0.538	ns	0.377	ns
Trifolium pratense	0.641	ns	0.107	ns
Trifolium repens	0.41	ns	0.165	ns
Trifolium spec.	0.301	ns	0.368	ns
Stellaria graminea	0.825	ns	0.34	ns
Vicia sativa	0.344	ns	0.223	ns
Veronica chamedrys	0.182	ns	0.895	ns

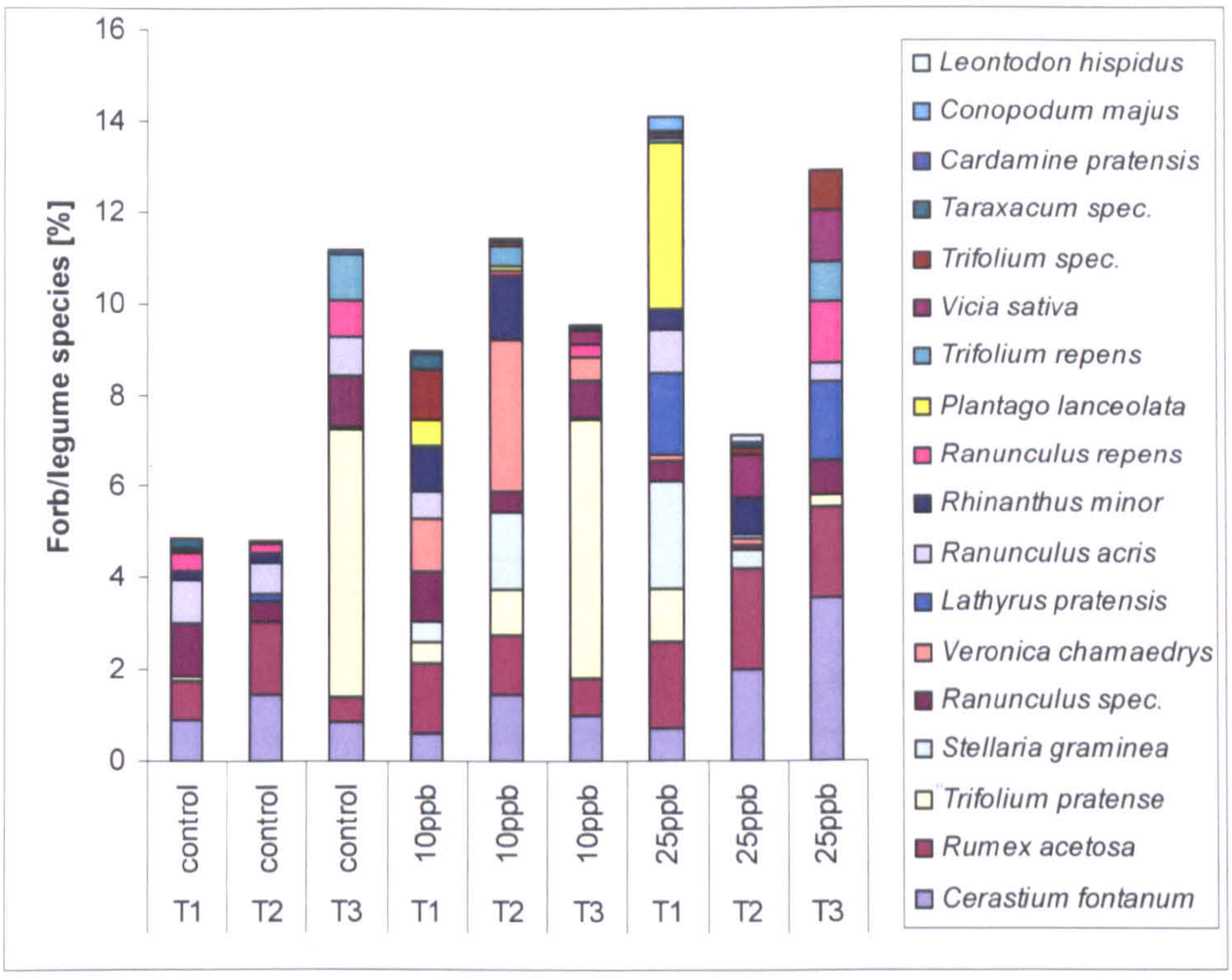


Figure 21: The percentage of the total aboveground biomass of major forb and legume species in all treatments in August 2007. Unidentified forb species are not shown.

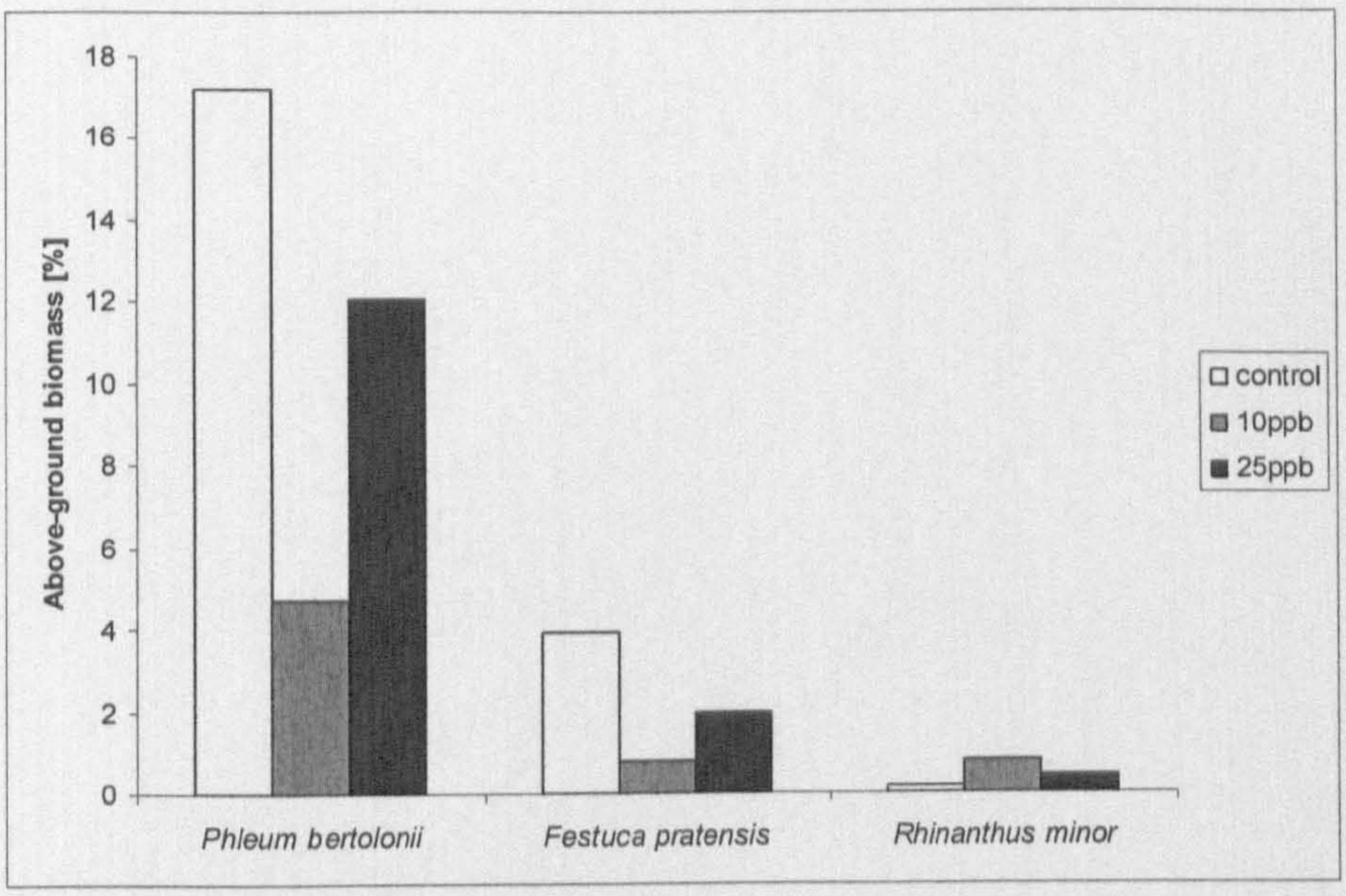


Figure 22: Significant O_3 effects on species biomass expressed as a percentage of total above-ground biomass in August 2007.

2.3.3 August 2008 harvest

Total above-ground biomass

The total biomass data in 2008, in contrast to 2007, showed no significant differences between transects (Table 16, Figure 23). Although the total biomass was not significant (with a P value of 0.08), there was a trend for greater total biomass in the third transect ($P< 0.1$), similar to that found in 2007. In comparison to 2007, there was much stronger evidence for an O_3 effect. In 2008, both elevated ozone treatments reduced the forb biomass (Figure 25); the forb fraction (excluding legumes) in the 10 ppb and the 25 ppb treatment were both significantly smaller than in the control treatment ($P= 0$). For the legumes, on the other hand, biomass was significantly greater in the control plots than in the 10 ppb plots ($P= 0.013$). A similar trend was observed for the 25 ppb plot, which also had a greater biomass than the control plots, although the P-value was only just significant at $P< 0.1$ (Figure 26). The grass/ forb ratio showed

quite different effects of O_3 in 2008 compared to 2007 (Figure 19, Figure 27). Both the 10 ppb and the 25 ppb treatments showed a higher grass/ forb ratio than the control plots, with the value in the 25 ppb treatment being significantly greater than the control treatment (P= 0.004).

Table 16: Results of the ANOVA of effects of ozone and transect on total above ground biomass, and the grasses, forbs and legumes in August 2008. Ration indicates grass/ forb ratio. Post-hoc differences are significant at P< 0.05 (ns = non significant)

	Effects of ozone	*Post-hoc*	*Effects of transect*	*Post-hoc*
total	0.333	ns	0.08	ns
grasses	0.362	ns	0.103	ns
ratio	**0.004**	Control < 25ppb	0.191	ns
forbs	**0.000**	Control > 10ppb, 25ppb	0.272	ns
legs	**0.013**	Control < 10ppb	0.489	ns

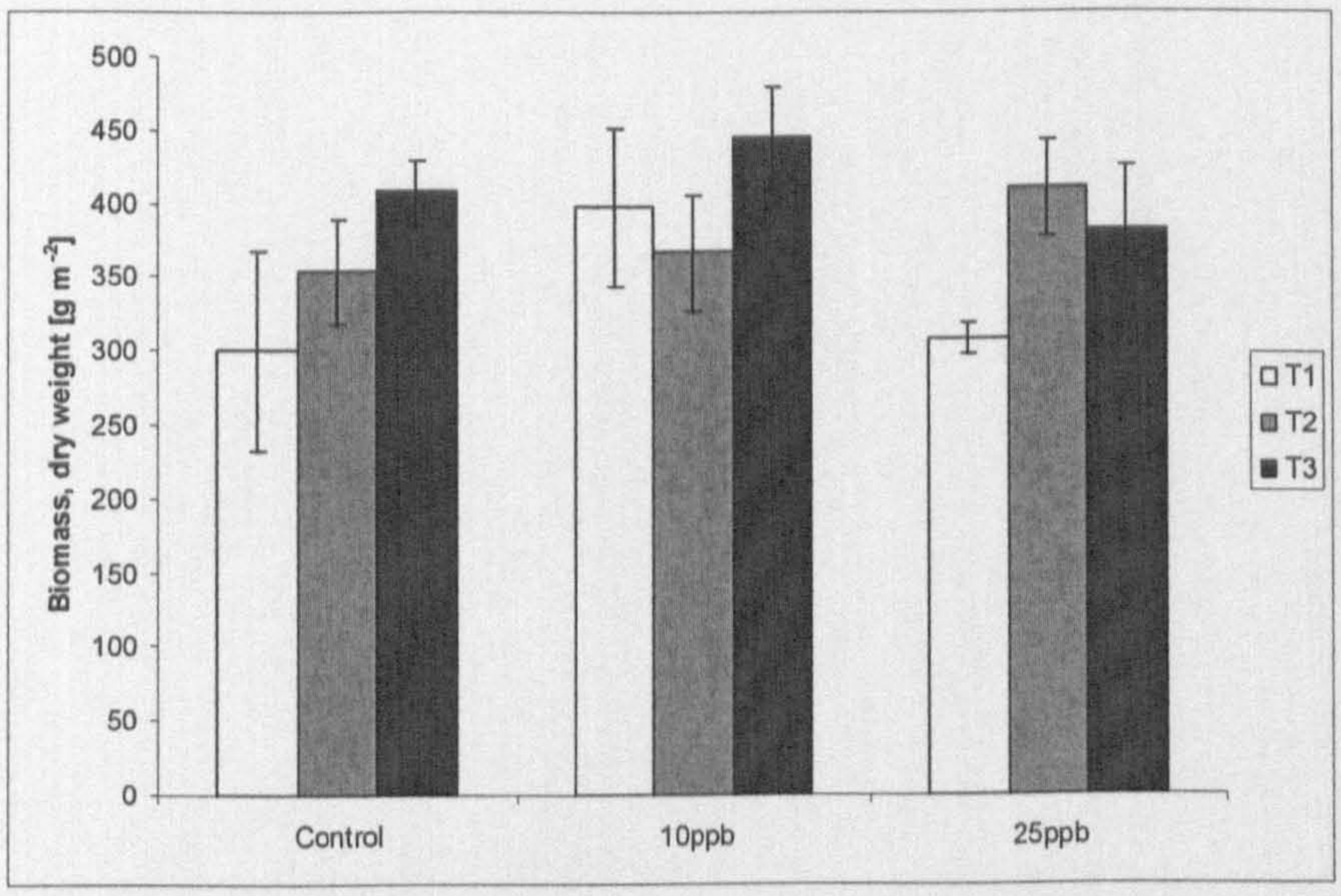


Figure 23: Mean total above ground biomass (g dry weight m^{-2}) in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2008. Error bars represent the standard error between replicate sub-plots.

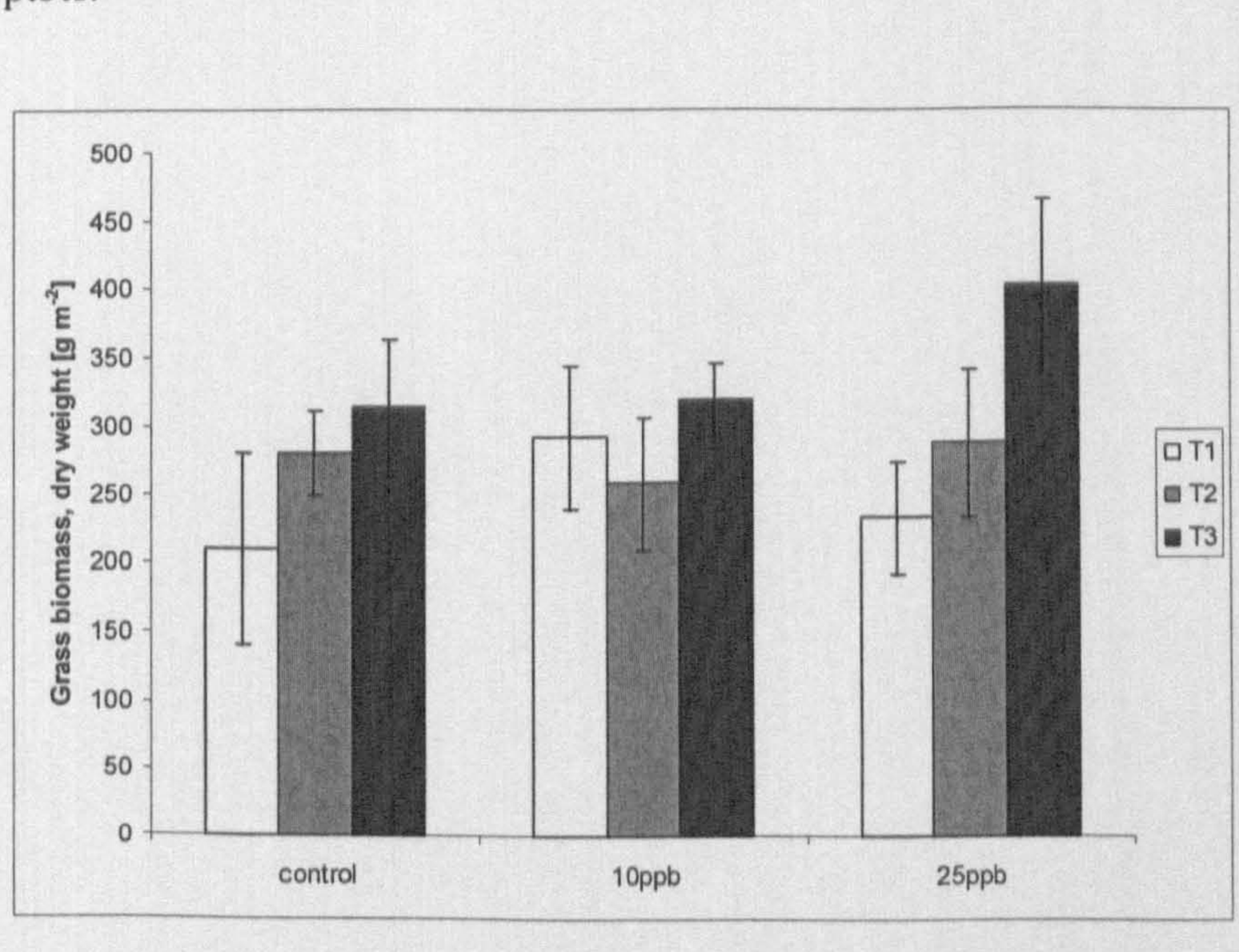


Figure 24: Mean grass biomass (g dry weight m^{-2}) in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2008. Error bars represent the standard error between replicate sub-plots.

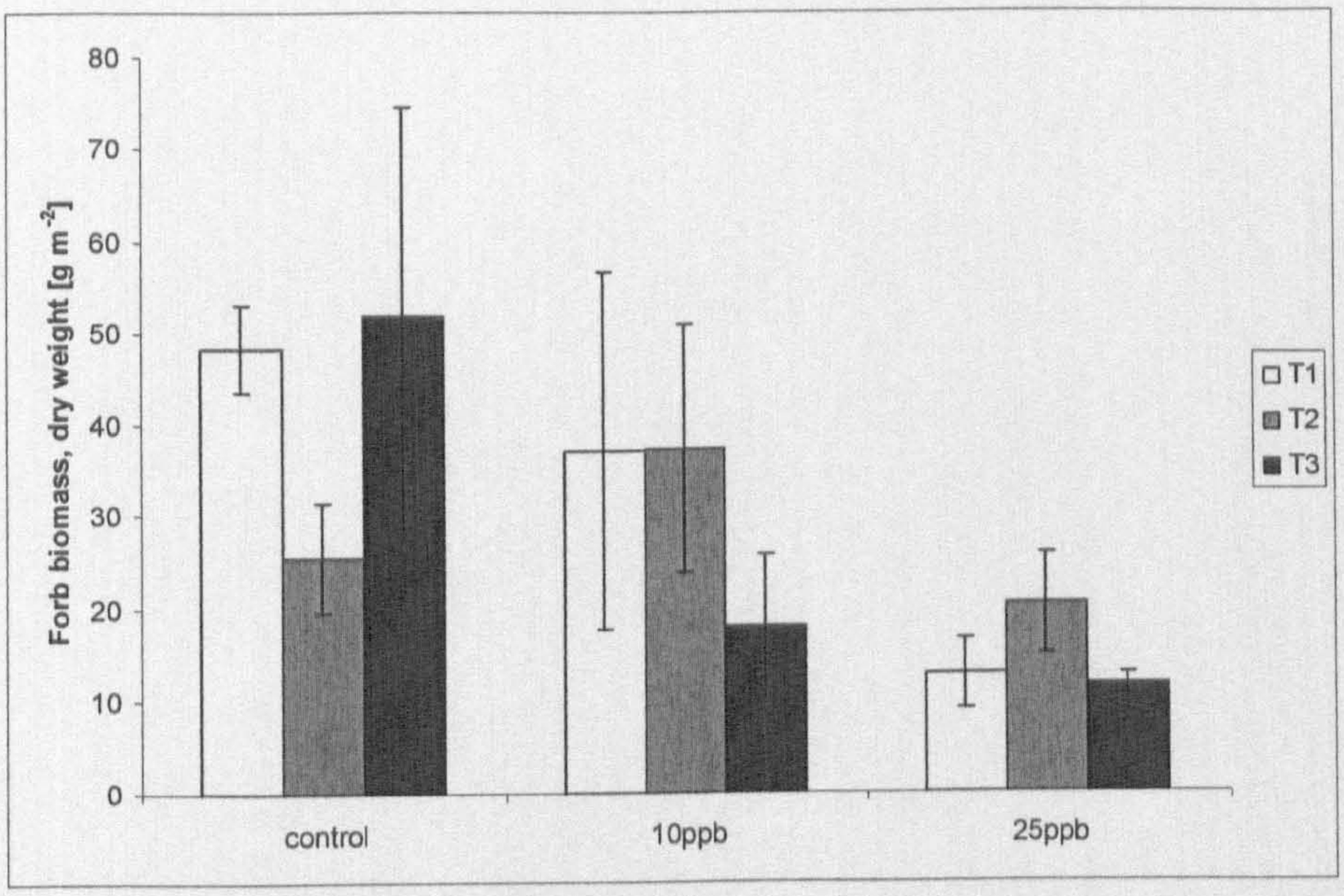


Figure 25: Mean forb biomass (g dry weight m^{-2}) in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2008. Error bars represent the standard error between replicate sub-plots.

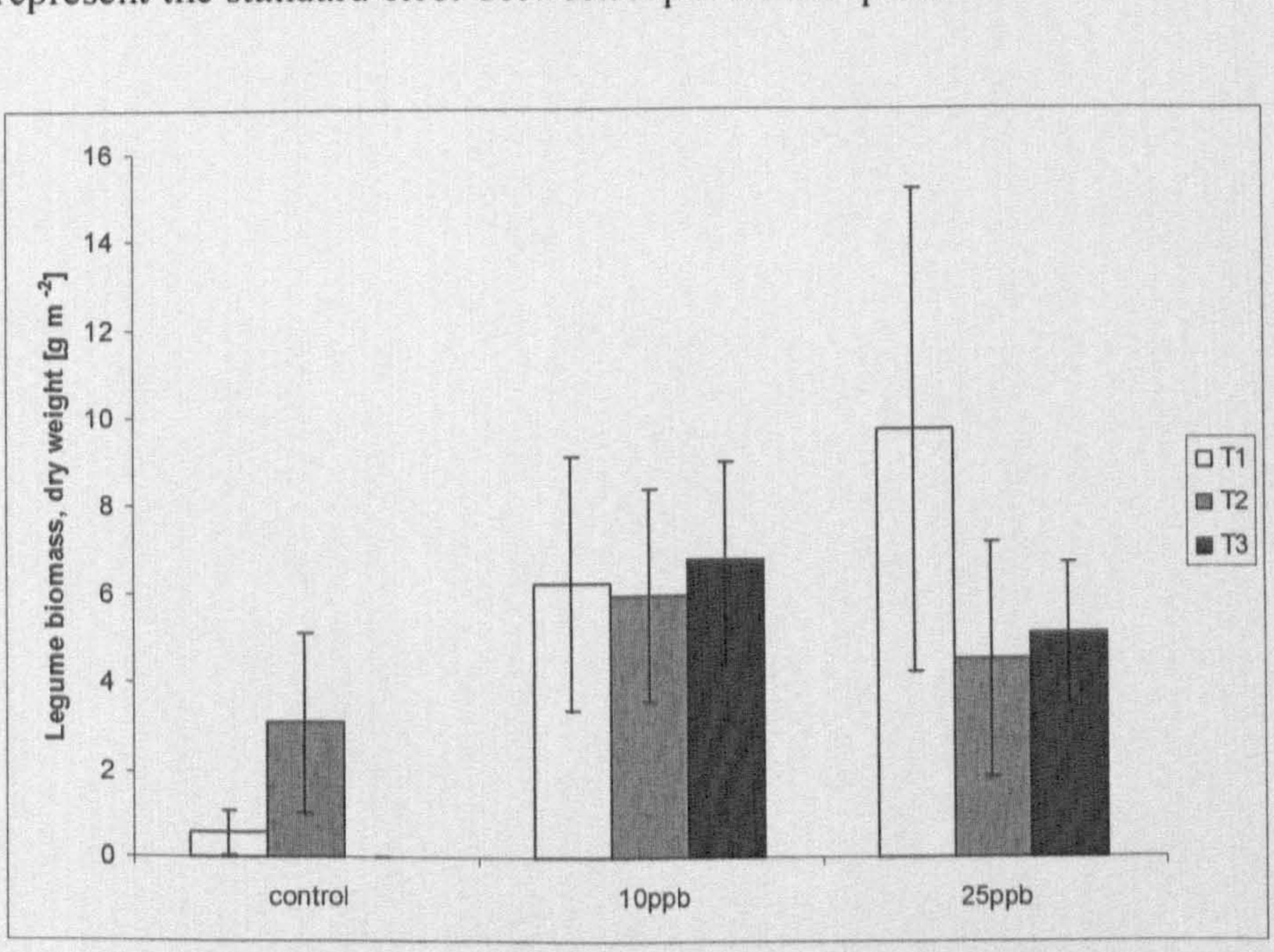


Figure 26: Mean legume biomass (g dry weight m^{-2}) in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2008. Error bars represent the standard error between replicate sub-plots.

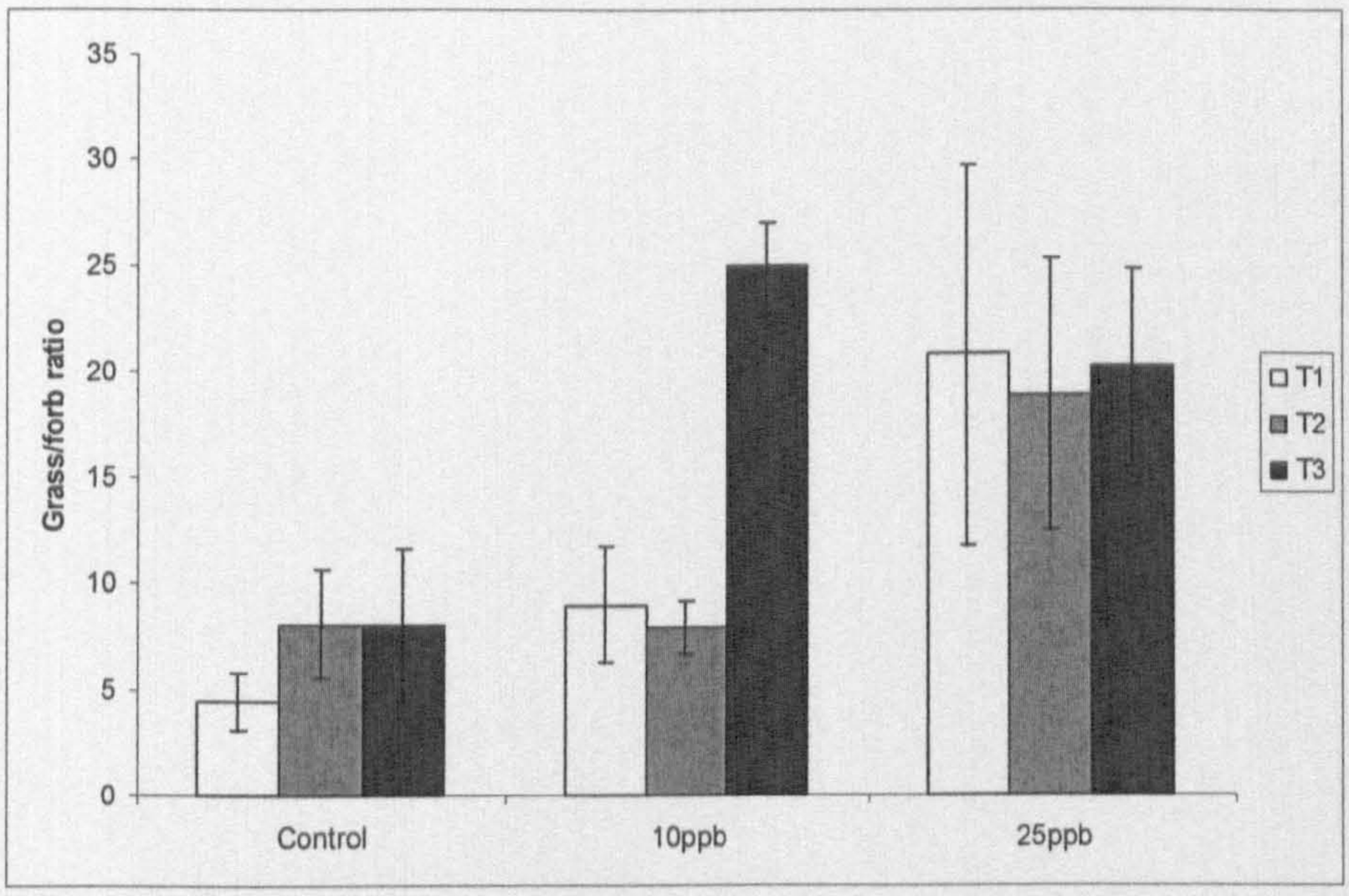


Figure 27: Grass/forb ratio in each treatment (Control, 10 ppb and 25 ppb) and transect (T1-T3) for the harvest of August 2008. Error bars represent the standard error between replicate sub-plots.

Species biomass

Table 17 and Table 18 summarise effects on all the biomass fractions of all the individual species which are shown in Figure 28 and Figure 29. The following species, showed significant effects of transect or ozone. *Trisetum flavescens* was the only grass species which showed significant differences between transects, with values in the 2nd and 3rd transect being significantly larger than in 1st transect (P= 0.008). Only one grass species showed a significant effect of ozone; the biomass of *Dactylis glomerata* (P= 0.004) was significantly greater in the 10 ppb and the 25 ppb treatments than in the control treatment (Table 17). Of the forbs, only *Rumex acetosa* showed a significant transect effect, with biomass being greater in the second transect compared to the first one (P= 0.0043). Two legume species showed significant effects of ozone. *Lathyrus pratensis* biomass was significantly greater in the 25 ppb than in the control treatment (P< 0.017). Significant differences were also found for *Trifolium repens*, with biomass being significantly greater in the 10 ppb plots

than in the control plots (P= 0.049). In contrast to these legume species, two forb species *Stellaria graminea* and *Ranunculus spec.* showed a significant negative effect of O_3 (Table 18). *S. graminea* showed lower biomass in the 10 ppb treatment than in the control treatments (P= 0.009), while *Ranunculus spec.* biomass was significantly smaller in the 10 ppb and the 25 ppb treatment than in the control treatment (P= 0.001). Similar observations were found for *Rhinanthus minor*, which declined in the elevated treatments, although the effect of O_3 was at the borderline of significance at P= 0.059 (Table 18). The interpretation of this as a real effect is supported by finding significant differences between the control and 25 ppb treatment after setting the P-level for the Kruskal-Wallis-test to P< 0.1, and for the Mann-Whitney-test to P< 0.03 (Bonferoni adjustment). *R. minor* biomass was significantly smaller in the 25 ppb treatment. Furthermore, *Rhinanthus minor* seemed to completely disappear in the 75 ppb treatment plots (Figure 31).

Significant differences for the 'transect*treatment' interaction were not observed for either the forbs or for the grasses. Significant main effects of ozone on individual species biomass are presented in Figure 30.

Table 17: The Results of the ANOVA of effects of ozone and transect on aboveground biomass of grass species in 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant).

	Effects of ozone	*Post-hoc*	*Effects of transect*	*Post-hoc*
Agrostis tenuis	0.330	ns	0.251	ns
Agrostis gigantea	0.124	ns	0.962	ns
Agrostis stolonifera	0.322	ns	0.629	ns
Alopecurus pratensis	0.374	ns	0.992	ns
Anthoxanthum odoratum	0.135	ns	0.664	ns
Dactylis glomerata	**0.004**	Control> 10ppb, 25ppb	0.110	ns
Festuca pratensis	0.060	ns	0.412	ns
Festuca rubra/ovina	0.512	ns	0.441	ns
Holcus lanatus	0.329	ns	0.089	ns
Lolium perenne	0.142	ns	0.268	ns
Phleum bertolonii	0.401	ns	0.656	ns
Phleum pratense	0.199	ns	0.061	ns
Poa pratensis	0.550	ns	0.952	ns
Trisetum flavescens	0.408	ns	**0.008**	T1< T3, T1< T2
Juncus effusus	0.391	ns	0.391	ns

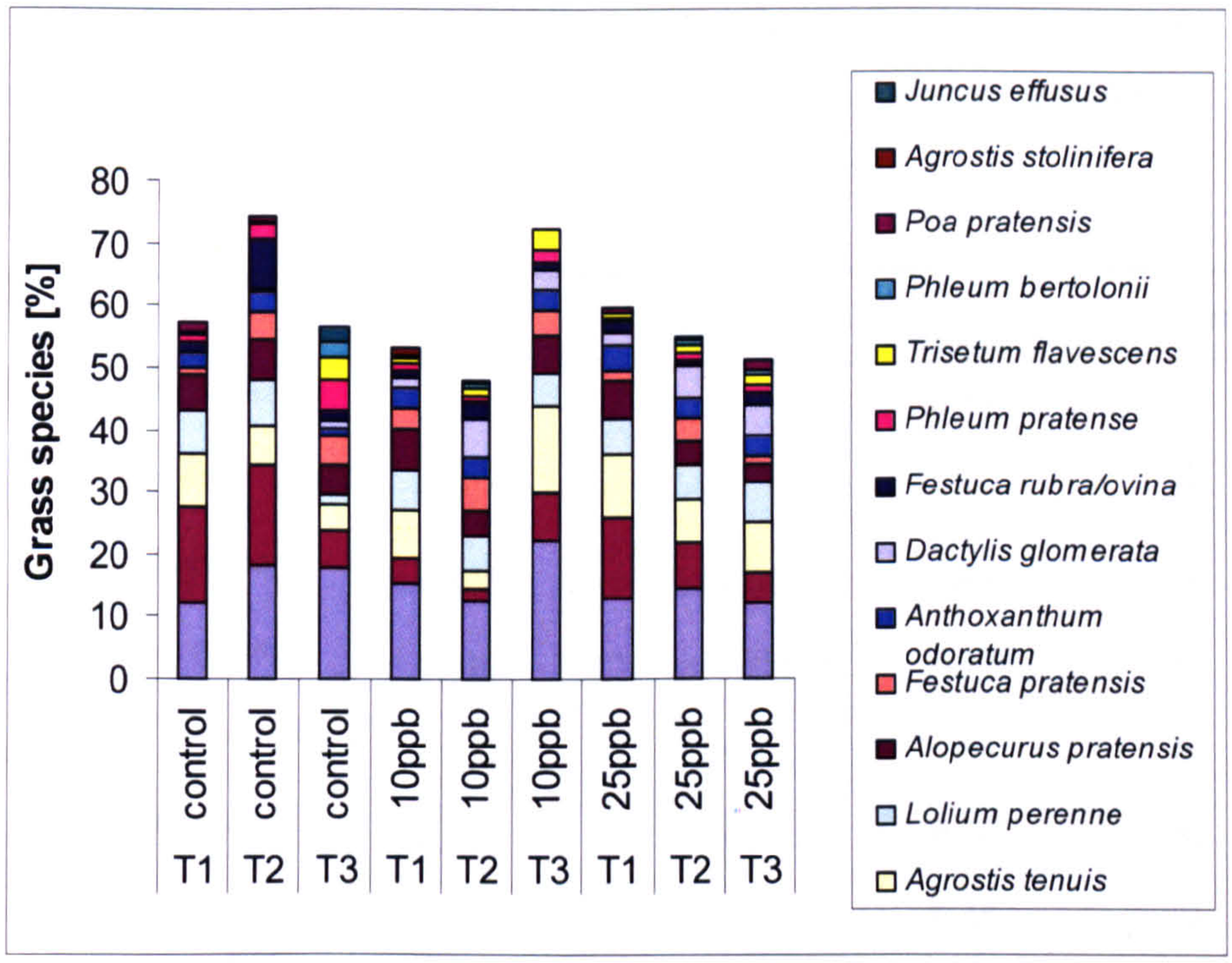


Figure 28: The percentage of the total aboveground biomass of major grass species in all treatments in August 2008. Unidentified grass species are not shown.

Table 18: The results of the ANOVA of effects of ozone and transect on aboveground biomass of forb species in 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant).

	Effect of ozone	*Post-hoc*	*Effect of transect*	*Post-hoc*
Cardamine pratensis	0.255	ns	0.342	ns
Cerastium fontanum	0.495	ns	0.320	ns
Conopodium major	0.326	ns	**0.042**	ns[7]
Lathyrus pratensis	**0.019**	Control< 25ppb	0.176	ns
Plantago lanceolata	0.373	ns	0.1	ns
Ranunculus acris	0.368	ns	0.368	ns
Ranunculus repens	0.368	ns	0.368	ns
Ranunculus spec.	**0.001**	Control> 10ppb, 25ppb	0.620	ns
Rhinanthus minor	0.059	ns	0.104	ns
Rumex acetosa	0.335	ns	**0.043**	T1< T2
Taraxacum spec.	0.761	ns	0.142	ns
Trifolium pratense	0.328	ns	0.402	ns
Trifolium repens	**0.041**	Control< 10ppb	0.149	ns
Stellaria graminea	**0.009**	Control< 10ppb	0.057	ns
Vicia sativa	**0.048**	ns[8]	0.216	ns
Veronica chamedrys	0.084	ns	0.503	ns

[7] Bonferoni adjustment to P<0.016
[8] Bonferoni adjustment to P<0.016

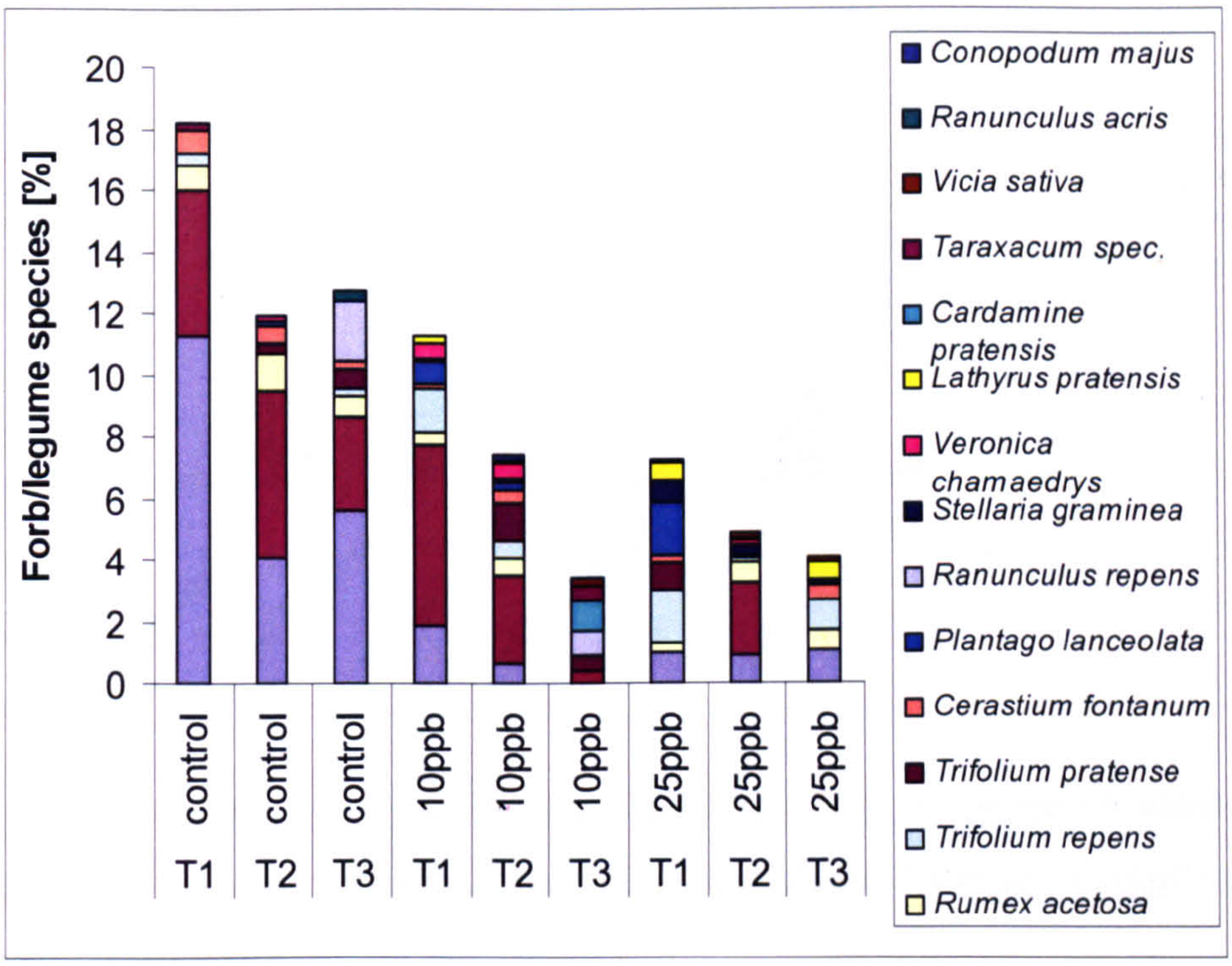


Figure 29: The percentage of the total aboveground biomass of major forb/ legume species in all treatments in August 2008. Unidentified forb species are not shown.

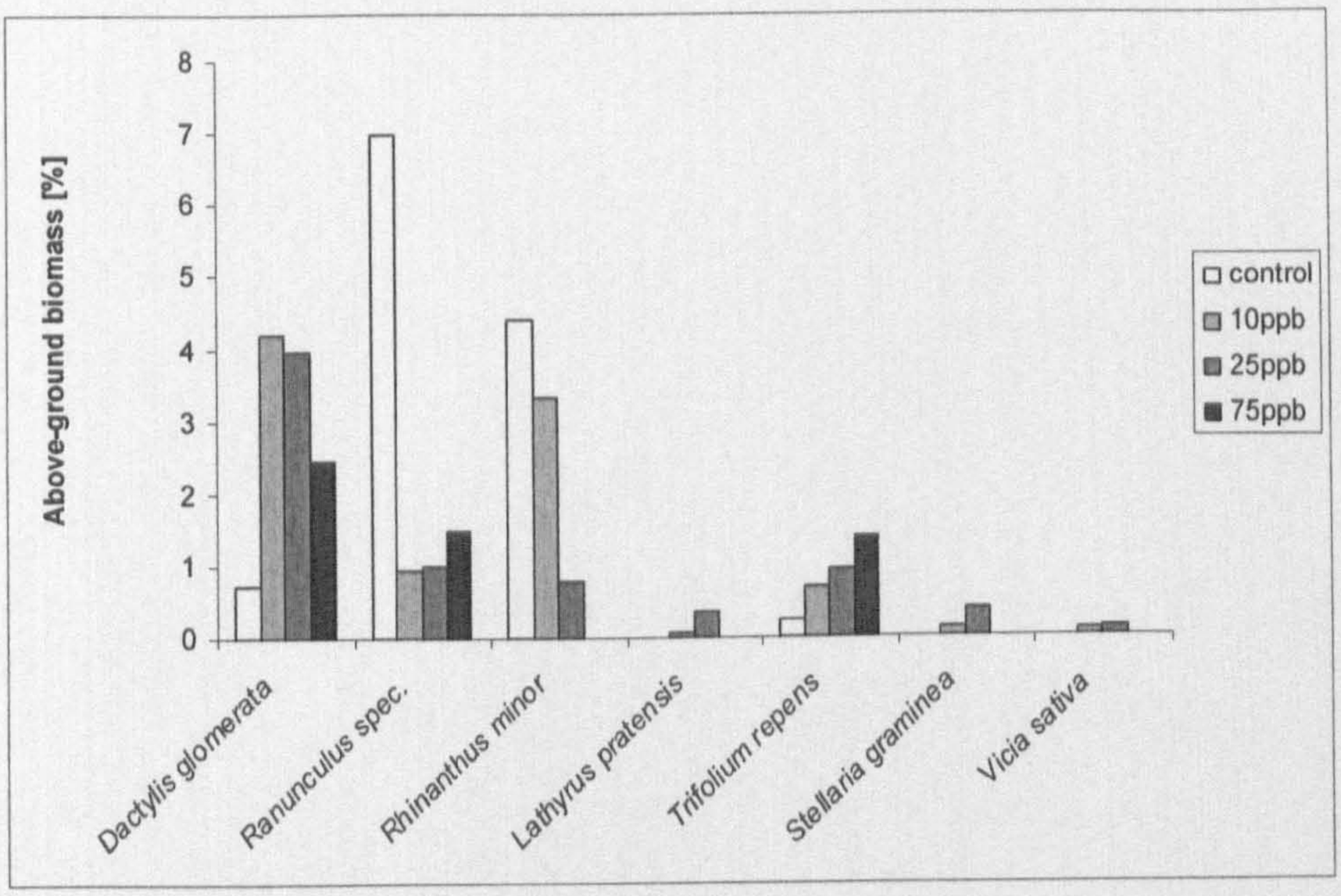


Figure 30: Effects of ozone on percentage of total above-ground biomass as individual species in August 2008, for those species showing a significant main effect of ozone.

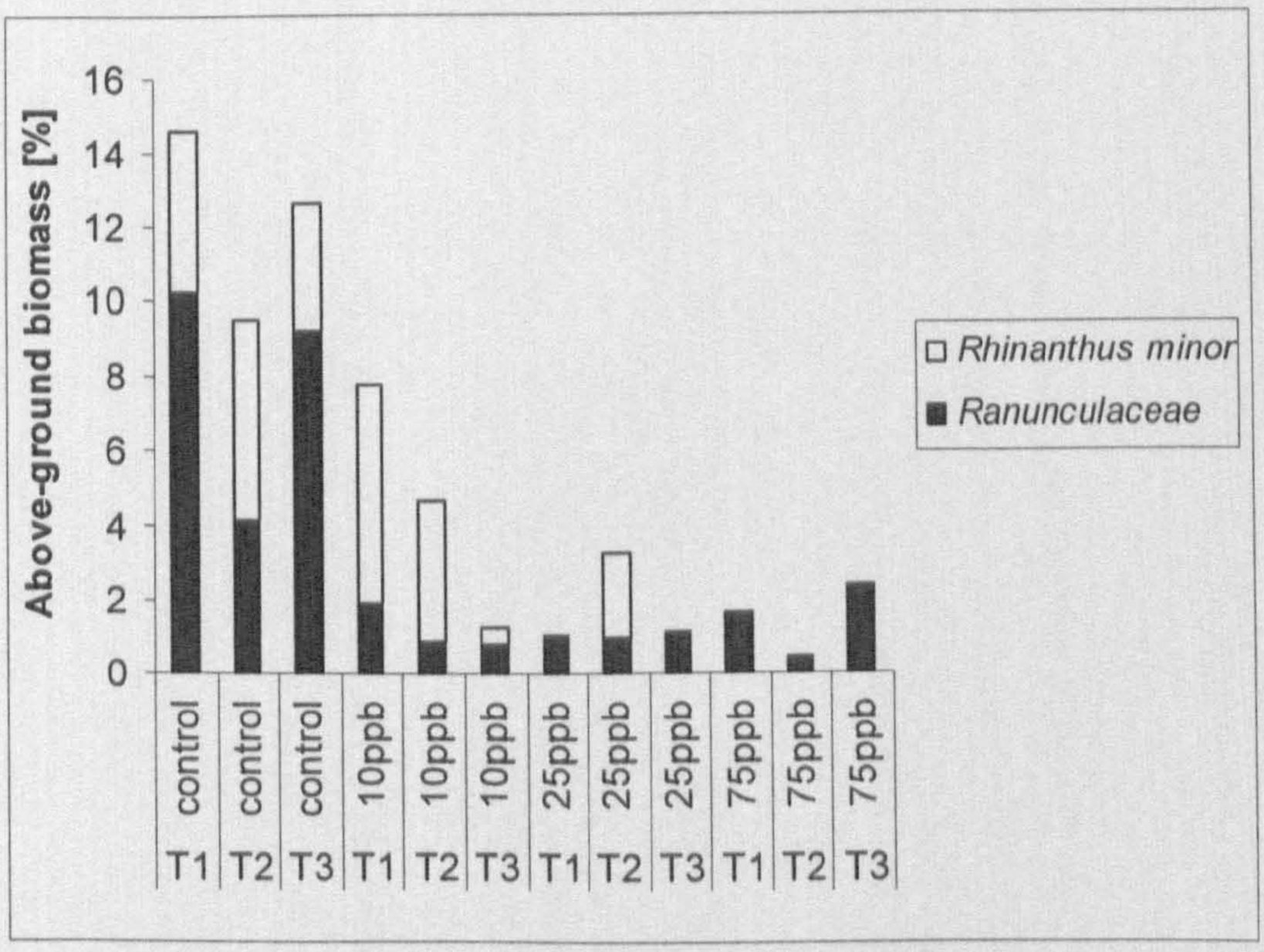


Figure 31: The percentage of the total above-ground biomass in *Ranunculus spec.* and *Rhinanthus minor* in August 2008 in all 12 plots. Data also includes the 75 ppb treatment plots.

2.3.4 Major changes between 2007 and 2008

The weather conditions on the site differed between the years 2007 and 2008. In general, 2008 was a much colder year, with a mean temperature for May-October of 10.8°C, compared to 15°C, in 2007 (Table 3 and Table 4). The ambient O3 concentrations were on average higher in 2008 than in 2007. A May-October mean of 30.7 ppb O3 were measured in 2008 whereas in 2007 a mean of 25.1 ppb of O3 was measured instead (see Section 2.23). As described in Section 2.22, the O3 levels achieved in 2007 differed very much from the levels achieved in 2008. In 2007, the transects fumigated successfully 26% of the time during the vegetation period. Average O3 concentrations achieved along Transect A during that period were about 33 ppb at 2.5 m, 29 ppb at 5 m and 26 ppb at 10 m downwind from the release line (Table 1, Figure 7). The system worked much better in 2008, although not all three transects released the same amount of O3; average O3 concentrations of 34 ppb (A), 33 ppb (B) and 33.9 ppb (C) were achieved (Table 2). Also the data from the Gradko tubes in 2008 shows a gradient of O3 concentrations along all three transects. O3 concentrations along transect A in 2008 were 61.2 ppb at 2.5 m, 41.5 ppb at 5 m and 28.9 ppb at 10 m (Figure 8 and Figure 9).

Overall, there was little change in the biomass of the different functional groups, and the total biomass, between 2007 and 2008 (Table 19, Figure 32). There almost no difference in the total biomass, in the grass biomass and in the legume biomass. The major changes found were for the forb biomass and the grass/ forb ratio. The forb biomass increased significantly from 2007 to 2008 (P= 0.006), whereas the grass/ forb ratio (P= 0.013) decreased significantly (Table 19).

Whereas in 2007, the main effects were due to significantly greater biomass in Transect 3, in 2008 the biomass was mainly affected by the O_3 treatment. Especially in 2008 the functional groups which were adversely affected by ozone were the forbs, and not the legumes and not the grasses. The forbs declined with increasing O_3 treatment in 2008, although their overall biomass increased compared with 2007. This result was supported by the

increasing grass/forb ratio in the high O_3 treatment. The grasses showed no significant effect of ozone, while the legumes increased with increasing O_3 concentration. However, most legumes except *Trifolium repens* were missing from the control plots in 2008, which was not the case in 2007. In comparison to the harvest of 2007 the following species have changed according to their abundance:

Holcus lanatus, the dominant grass species in 2007 was still dominant in 2008 but was reduced and so were *Alopecurus pratensis* and *Phleum bertolonii,* whereas *Agrostis gigantea* and *Lolium perenne* increased in biomass. The major forb species *Rumex acetosa* and *Cerastium fontanum* declined, whereas the legume species *Trifolium repens* increased compared to 2007.

The biggest significant decline with increasing O_3 was observed for the forbs *Ranunculus spec.* and *Rhinanthus minor.* In 2007 *R. minor* not only showed significant higher biomass in the 10ppb plots than in the control plots but was not present at all in the third transect. In contrast, in 2008, not only was significantly higher biomass found in the control plots than in the 25ppb plots, but also *R. minor* occurred in the third transect. In addition, the biomass of the species of the *Ranunculaceae* familiy was more affected by the O_3 treatment in 2008. Whereas in 2007, there was a significant transect effect observed for *Ranunculaceae* between transects, with Transect 2 biomass being significantly lower than Transect 3 (P= 0.015), in 2008, significant differences in the biomass were found with the biomass in the control treatments being greater than in the 10 ppb and 25 ppb treatment (P= 0.000). The overall biomass of *Ranunculacae* and *R. minor* increased from 2007 to 2008 (Figure 33 and Figure 34).

Table 19: Results of the independent samples test of the above-ground biomass between the years 2007 and 2008. P-value is significant at $P < 0.05$.

t-test	*P*
total	0.508
grasses	0.432
grass/ forb ratio	**0.013**
forbs	**0.006**
legumes	0.342

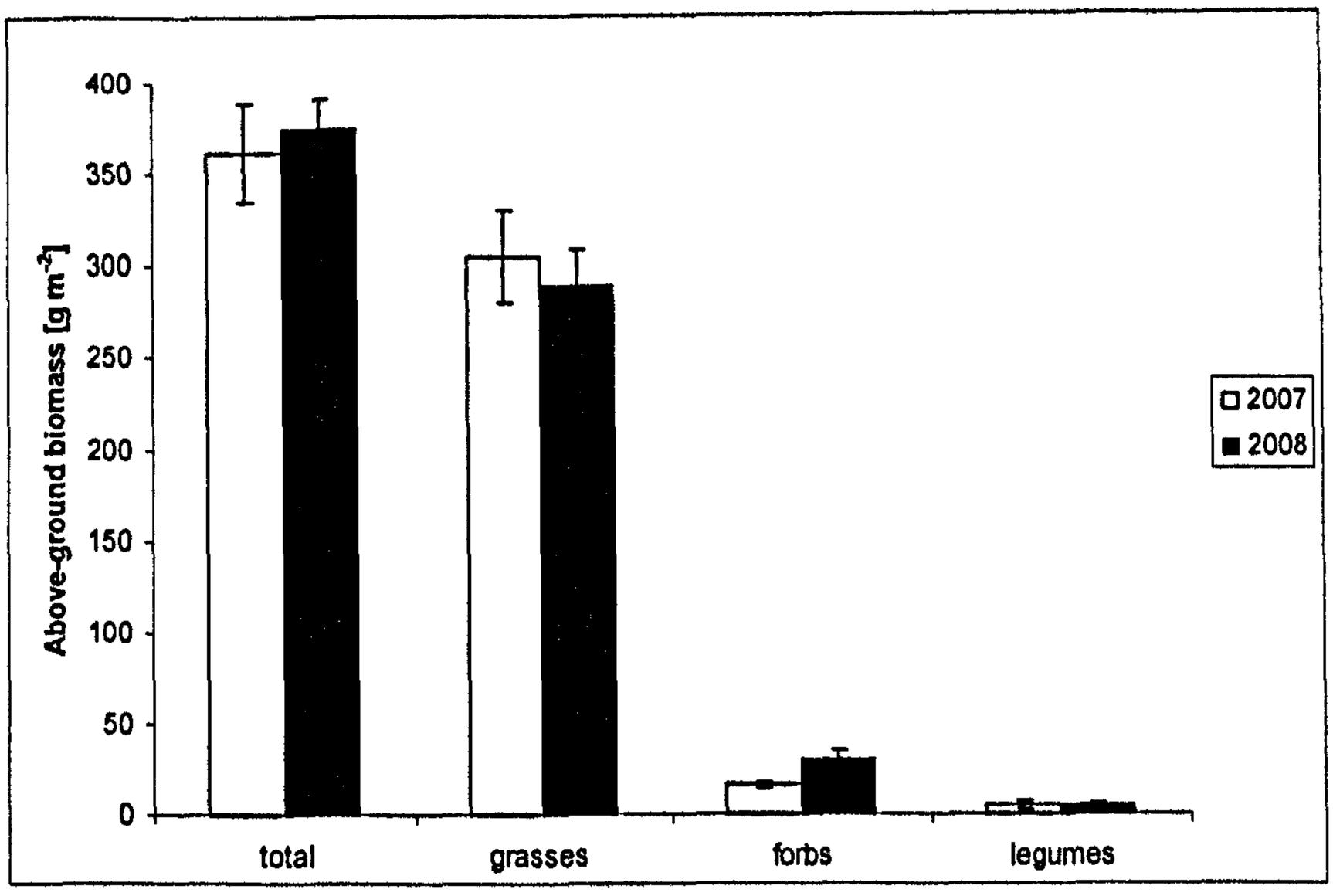


Figure 32: Comparison of the total, grass, forb and legume above-ground biomass between the harvests of 2007 and 2008. The total biomass also includes the unidentified plant species. Error bars represent standard errors between replicate sub-plots.

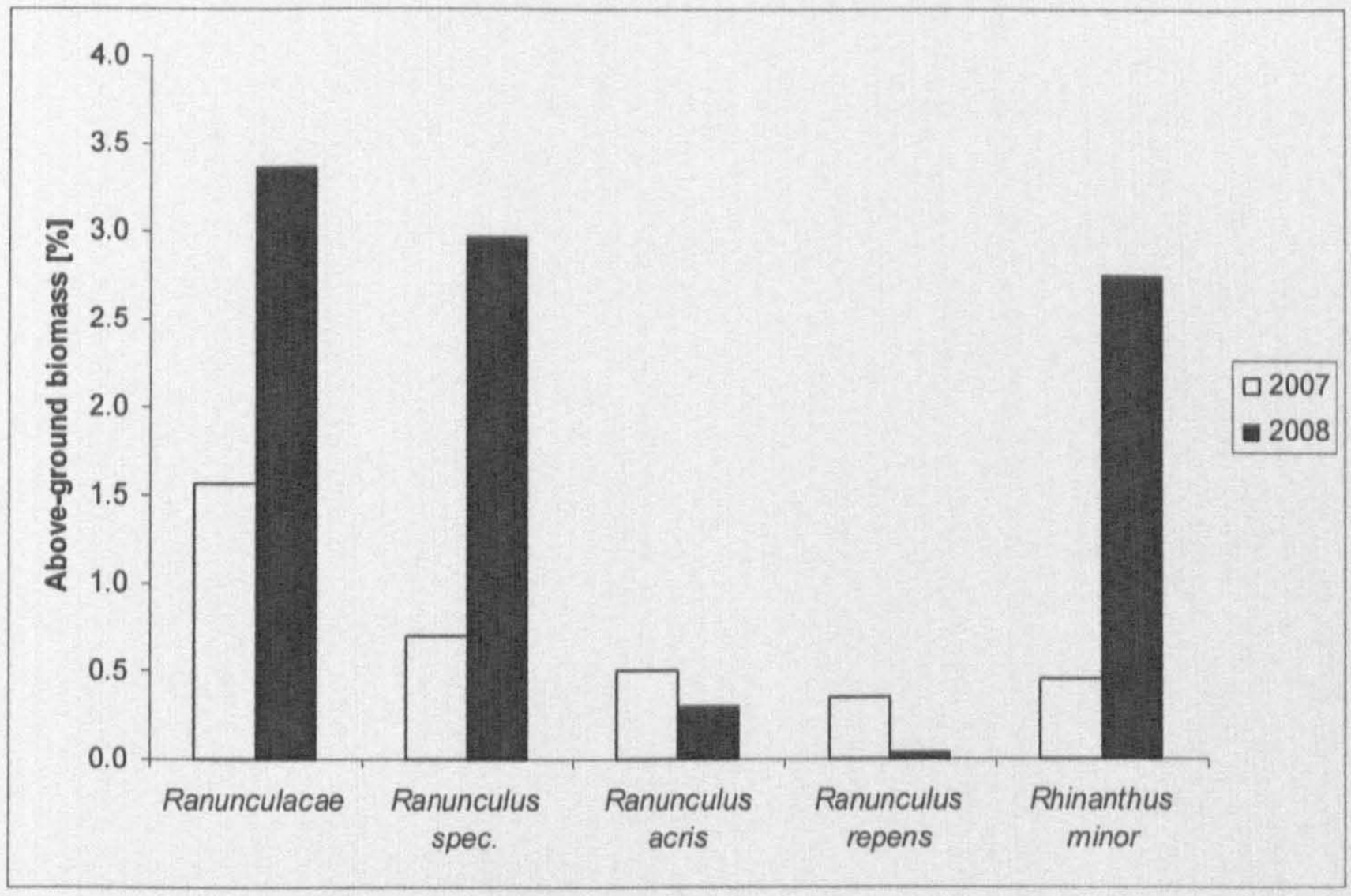


Figure 33: The percentage of *Ranunculacae* and *Rhinanthus minor*, expressed as a percentage of the total above-ground biomass, in August 2007 and in August 2008.

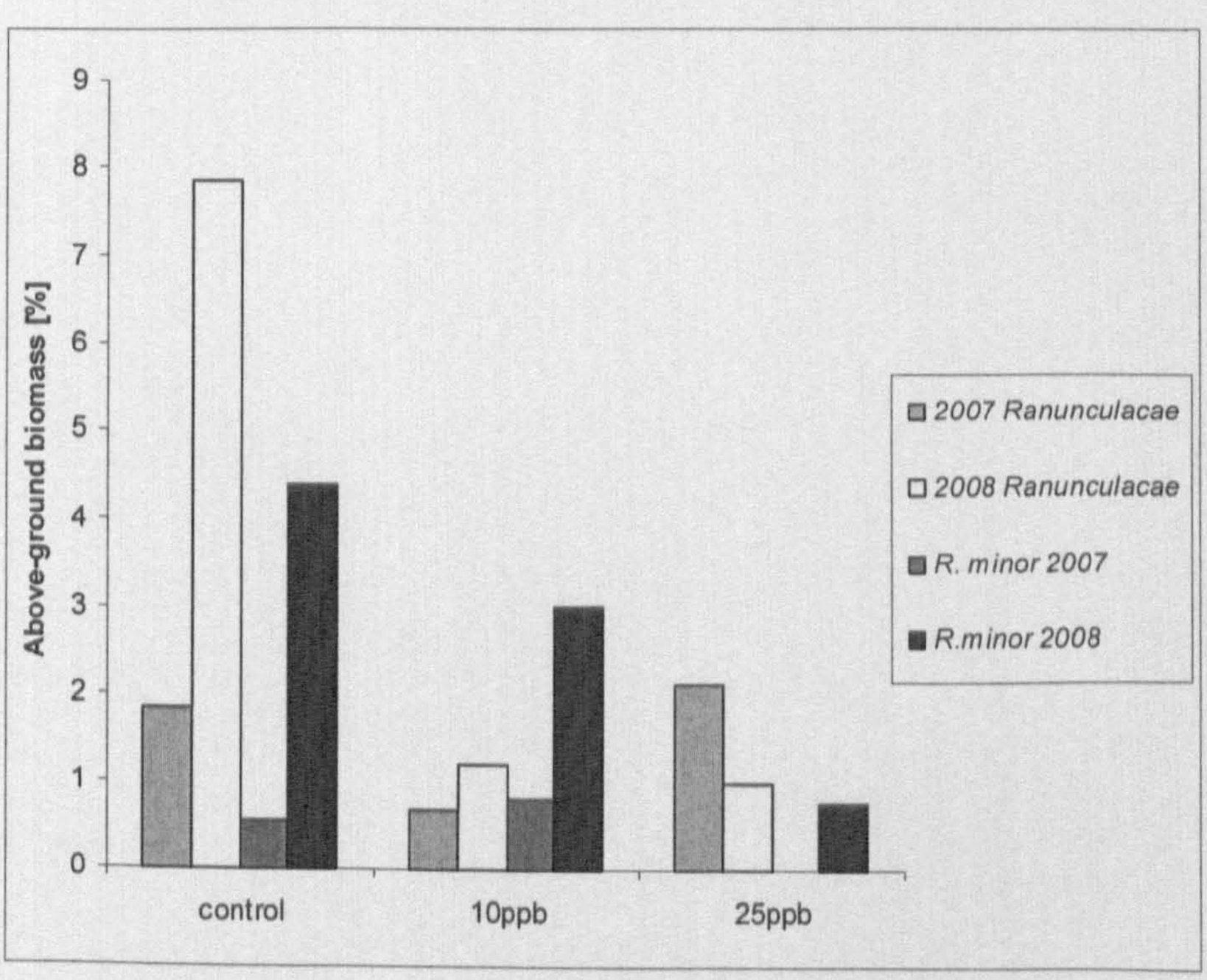


Figure 34: The percentage of *Ranunculacae* and *Rhinanthus minor*, expressed as a percentage of the total above-ground biomass in the different treatment plots (Control, 10 ppb and 25 ppb) in 2007 and 2008.

2.3.5 Multivariate analysis

Grasses

Three RDA analyses were conducted, for each of 2007 and 2008 one with only accumulated O_3 as an environmental variable, the second with all environmental variables except ozone measured at 0-10 cm, and the third with all environmental variables except ozone measured at 10-20 cm. The redundancy analysis (RDA) of the two harvests revealed differences between the two depths. O_3 explained only 4.3% of the species composition in 2007 (P= 0.66) (Appenidx 7.2, Figure 102), whereas all the other environmental variables account for 71.5% using data from 0-10 cm (Figure 35), and for 88% using data from 10-20 cm (Figure 36) of the species composition.

The cover of some of the grass species was associated with positive values on the accumulated ozone axis (*Alopecurus pratensis, Anthoxanthum odoratum, Festuca rubra/ovina, Lolium perenne* and *Poa pratensis*), whereas that of the other species was associated with negative values on the accumulated ozone axis (Agrostis *gigantea, Festuca pratensis, Holcus lanatus, Phleum bertolonii* and *Poa spec.*).

Now in 2008, O_3 accounts for 6.2% (P= 0.388) (Appenix 7.2, Figure 104), while all the other environmental variables explain 75.7% of the species variation using data from 0-10 cm (Figure 37) and 70.8% using data from 10-20 cm (Figure 38) of the species composition. Overall there was a small change between the two years; more variation in the species composition can be attributed to O_3 in 2008 than in 2007, although still most of the variation is due to all the other environmental variables.

The cover of some of the grass species was associated with positive values on the accumulated ozone axis (*Anthoxanthum odoratum, Dactylis glomerata* and *Lolium perenne*), whereas that of the other species was associated with negative values on the accumulated ozone axis (*Agrostis gigantea, Alopecurus pratensis, Festuca pratensis, Festuca rubra/ovina, Holcus lanatus, Lolium perenne, Phleum pratense* and *Trisetum flavescens*)

In 2007, ozone treatments differentiated mainly on Axis 1 transects mainly on Axis 2. Strong association of soil variables was found with Axis 1. Similar patterns were observed in 2008.

For ozone, some species are associated with the ozone axis (*A. oderatum, L. perenne)* in both years; main change is a stronger association with *Dactylis glomerata* in 2008.

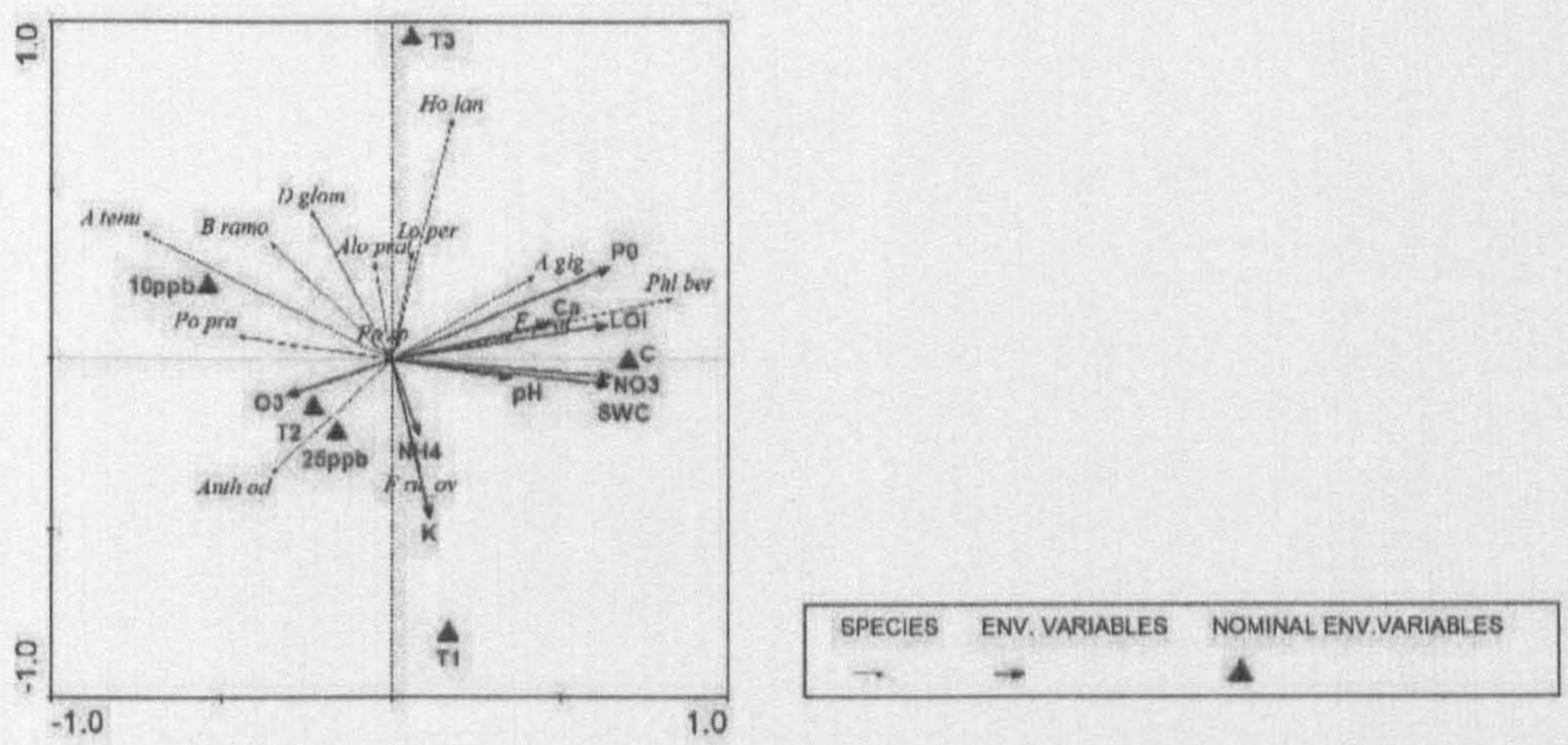


Figure 35: Redundancy analyses of the harvest in 2007. Environmental variables are soil parameters measured at 0-10 cm (SWC= soil water content, LOI= organic matter content, NO3= nitrate, NH4= ammonium, P0= phosphorus, K= Potassium, Ca= Calcium, O3= ozone). Transects (T1, T2, T3) and plots (C, 10 ppb, 25 ppb) at the field site are represented by nominal environmental variables. Species codes are: *A tenu=Agrostis tenuis, A gig= Agrostis gigantea, A stol= Agrostis stolonifera, Alo prat= Alopecurus pratensis, Anth od= Anthoxanthum odoratum, D glom= Dactylis glomerata, F prat= Festuca pratensis, F ru_ov= Festuca rubra/ovina, Ho lan= Holcus lanatus, Lo per= Lolium perenne, Phl ber= Phleum bertolonii, Phl pra= Phleum pratense, Po pra= Poa pratensis, Tris fla= Trisetum flavescens, Ju eff= Juncus effusus*

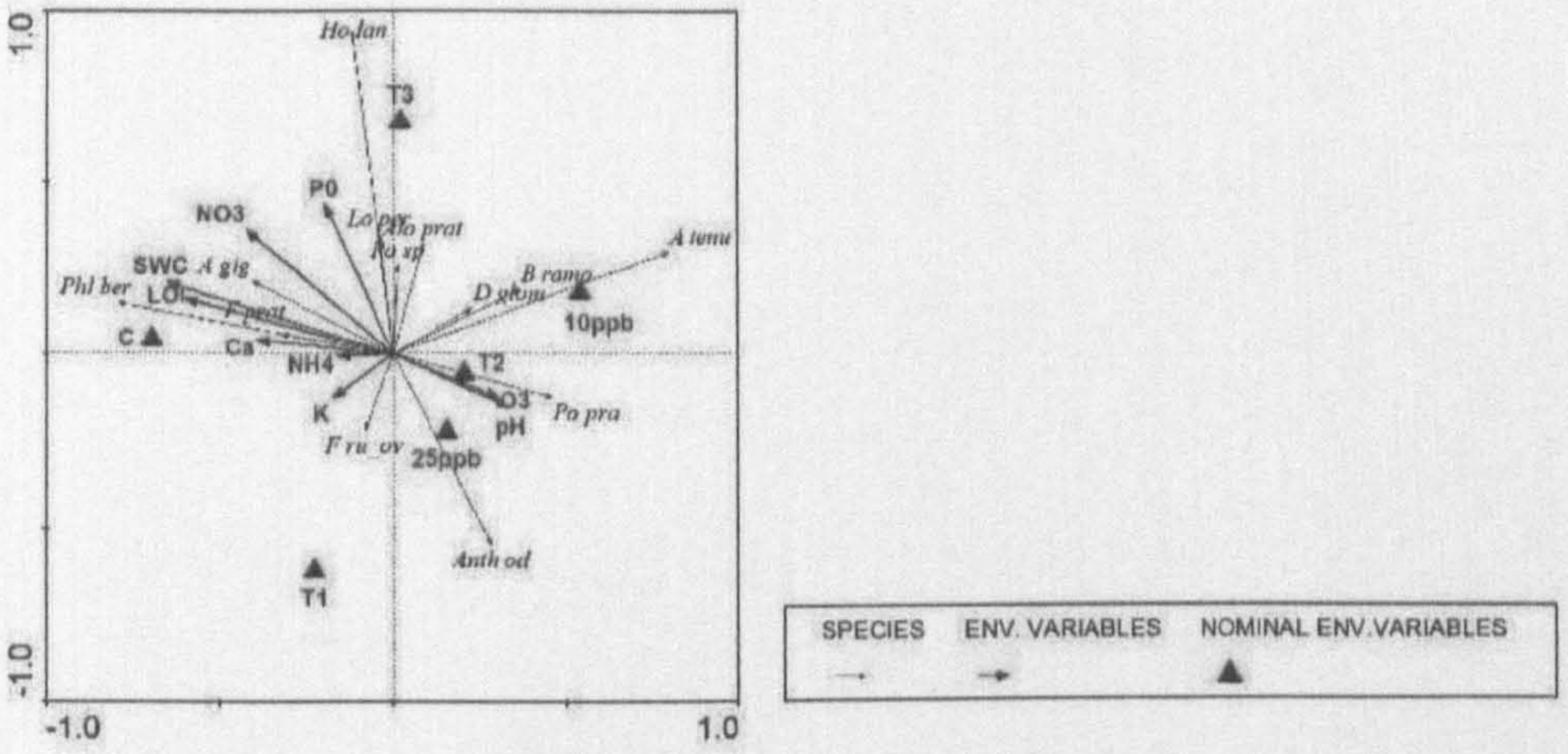


Figure 36: Redundancy analyses of the harvest in 2007. Environmental variables are soil parameters measured at 10-20 cm (SWC= soil water content, LOI= organic matter content, NO3= nitrate, NH4= ammonium, P0= phosphorus, K= Potassium, Ca= Calcium, O3= ozone). Nominal environmental variables present transects (T1, T2, T3) and plots (C, 10 ppb, 25 ppb) at the field site. Species codes are: *A tenu= Agrostis tenuis, A gig= Agrostis gigantea, A stol= Agrostis stolonifera, Alo prat= Alopecurus pratensis, Anth od= Anthoxanthum odoratum, D glom= Dactylis glomerata, F prat= Festuca pratensis, F ru_ov= Festuca rubra/ovina, Ho lan= Holcus lanatus, Lo per= Lolium perenne, Phl ber= Phleum bertolonii, Phl pra= Phleum pratense, Po pra= Poa pratensis, Tris fla= Trisetum flavescens, Ju eff= Juncus effusus*

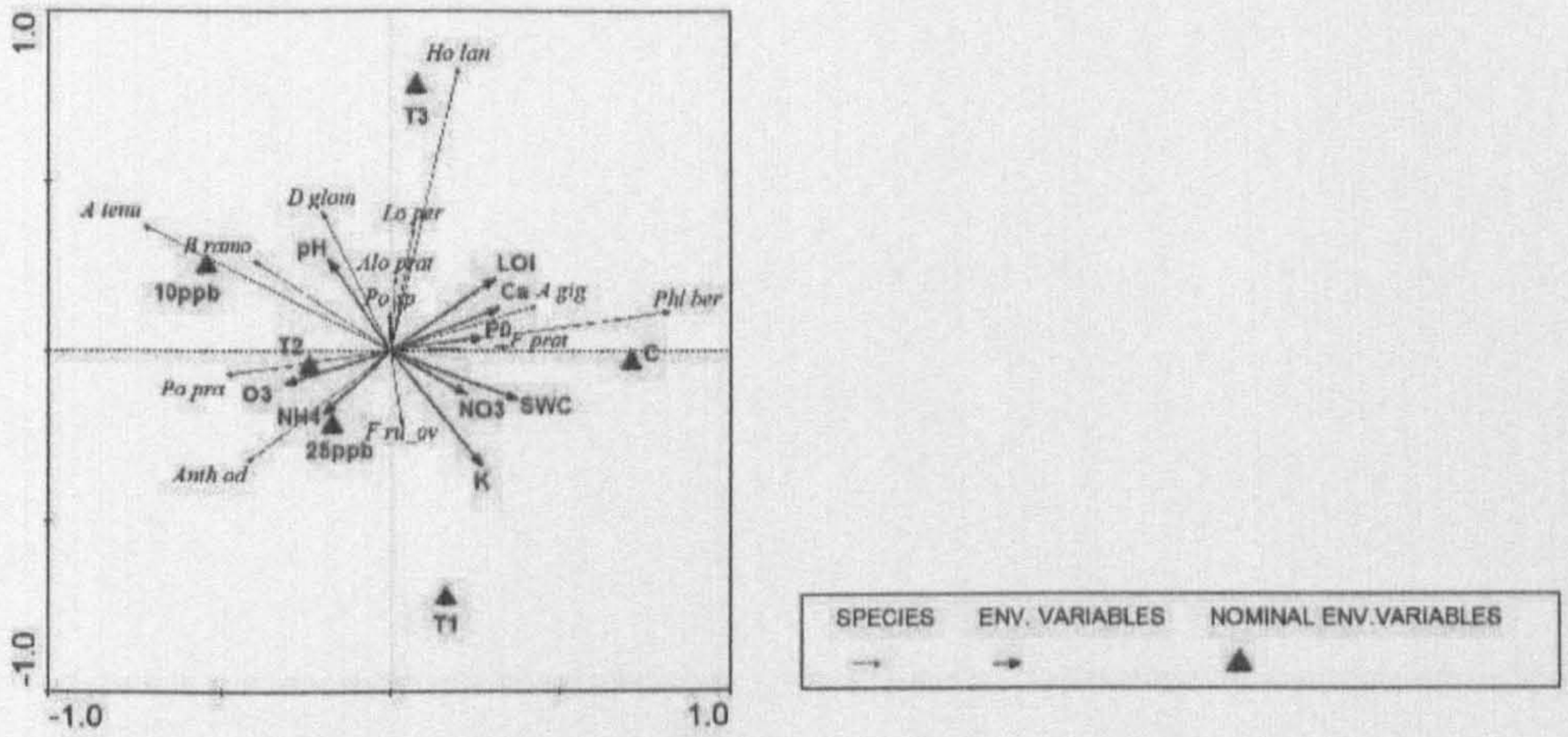


Figure 37: Redundancy analyses of the harvest in 2008. Environmental variables are soil parameters measured at 0-10 cm (SWC= soil water content, LOI= organic matter content, NO3= nitrate, NH4=ammonium, P0=phosphorus, K= Potassium, Ca= Calcium, O3= ozone). Nominal environmental variables present transects (T1, T2, T3) and plots (C, 10 ppb, 25 ppb) at the field site. Species codes are: *A tenu= Agrostis tenuis, A gig= Agrostis gigantea, A stol= Agrostis stolonifera, Alo prat= Alopecurus pratensis, Anth od= Anthoxanthum odoratum, D glom= Dactylis glomerata, F prat= Festuca pratensis, F ru_ov= Festuca rubra/ovina, Ho lan= Holcus lanatus, Lo per= Lolium perenne, Phl ber= Phleum bertolonii, Phl pra= Phleum pratense, Po pra= Poa pratensis, Tris fla= Trisetum flavescens, Ju eff= Juncus effusus.*

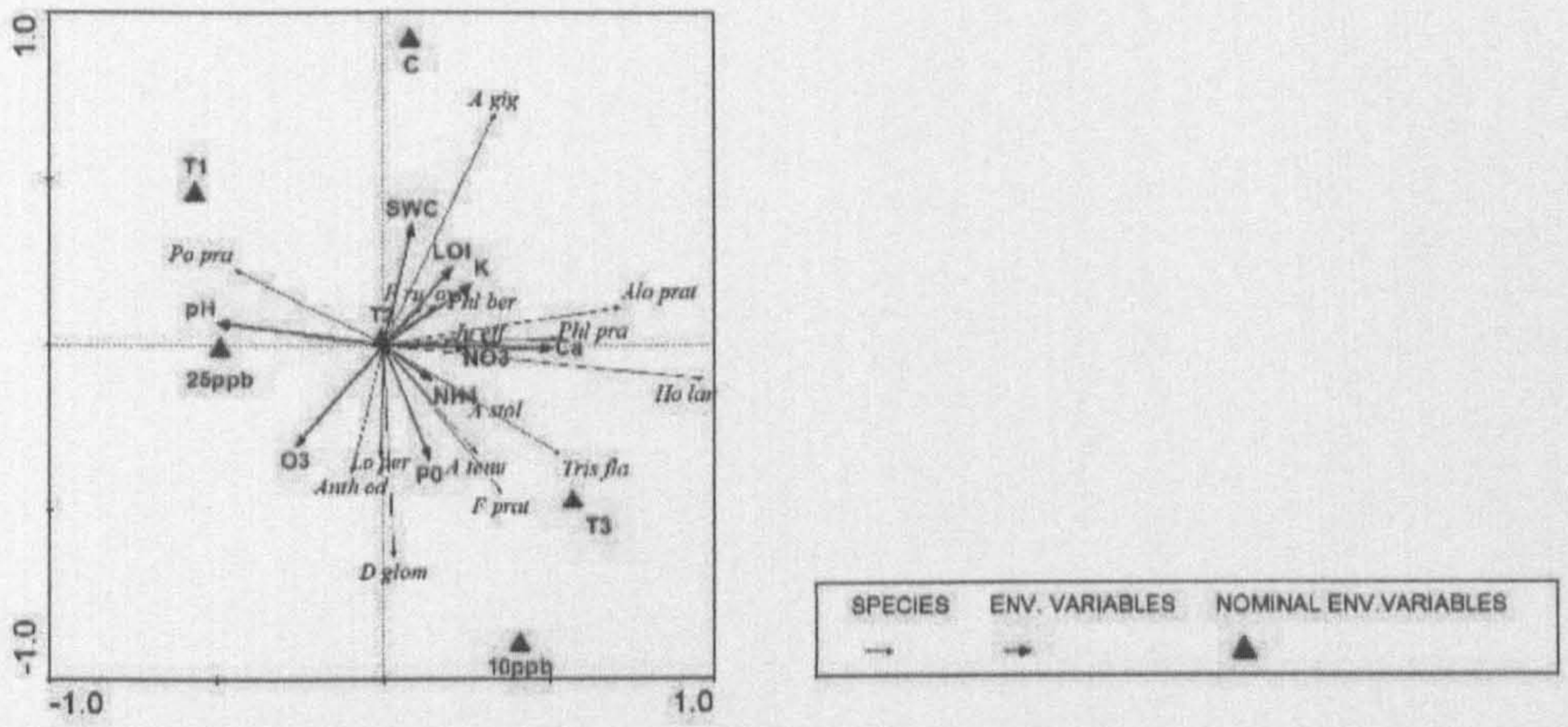


Figure 38: Redundancy analyses of the harvest in 2008. Environmental variables are soil parameters measured at 10-20 cm (SWC= soil water content, LOI= organic matter content, NO3= nitrate, NH4=ammonium, P0=phosphorus, K= Potassium, Ca= Calcium, O3= ozone). Nominal environmental variables present transects (T1, T2, T3) and plots (C, 10 ppb, 25 ppb) at the field site. Species codes are: *A tenu= Agrostis tenuis, A gig= Agrostis gigantea, A stol= Agrostis stolonifera, Alo prat= Alopecurus pratensis, Anth od= Anthoxanthum odoratum, D glom= Dactylis glomerata, F prat= Festuca pratensis, F ru_ov= Festuca rubra/ovina, Ho lan= Holcus lanatus, Lo per= Lolium perenne, Phl ber= Phleum bertolonii, Phl pra= Phleum pratense, Po pra= Poa pratensis, Tris fla= Trisetum flavescens, Ju eff= Juncus effusus*

Forbs

In 2007 O_3 explained only 4.0% of the species composition (P= 0.2) (Appenix 7.2, Figure 103), whereas all the other environmental variables account for 33.8% using data from 0-10 cm (Figure 39) and for 41.6% using data from 10-20 cm (Figure 40) of the species composition.

The cover of some of the forb species was associated with positive values on the accumulated ozone axis (*Cardamine pratensis, Conopodium majus, Leontodon hispidus, Lathyrus pratensis, Plantago lanceolata, Ranunculus spec., Rhinanthus minor, Stellaria graminea* and *Vicia sativa*), whereas that of the other species was associated with negative values on the accumulated ozone axis (*Taraxacum spec., Trifolium pratensis* and *Veronica chamaedrys*).

Now in 2008, O_3 accounts for 9.5% (P= 0.01) (Appenix 7.2, Figure 105), while all the other environmental variables explain 50.8% using data from 0-10 cm (P= 0.01) (Figure 41) and 50% using data from 10-20 cm of the species composition (P= 0.008) (Figure 42).

The cover of some of the forb species was associated with positive values on the accumulated ozone axis (*Cardamine pratensis, Lathyrus pratensis, Stellaria graminea, Trifolium repens* and *Vicia sativa*), whereas that of the other species was associated with negative values on the accumulated ozone axis (*Ranunculus spec., Rhinanthus minor, Rumex acetosa, Taraxacum spec., Trifolium pratensis* and *Veronica chamaedrys*).

In 2007 there was a strong separation of T3 from T1 and T2, which was associated with legume species, while in 2008 a separation was found of T1 from T2 and T3, which was not associated with only legumes.

For ozone, most forb and legume species moved away from the accumulated ozone axis, just a few species such as *S. graminea*, *C. pratensis* and *V. sativa* were also associated with the ozone axis.

Overall there was a distinct change between the two years; more variation of the species composition can be attributed to O_3 in 2008 than in 2007. Despite the small proportion of the explained variance, fitting the cumulative ozone exposure as the explanatory variable was significant in 2008.

Furthermore, some of the few species seemed to depend on certain soil features. *Lathyrus pratensis* was negatively correlated with the organic matter content (LOI) at 0-10 cm; *Agrostis gigantea* was positively correlated with the soil water content (SWC) at 10-20 cm,

Festuca rubra/ovina positively with potassium at 0-10 cm and *Poa pratensis* positively with the pH at 10-20 cm both in 2007 and 2008. *Phleum bertolonii* and *Festuca pratensis* were correlated positively with calcium at 0-10 cm and 10-20 cm and *Poa pratensis* with the pH in 2007. *Agostis tenuis* was correlated positively with the 10 ppb plot at 0-10 cm and *Anthoxanthum odoratum* with the 25 ppb plot in both years.

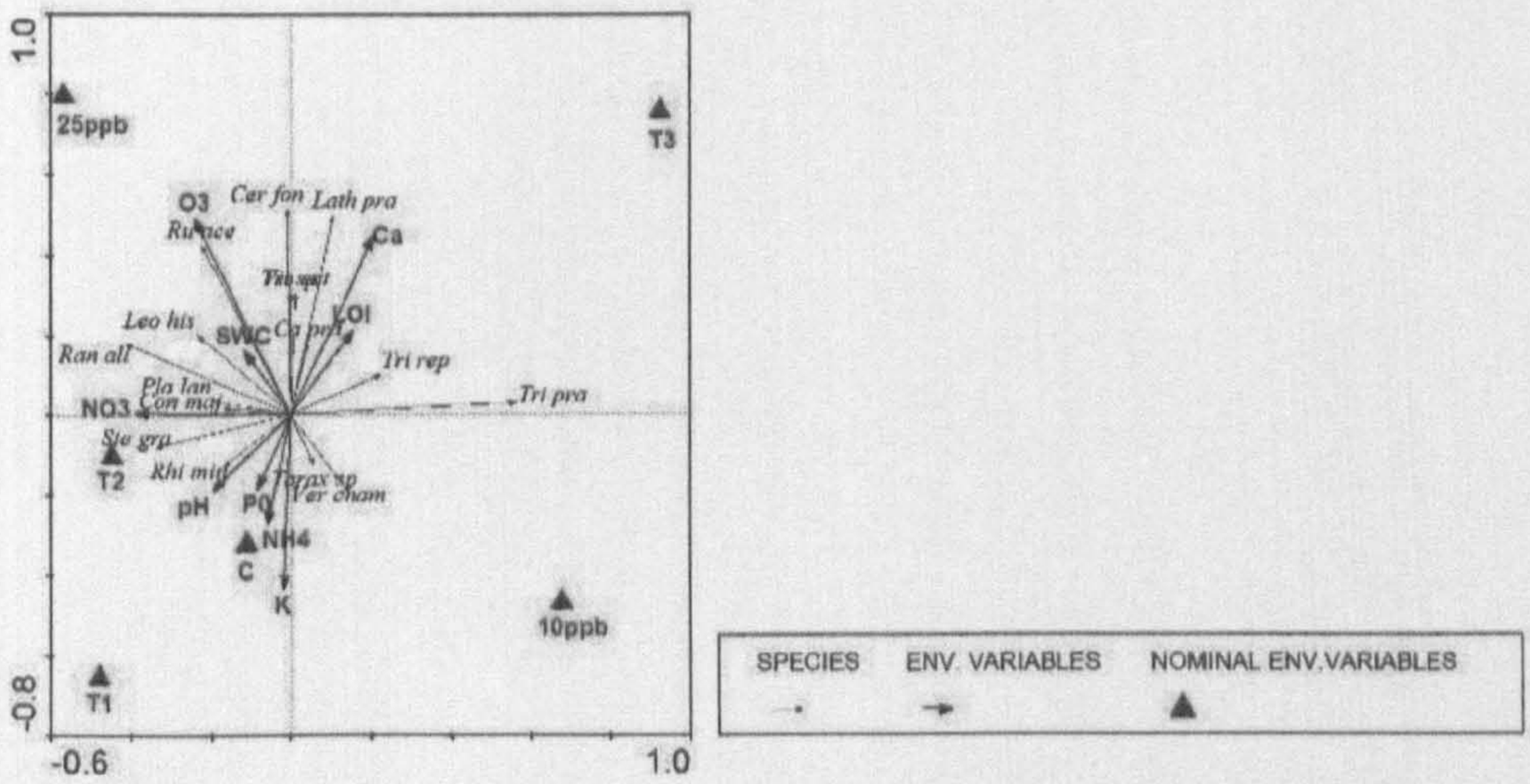


Figure 39: Redundancy analyses of the harvest in 2007. Environmental variables are soil parameters measured at 0-10 cm (SWC= soil water content, LOI= organic matter content, NO3= nitrate, NH4= ammonium, P0= phosphorus, K= Potassium, Ca= Calcium, O_3= ozone). Nominal environmental variables present transects (T1, T2, T3) and plots (C, 10 ppb, 25 ppb) at the field site. Species codes are: *Ca pra= Cardamine pratensis, Cer fon= Cerastium fontanum, Con maj= Conopodium majus, Lath pra= Lathyrus pratensis, Leo his= Leontodon hispidus, Pla lan= Plantago laneolata, Ran all= Ranunculacae, Rhi min= Rhinanthus minor, Ru ace= Rumex acetosa, Tarax sp= Taraxacum spec., Tri pra= Trifolium pratense, Tri rep= Trifolium repens, Ste gra= Stellaria graminea, Vic sat= Vicia sativa, Ver cham= Veronica chamaedrys.*

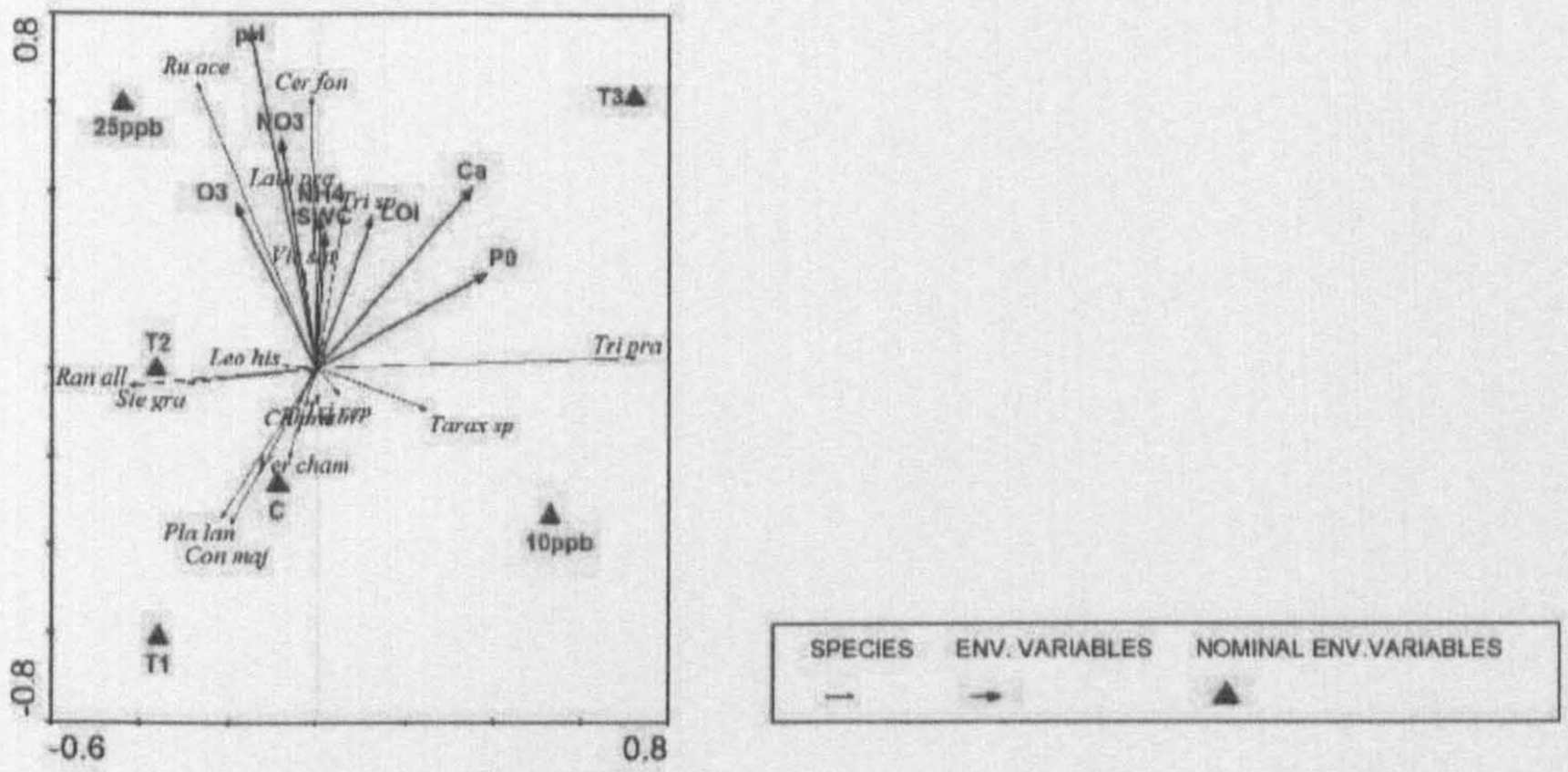


Figure 40: Redundancy analyses of the harvest in 2007. Environmental variables are soil parameters measured at 10-20 cm (SWC= soil water content, LOI= organic matter content, NO3= nitrate, NH4= ammonium, P0= phosphorus, K= Potassium, Ca= Calcium, O3= ozone). Nominal environmental variables present transects (T1, T2, T3) and plots (C, 10 ppb, 25 ppb) at the field site Species codes are: *Ca pra= Cardamine pratensis, Cer fon= Cerastium fontanum, Con maj= Conopodium majus, Lath pra= Lathyrus pratensis, Leo his= Leontodon hispidus, Pla lan= Plantago laneolata, Ran all= Ranunculacae, Rhi min= Rhinanthus minor, Ru ace= Rumex acetosa, Tarax sp= Taraxacum spec., Tri pra= Trifolium pratense, Tri rep= Trifolium repens, Ste gra= Stellaria graminea, Vic sat= Vicia sativa, Ver cham= Veronica chamaedrys.*

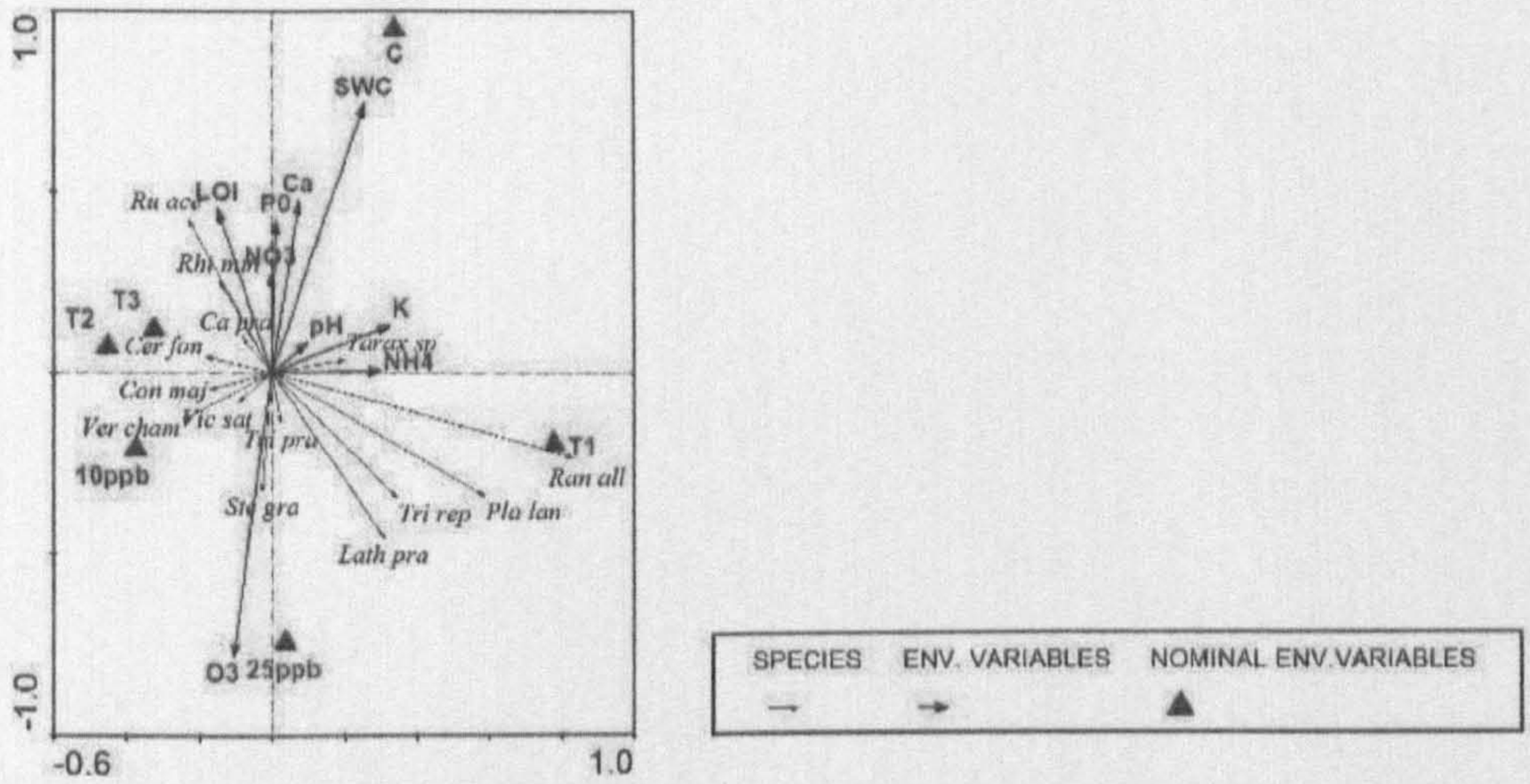


Figure 41: Redundancy analyses of the harvest in 2008. Environmental variables are soil parameters measured at 0-10 cm (SWC= soil water content, LOI= organic matter content, NO3= nitrate, NH4= ammonium, P0= phosphorus, K= Potassium, Ca= Calcium, O3= ozone). Nominal environmental variables present transects (T1, T2, T3) and plots (C, 10 ppb, 25 ppb) at the field site. Species codes are: *Ca pra= Cardamine pratensis, Cer fon= Cerastium fontanum, Con maj= Conopodium majus, Lath pra= Lathyrus pratensis, Leo his= Leontodon hispidus, Pla lan= Plantago laneolata, Ran all= Ranunculacae, Rhi min= Rhinanthus minor, Ru ace= Rumex acetosa, Tarax sp= Taraxacum spec., Tri pra= Trifolium pratense, Tri rep= Trifolium repens, Ste gra= Stellaria graminea, Vic sat= Vicia sativa, Ver cham= Veronica chamaedrys.*

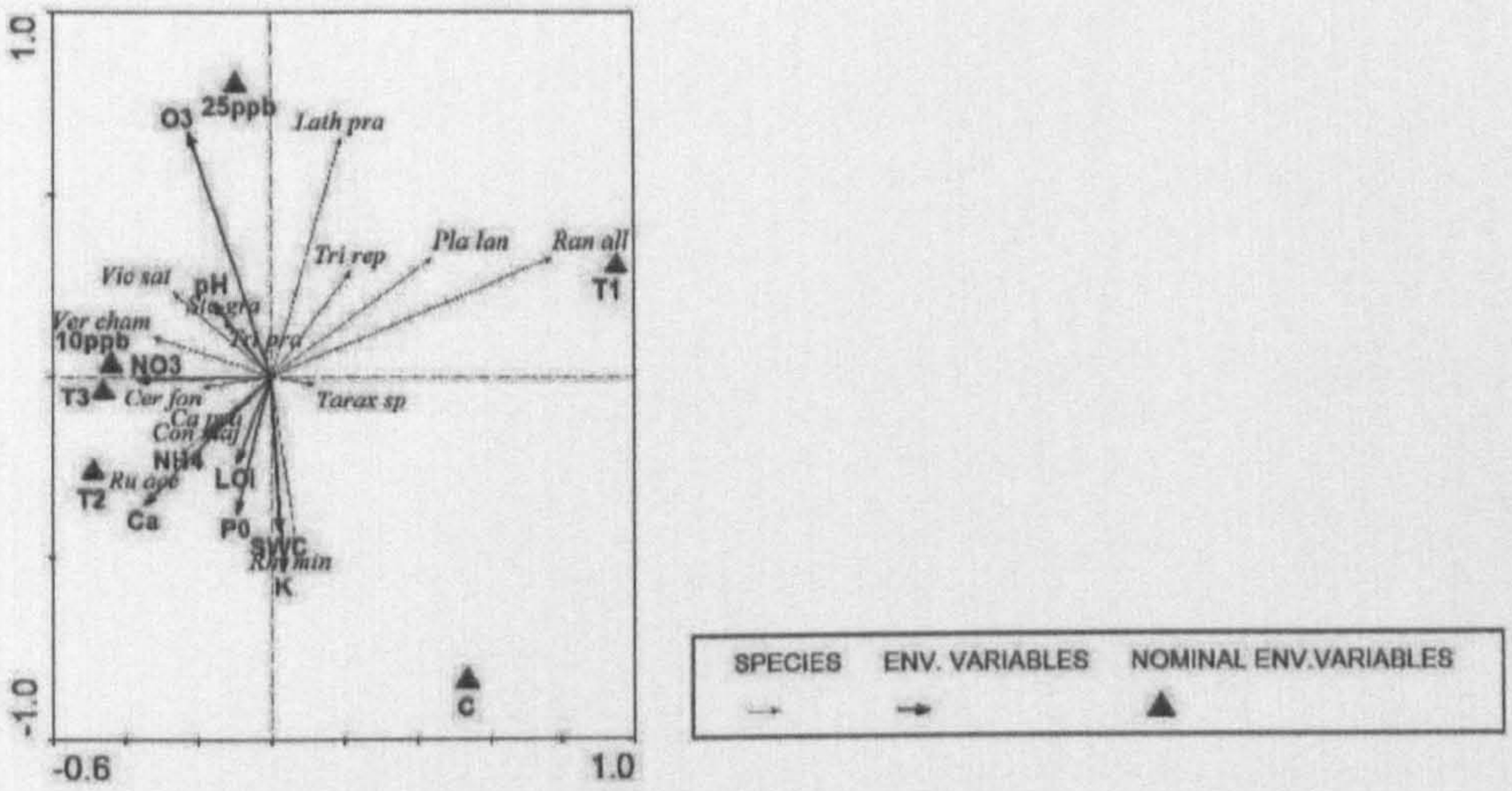


Figure 42: Redundancy analyses of the harvest in 2008. Environmental variables are soil parameters measured at 10-20 cm (SWC= soil water content, LOI= organic matter content, NO3= nitrate, NH4= ammonium, P0= phosphorus, K= Potassium, Ca= Calcium, O_3= ozone). Nominal environmental variables present transects (T1, T2, T3) and plots (C, 10 ppb, 25 ppb) at the field site Species codes are: *Ca pra= Cardamine pratensis, Cer fon= Cerastium fontanum, Con maj= Conopodium majus, Lath pra= Lathyrus pratensis, Leo his= Leontodon hispidus, Pla lan= Plantago laneolata, Ran all= Ranunculacae, Rhi min= Rhinanthus minor, Ru ace= Rumex acetosa, Tarax sp= Taraxacum spec., Tri pra= Trifolium pratense, Tri rep= Trifolium repens, Ste gra= Stellaria graminea, Vic sat= Vicia sativa, Ver cham= Veronica chamaedrys*

Table 20 and Table 21, the results of a stepwise canonical analysis of the main environmental and species variables at 0-10 cm and 10-20 cm are presented. On the first axis soil water content, calcium and nitrate are positively whereas ozone is negatively correlated. On the second axis only soil water content is negatively correlated while the other three environmental variables are positively correlated. The first two axes are quite distinct from each other. Ozone is the only major environmental

variable on axis 2, whereas it has less impact than SWC, NO_3^- and Ca_2^+ on axis 2. The species which are associated with axis 2 are *S. graminea* and *L. pratensis* being positively correlated with ozone, and *R. minor* and *Ranunculacae* being negatively correlated with ozone. However, the environmental variables explain more of the species variability on axis 1 than they do on axis 2, and the third and fourth axes explain even less. Likewise, ozone is an important driver here, although soil water content, calcium and nitrate play a bigger role in the species variation.

Table 20: showing the results from the canonical correspondence analysis of the harvest in 2008 at 0-10 cm. The first four species axes (only forbs) and the first four environmental axes are listed. The four chosen environmental variables are all significant and explain most of the species variability.

SPEC AX1	1.00							
SPEC AX2	0.08	1.00						
SPEC AX3	0.24	-0.15	1.00					
SPEC AX4	-0.13	0.00	-0.13	1.00				
ENVI AX1	0.76	0.00	0.00	0.00	1.00			
ENVI AX2	0.00	0.67	0.00	0.00	0.00	1.00		
ENVI AX3	0.00	0.00	0.59	0.00	0.00	0.00	1.00	
ENVI AX4	0.00	0.00	0.00	0.57	0.00	0.00	0.00	1.00
O3	**-0.47**	**0.50**	**0.12**	**-0.06**	**-0.62**	**0.75**	**0.21**	**-0.11**
SWC	**0.61**	**-0.04**	**-0.32**	**0.13**	**0.81**	**-0.06**	**-0.54**	**0.22**
NO3	**0.70**	**0.07**	**0.08**	**-0.20**	**0.92**	**0.10**	**0.13**	**-0.35**
Ca	**0.52**	**0.02**	**-0.43**	**-0.07**	**0.69**	**0.03**	**-0.72**	**-0.11**
	SPEC AX1	SPEC AX2	SPEC AX3	SPEC AX4	ENVI AX1	ENVI AX2	ENVI AX3	ENVI AX4

Table 21: showing the results from the canonical correspondence analysis of the harvest in 2008 at 10-20 cm. The first four species axes (only forbs) and the first four environmental axes are listed. The four chosen environmental variables are all significant and explain most of the species variability.

SPEC AX1	1.00							
SPEC AX2	0.21	1.00						
SPEC AX3	0.10	0.26	1.00					
SPEC AX4	-0.14	-0.09	0.14	1.00				
ENVI AX1	0.78	0.00	0.00	0.00	1.00			
ENVI AX2	0.00	0.63	0.00	0.00	0.00	1.00		
ENVI AX3	0.00	0.00	0.50	0.00	0.00	0.00	1.00	
ENVI AX4	0.00	0.00	0.00	0.38	0.00	0.00	0.00	1.00
O3	**-0.48**	**0.48**	**-0.01**	**0.07**	**-0.61**	**0.77**	**-0.02**	**0.18**
SWC	**0.69**	**-0.03**	**0.14**	**0.13**	**0.89**	**-0.04**	**0.28**	**0.35**
NO3	**0.66**	**0.27**	**-0.11**	**-0.07**	**0.86**	**0.43**	**-0.22**	**-0.19**
Ca	**0.60**	**-0.03**	**-0.20**	**0.19**	**0.78**	**-0.04**	**-0.40**	**0.49**
	SPEC AX1	SPEC AX2	SPEC AX3	SPEC AX4	ENVI AX1	ENVI AX2	ENVI AX3	ENVI AX4

2.4 Discussion

Drivers on the site

Despite the low ozone exposures, with an average of 33.6 ppb during May to August surprisingly large effects of O_3 were observed on forbs above-ground biomass, grass/ forb ratio and also individual forb contributions were affected by O_3 in 2008. Even more surprising was the impact of fumigation in 2007 when O_3 concentrations achieved along the gradient were 33 ppb at 2.5 m, 29 ppb at 5 m and 26 ppb at 10 m downwind from the release line (Transect 1).

Climatic differences between the two years probably had a major effect on species composition at the field site. The temperatures in 2007 were on overall substantially higher than in 2008, but the relative humidity did not vary much, and neither did rainfall. PAR was only slightly higher in 2008, and the ambient O_3 concentrations were in general around 5 ppb higher. This leads to the conclusion that the higher temperature in 2007 would have the greater climatic influence on species composition, although it does not explain why in some plots the biomass was greater, and in other plots the biomass was lower in 2008 compared with 2007. However, the colder climate in 2008 might be responsible for the smaller effects of transect on biomass production in 2008.

One of the main drivers on the site is the soil conditions. The soil analysis revealed significant differences between the transects, showing significantly greater concentrations of calcium and ammonium at 0-10 cm in Transect 3. At 10-20 cm, phosphorus, calcium, and nitrate concentrations as well as soil water content and organic matter content, were also significantly greater in Transect 3. The higher availability of nutrients in this transect may have favoured increased growth, and is likely an explanation for the significantly higher biomass in the third transect in 2007. Significantly higher concentrations of calcium and nitrate, and higher soil water contents and organic matter contents were found at the control plots at 0-10 cm. So, overall, the third transect and the control plots were more nutrient rich and

wetter than the other transects and plots of the field site. Hence the gradient in soil conditions between the plots confounds the potential for any significant effect of ozone, and which can also explain the position of some species.

Analysis of the data at the community level showed that, overall, the cumulative ozone exposure explained only a small proportion of the variation in the partial redundancy analysis. Only 4.3% of the variation grass biomass and 4% of the variation forb biomass were explained by O_3 in 2007. This increased to 6.2% of the grasses and 9.5% of the forbs in 2008 (Appendix 2). Although the axis of cumulative ozone exposure was significant for the forbs in 2008, the other environmental axes were much more significant, explaining about 50% of the variation. Nitrate, soil water content and calcium were the most important variables in explaining the species variation, but also ozone was a significant factor in 2008. Taking this all in consideration, the soil conditions at the site account for most of the variation in species composition in the harvests of 2007 and 2008, but the shift in species composition, and the increased proportion of variation explained by ozone in 2008, also suggests that O_3 has become a more important driver.

Overall effects of O_3 on total biomass and functional groups

The total aboveground biomass data of 2007 and 2008 showed no effect of ozone; the only significant differences found in 2007 were between transects. These significant transect differences were also observed for the different functional groups, with legumes and grasses favouring the third transect. As discussed above, these observed effects probably have to do with the differences in nutrient availability in the three transects, with Transect 3 having the highest nutrient levels. Also, the two productive grass species *Lolium perenne* and *Poa pratensis* showed significantly higher biomass in the third transect in 2007. There was a trend of higher forb biomass in the 25 ppb plots than in the control plots in 2007, which might be related to the higher soil water content of the control plots at both soil depths, and which reflects the plant distribution across the field site.

In 2008 a change in the balance of grasses and forbs was observed. Despite a trend to higher biomass in Transect 3, which was not so pronounced, the functional groups were more affected by the ozone treatment. There were only two species which showed a transect effect; *Trisetum flavescens* with a significantly higher biomass in T3 and T2, and *Rumex acetosa* which was significantly higher in T2. The ozone treatment affected especially the forbs, which had a significantly higher biomass in the control plots than at the 25 ppb plots. Whereas in 2007, the forb biomass was lower in the wetter control plots, in 2008 they were less abundant in the 25 ppb treatment. Although the lower biomass of at the 25 ppb plots might be still due to the soil gradient between the plots, the impact of the greater O_3 exposure on the forbs biomass has to be considered also.

Effects on grass/forb ratio

The forb/ grass ratio was only affected by the O_3 treatment, and not by transects which were significantly greater in the control plots than at the 25 ppb plots in 2007. This trend was reversed in 2008. The grass/ forb ratio was significantly smaller at the control plots than in the 25 ppb plots. As there was no significant effect on the grass biomass, and the relatively small legume biomass increased with increasing O_3, the change of the grass/ forb ratio was mainly due to a reduction of forb biomass in the 10 ppb and 25 ppb plots. When comparing the forb biomass data of 2007 with 2008, it is apparent that the forb biomass production did not decline overall, the forb biomass rather increased about 50% in the control plots, whereas in the elevated O_3 plots the forbs biomass decreased by only 5%.

Other studies of grassland communities have also examined effects of functional groups. In a lowland hay meadow mesocosm experiment, Ramö *et al.* (2006) exposed seven species both to O_3 and CO_2 in open-top chambers over a three year period. Also in their study, the functional group that was most sensitive to ozone in terms of growth reduction was also the group of non-leguminous herbs (*Fragaria vesca* L., *Campanula rotundifolia* L.), while the leguminous species showed visible ozone injuries

(*Trifolium medium* L., *Vicia cracca* L.) but little effect on biomass. The total biomass was decreased by 40% in the study of Ramö *et al.* (2006). This is in contrast to the results of this study, which may be due to the much greater dominance of grass biomass at the High Keenley Fell site. Basin *et al.* (2007) found in their study on nitrogen deposition and ozone effects on the productivity and community composition of a subalpine grassland that after three years of increased ozone exposure, no effects of ozone or ozone*nitrogen were observed for grasses, legumes and forbs. This suggested considerable ozone tolerance of the system and inherent community characteristics. However, their ozone levels were greater than in this study, and despite the lower O_3 levels used in this study the mature grassland community showed effects of an altering community under the ozone regime.

Volk *et al.* (2006) studied a semi-natural grassland in a five year free-air ozone fumigation period. They found that the total biomass in the elevated O_3 plots decreased by 23% in comparison to the control plots, and that was accompanied by a change in biomass production in the functional groups. In this case especially the legumes rather than forbs showed a strong negative response. This strong negative response of the legume fraction was not observed in the current study, although this was a very small proportion of the total biomass. The main reduction in 2008 was more due to the forb fraction than to the legume fraction, but the fractions were only exposed to O_3 for a two year period, with the first period not having received much O_3, and therefore stronger negative responses may still occur in years to come. Wilbourn *et al.* (1995) exposed a grass-clover mixture to elevated O_3 concentrations in a free air ozone fumigation system for a year. They found that most of the yield loss was due to effects on *Trifolium repens*, but as this study was a two species mixture, the ecosystem is rather different from this study. Also, in a study on the composition of artificial grassland communities affected by O_3 and cutting, Ashmore & Ainsworth (1995) observed that the biomass of *T. repens* declined both in cut and uncut pots under elevated O_3 concentrations. This was also confirmed by a study of Fuhrer *et al.*, (1994) who found that ozone reduced the yield of two clover

species, by 24% in the 2nd year of the experiment. Legumes have been consistently reported to be ozone sensitive (Mills *et al.*, 2006), and hence their response of increasing biomass at higher ozone levels in this study is not consistent with data for individual species, although Bassin *et al.* (2009) did not report any O_3 effects on the legumes in their alpine grassland study either.

In this study the legumes increased with increasing O_3 concentrations but one has to consider the low O_3 concentrations received. Another reason could have been the lower soil water content of the 10 ppb compared to the control plots. In addition, most of the legume species were absent from the control plots, which was not the case in the year before. Therefore, this unusual response of the legumes in 2008 is probably more influenced by the soil conditions than the actual O_3 concentration.

Individual species

A number of individual species showed significant effects of ozone on biomass in the 2008 harvest. Here these species are considered in relation to the literature. *Ranunculus* species were reduced in biomass by elevated ozone. Mortensen *et al.* (1993) found similar results for *Ranunculus acris,* which showed a highly significant growth reduction at an O_3 concentration of 90 ppb in a controlled environmental chamber study. The O_3 concentrations in their study were much higher than O_3 concentrations in the current study but despite the low O_3 concentration of 10 and 25 ppb used, the effects of elevated ozone on *Ranunculus* spec. were significant. In top of that not only *Ranunculus spec.* but also family *Ranunculacae* was overall significantly reduced at the 10 ppb and 25 ppb treatment compared to the control treatment. *Ranunculacae* was also negatively correlated with the axis of cumulative ozone exposure, and a positive correlation with other environmental variables was not found, although the higher soil water content and nutrient availability in the control plots might have benefited them.

R. acris was also studied by Rämö *et al.* (2006) in a lowland hay medow mesocosm experiment, exposed both to O_3 and CO_2 in OTCs. Although

R. acris had been reported to show not only visible injuries but also growth reductions at high levels of O_3, these effects did not occur at lower O_3 concentrations of 40-50 ppb. Since in this study, effects of O_3 could only be reported for *Ranunculus* spec. and for the whole family *Ranunculacae*, the effects on individual *Ranunculus* species might still be quite different.

For *Rhinanthus minor* the study suggested effects of a declining biomass of *R. minor* at higher O_3 concentrations. Thwaites (1996) found a decline in the biomass of *R. minor* when exposed to elevated O_3. As yet, there is little literature on the effects of elevated O_3 on *R. minor*, although *R. minor* is an important hemi-parasite in maintaining species diversity within the grassland.

Dactylis glomerata was stimulated by higher O_3 concentrations. Fuhrer *et al.* (1994) studied the effect of ozone on a managed pasture. Their findings of a decline in the legumes were associated with a slight increase in the biomass of the grasses mainly caused by *D. glomerata.* Similar effects on *D. glomerata* were reported by Thwaites *et al.* (2006). They studied O_3 effects on the species dynamics of calcareous grasslands, and observed an increase in the biomass of *D. glomerata* under elevated O_3. Taking this into account, and that *D. glomerata* has been reported to be insensitive to O_3 (relative sensitivity: 0.992) (Hayes *et al.*, 2006), the observed stimulation by ozone in 2008 in the elevated treatments might be real, and could be influenced by decreased competition from more sensitive grass species. Since *D. glomerata* is a potentially nutrient demanding dominant grass species, it should favour the higher nutrient levels of the control plots (Thwaites *et al.*, 2006).

The legumes *Lathyrus pratensis* and *Trifolium repens,* and the forb *Stellaria graminea,* also showed a stimulation of their biomass at elevated O_3 concentrations. Despite belonging to the *Fabaceae* family, *L. pratensis* has not been reported to be sensitive to O_3, but effects on the shoot-root ratio have been observed. Power and Ashmore (2002) found, in a study of the responses of fen and fen-meadow communities to O_3, that below-ground biomass was more affected than the above-ground biomass, which was reflected in a general trend for lower root-shoot ratios. In the case of *L. pratensis* the root-shoot ratios had been altered in favour of the shoot by

ozone, thereby probably compensating for the loss of effective leaf area by O_3. Although the root biomass was not investigated in the current study, this could explain why *L. pratensis* was not affected negatively by the 10 ppb treatment. In addition to this, stimulations of shoot growth in response to O_3 have been shown for some species in other studies (e.g. Franzaring *et al.*, 2000 and Bungener *et al.*, 1999b). Franzaring *et al.* (2000) observed a significant stimulation of growth in *Molinia caerulea*. As the root or shoot biomass of other species such as *Achillea ptarmica* and *Cirsium dissectum* was decreased due to ozone, this might have caused a changed competitive ability of the species. *M. caerulea* probably increased its competitive ability under rising levels of ozone.

Trifolium repens showed a positive response to ozone, which is quite an unusual reaction for this species and is not consistent with the literature. However, the significantly higher biomass at the 10 ppb was found at a low P value of 0.049, which was only a marginally significant effect. There are numerous studies of responses of *T. repens* to elevated O_3 concentrations. Most of them found that *T. repens* reacted negatively, when fumigated with O_3. Wilbourn *et al.* (1995), Ashmore & Ainsworth (1995) and Fuhrer *et al.* (1994) all observed a decline in the biomass of *T. repens* at higher O_3 concentrations. Also, legumes have been reported to be very ozone sensitive (Mills *et al.*, 2006) and *T. repens* has a relative ozone sensitivity index of 0.858 (Hayes *et al.*, 2006). Nonetheless, in this study these results were the outcome of the second year, and there was not much fumigation of ozone in the first year; another year of fumigation might show a strong negative response.

Stellaria graminea responded in the same way to elevated O_3 as *L. pratensis*. Positive correlations were only found for ozone and pH. Also in this case, it might be an avoidance of the lower pH at the control plots. *S. graminea* belongs to the *Caryophyllacaea* family, which was reported with relative sensitivity of 0.98 as insensitive by Hayes *et al.* (2006), but there is noliterature on the effects of ozone on *Stellaria graminea*.

The main effects of ozone and the potential confounders at the High Keenley Fell site are summarised in Table 22.

Table 22: Summary of the ozone effects on individual species. Ozone sensitivity index refers to Hayes *et al.* (2006) (n.a = not available).

Species	***Effects of ozone at Keenley***	***Potential confounders***	***Effects reported in other studies***	***Ozone sensitivity index***
Ranunculacae	Decline	No correlations found	Decline	n.a.
Rhinanthus minor	Decline	Organic matter, soil water content	Decline	n.a.
Trifolium repens	Stimulation	No correlations found	Decline	0.858
Stellaria graminea	Stimulation	pH	Not reported	0.98
Lathyrus pratensis	Stimulation	Calcium, organic matter	Stimulation	n.a.
Dactylus glomerata	Stimulation	Phoshorus	Stimulation	0.992

2.5 Conclusion

This study showed that even low enhancements of the ambient ozone concentrations can have significant and substantial effects

1. On the balance of functional groups. A significant decline was found in the forb biomass at elevated O_3 concentrations. There was no change in the grass fraction, which lead to an increase in the grass/forbs ratio at the ambient plots and to a decrease at the 25 ppb plots.
2. On the frequency of some individual species. As the fumigation differed between the two years, and the soil conditions varied across the plots, not all of the changes in biomass can be attributed entirely to the ozone treatment, although there is strong evidence from 2008 that O_3 played a role in influencing species composition. These changes could point towards a shift in species dominance, favouring the grasses over the forbs, and even lead to an alteration of the species present in the community.
3. The responses to ozone of the individual species in this study only partly reflected the response of the plant community to ozone. Whereas *Dactylis glomerata, Lathyrus pratensis, Trifolium repens* and *Stellaria graminea* seemed to be stimulated significantly at higher O_3 concentrations, *Ranunculus spec.* and *Rhinanthus minor* showed a significant decrease in their biomass.

The findings reported in this study are consistent with some of the literature, as forbs and forb species have been reported to decline in their biomass when exposed to elevated ozone, but effects are not consistent with reports from other studies of ozone effects on legumes. Legumes, and especially *T. repens,* have been reported to be ozone sensitive, and in many studies their biomass decreased with increasing ozone exposure. However, most of the reported studies used higher ozone concentrations, and very few studies were dealing with long-established communities under free air fumigation. In this study, the ozone gradient fumigation is more likely to lead to higher

peaks of ozone than to constant ozone concentration. Therefore, effects on species biomass in the 25 ppb plots and especially in the 75 ppb plots are very likely due to ozone, whereas the responses at the lower 10 ppb plots are less likely to be attributable to ozone. Nonetheless, this also depends on the species sensitivity to ozone, and the position of that specie within the canopy. Species living in the lower canopy might be less exposed to the elevated O_3 concentrations (Jaeggi *et al.*, 2006). *T. repens* for instance occupys the lower part of the canopy, and has shown to recover even quickly after cuts during ozone exposure due to a loss of stolon density, using its resources for biomass production. Also Nussbaum *et al.* (1995) and Fuhrer *et al.* (1994) observed a recovery of *T. repens* after ozone exposure.

Although *T. repens* seems to be able to recover due to its stolons, other species such as *Rhinanthus minor* and *Ranunculaceae* might not be able to sustain under higher ozone exposure. In this study *R. minor* and *Ranunculaceae* were negatively affected by the higher ozone treatment. As the negative effects were already observed at elevated background concentrations, this could mean their loss from ecosystems such as uplands that are in general exposed to higher ozone background concentrations.

The negative development of the forbs and the species *Rhinanthus minor* and *Ranunculaceae* show how important ongoing long-term studies are and that these will be necessary to better understand the impacts of ozone in these ecosystems. In addition, it is not enough to consider only above-ground biomass effects, as some studies (e.g. Power and Ashmore, 2002) have shown effects on the below-ground biomass even when there were no changes to be seen in the above-ground biomass. Such studies have to be examined at different levels of the whole ecosystem.

3. Stomatal flux

3.1 Introduction

When assessing the risks that ozone poses to vegetation across Europe, the most commonly used index for linking ozone concentrations to plant growth and yield has so far been the AOT40 index (Karlsson *et al.*, 2003). However, it is now widely acknowledged that impacts of ozone on plants are more closely related to the absorbed ozone dose through the stomata rather than to external ozone exposure (Fuhrer *et al.*, 1997; Bassin *et al.*, 2007). The absorbed ozone dose can be estimated using flux models that are based on stomatal conductance measurements; these models account for the influence of the local micro-climate on the stomatal function and hence stomatal uptake of ozone. This flux-based approach has to date been mainly applied for the assessment of ozone effects on major crop (Georgiadis *et al.*, 1995; Grantz *et al.*, 1997; Ewert & Porter, 2000; Nussbaum *et al.*, 2003) and tree species (Fuentes *et al.*, 1992; Pleijel *et al.*, 1994; Ro-Poulsen *et al.*, 1998; Baumgarten *et al.*, 2000; Matyssek *et al.*, 2004), whereas its application to semi-natural vegetation still faces challenges due to the more complex and heterogeneous canopy of these ecosystems (Fuhrer *et al.*, 2003), but there are some modelling studies that present first attempts of flux modelling to grasslands (e.g. Bassin *et al.*, 2004; Ashmore *et al.*, 2007). Data currently available on the stomatal response of key grassland species to the prevailing micro- climate and ozone concentration are limited and there is a lack of understanding of the processes which influence ozone fluxes within complex plant communities during the growing season (Jaeggi *et al.*, 2006).

The effect of O_3 on plants depends on the O_3 uptake by leaves, which is directly coupled to the leaf gas exchange (Reich, 1987). After its uptake, O_3 can reduce stomatal conductance directly (Reiling & Davison, 1995; Nussbaum *et al.*, 2000, Bassin *et al.*, 2009), although not every study so far showed this. Jaeggi *et al.* (2005) conducted a study of leaf $\delta^{13}C$ and leaf conductance of plant

species which were grown in a semi- natural grassland with or without irrigation. Changes in stomatal conductance (g_s) and/ or carboxylation efficiency are reflected by shifts in the stable carbon isotope signature of C3 plant matter ($^{13}C/^{12}C$ ratio expressed as $\delta^{13}C$), which has been widely used as an integral signal of leaf gas exchange (Farquhar *et al.*, 1989). $\delta^{13}C$ is inverse proportional to the ratio of the internal CO_2 concentration (c_i) to the atmospheric CO_2 concentration (c_a) (Farquhar *et al.*, 1989). This is is because Rubisco discriminates against $^{13}CO_2$, the heavier isotope, which becomes concentrated in the intercellular air space as ci decreases. The analysis of $\delta^{13}C$, combined with measurements of g_s, can help to clarify the type and magnitude of combined effects of O_3 and water stress. Jaeggi *et al.* (2005) found that elevated O_3 reduced stomatal conductance and increased $\delta^{13}C$ in *Plantago lanceolata, Holcus lanatus* and *Trifolium pratense*, irrespective of irrigation.

Recent studies by Mills *et al.* (2009) showed that even small increases in background O_3 concentrations had an effect in increasing the stomatal conductance of *Leontodon hipidus* and *Dactylis glomerata.* A transpiration bioassay from the same study revealed that leaves of *L. hispidus* from the highest O_3 treatment were not able to respond to abscisic acid (ABA), which normally induces stomatal closure and reduced transpiration. They also looked at the effect of O_3 on the response of stomata to a range of abscisic acid concentrations. *L. hispidus, Ranunculus acris* and *Rumex acetosa* showed a smaller reduction in transpiration in response to ABA in the O_3 treatment. Ozone therefore appeared to disrupt the ABA- induced signal transduction pathway for stomatal control thereby reducing the ability of plants to respond to drought.

According to studies in forests, there is a potential for ozone uptake within the canopy to influence O_3 exposure and flux to different species (Enders, 1992; Fontan *et al.*, 1992; Lorenzini & Nali, 1995; Joss & Graber, 1996; Samuelson & Kelly, 1997; Davison *et al.*, 2003). One of the important elements in multi-species communities is the position of the species within the canopy. As

individual species inside a grassland canopy typically occupy layers at different heights, they might be exposed to different levels of ozone. The O_3 concentration profile is controlled by the strength and distribution of sinks such as uptake via stomata, destruction at surfaces, or gas- phase reactions, and also by the turbulent mixing of air into the canopy. However, quantitative data on the in-canopy distribution of ozone is scarce, and studies have mainly been carried out in tall forest canopies (Enders, 1992; Fontan *et al.*, 1992; Joss & Graber, 1996). Yet, Veit & Henning-Müller (2001) studied the influence of different meteorological parameters on the development of an ozone gradient within a grassland canopy. The vertical ozone profiles in their study of a grass canopy clearly showed lower O_3 concentrations within the canopy than at 250 cm above it. They also found that O_3 concentrations at different heights in the canopy were correlated well over time with the O_3 concentrations at the top of the canopy. Short term variation in the ratio of O_3 concentrations at 1 cm, compared to the O_3 concentrations at 250 cm, was mostly influenced by the wind speed.

Another study supporting this was carried out by Jaeggi *et al.* (2006) on a productive grassland in Switzerland. They suggested that, in multi- species grassland communities, ozone exposure of individual species may differ due to their location inside the canopy. Strong vertical gradients in ozone concentrations were found, which they attributed to the leaf density being much higher than in crops or forests. They assessed how the ozone profile inside a grassland canopy is influenced by the vertical distribution of leaf area index and by meteorological parameters, especially by the turbulence intensity. It was found that the shape of the ozone profile was not affected by increasing leaf area index (LAI), or by the changing vertical distribution of LAI, but was affected by the turbulence intensity. They also concluded that turbulence is a more appropriate controlling parameter than the wind speed above the canopy, due to the different stability regimes in the atmosphere during day and night. During the night the turbulence is only a function of wind speed (i.e. generated

by mechanical friction); while during the day it is additionally influenced by the radiative heating of the surface i.e. turbulence by thermal convection.
Nonetheless, more information is needed on O_3 concentration profiles within grassland canopies in order to provide a sound basis for a flux based approach. There are not many studies on how O_3 affects the stomatal conductance of different functional groups and how far O_3 exposure interferes with stomatal conductance.

Hence, the main aim in this study was to provide a basis for modelling of ozone fluxes to a semi- natural upland grassland with different O_3 treatments. The work focused on one or two dominant grass, forb and legume species. The development of vertical gradients of ozone concentrations and stomatal conductance, and hence ozone flux, within the canopy were a particular focus. This study was mainly carried out at the free air fumigation system at High Keenley Fell in 2008. More details about the field site, its management, the technical system and the experimental set up is provided in Chapter 2. In addition, measurements of stomatal conductance were made in the long- term grassland mesocosm experiment which has been run by Newcastle University at the Close House site. Based on the findings of the project for the last four years, measurements were focused on the grass species, most affected by the ozone treatment.

The following specific hypotheses were tested:

i. Elevated ozone concentrations reduce the stomatal conductance of certain grass, forb and legume species,

ii. The ozone concentration within the canopy shows a vertical gradient and differs from the ozone concentration above the canopy due to the vertical gradient in LAI; this may lead to varying ozone fluxes to different species according to canopy height and canopy density.

3.2 Materials and Methods

3.2.1 Stomatal conductance and other relevant measurements

Stomatal conductance was measured with a cycling porometer (type AP4, ΔT Devices, Cambridge) in two main measurement periods, in June and in July 2008. The porometer measures the stomatal resistance of plant leaves which is a measure of the resistance to loss of water vapour through the stomata. The instrument works by measuring the time it takes for a leaf to release sufficient water vapour to change the relative humidity in a small chamber by a fixed amount. This is compared with a calibration plate of known resistance (Bragg, 2004[9]).

Before starting the actual measurements, the porometer had to be calibrated. To do this, a damp filter paper was placed on the back of the calibration plate to cover all the calibration holes in order to mimic the conditions of a real leaf. As calibration settings the following parameters were chosen: slotted cup type, conductance measured in mmol m^{-2} s^{-1}, the pressure set to 1000 hPa, and the order ascending from 1 to 6. The settings were adjusted to the apparent humidity conditions, then the head unit of the porometer was clipped onto the calibration plate and the calibration cycle was started. After performing six calibration cycles (from smallest calibration holes to largest ones) a calibration curve was calculated. If the differences from the true values were less than 10% the calibration was accepted and the stomatal conductance measurements were started.

The measurements were focused on three different functional groups: grasses, legumes and forbs. Four species were selected for detailed measurement at Keenley Fell, because of the ease of measurement and identification, and because they were consistently present in the experimental plots. These were

[9] http://www.delta-t.co.uk/support-article.html?article=faq2005100702977

the grasses *Holcus lanatus* and *Phleum bertolonii*, the forb species *Rumex acetosa* and the legume species *Trifolium repens*. In addition to the long- term monitoring plots with control, +10 ppb and +25 ppb target concentrations, additional plots with +75 ppb target concentrations were used to better evaluate possible impacts of ozone.

In June, the stomatal conductance measurements concentrated on establishing a profile of each species using all 42 plots (36 + 6 additional at +75 ppb). Measurements followed a certain order. Measurements were started in Transect 1, control treatment in one subplot. In each subplot, two plants of each species were measured (only one leaf per plant). Then measurements continued along this transect, from the 75 ppb treatment to the 25 ppb and the 10 ppb treatments. The same procedure was followed in Transect 2 and 3. The three species were measured in sequence after completion of the measurement cycle for the first species. For *Trifolium repens* both sides of the leaf (of a surface, directed towards the axis (adaxial); directed away from the axis (abaxial)) were measured, whereas for *Holcus lanatus* (the stomata of most grasses are equally distributed on both surfaces of the leaf) and *Rumex acetosa* (hardly any literature was available on the stomata distribution was available) the measurements were carried out mainly on the abaxial surface. During each cycle, photosynthetically active radiation (PAR) readings were recorded with the porometer, and air temperature and humidity readings were noted with a whirling hygrometer (ETI 817-001, Electronic Temperature Instruments Ltd). The temperature and humidity were taken before the stomatal conductance measurements were started, then in each treatment and at the end of each transect. Measurements dates are listed in Table 23.

Table 23: Measurement dates of stomatal conductance of *R. acetosa*, *H. lanatus* and *T. repens* in 2008 at High Keenley Fell.

Species	*Measurement dates*
R. acetosa	05-06/06, 16/07, 22-24/07 and 27-28/07
H. lanatus	05-06/06, 16/07, 22-24/07 and 27-28/07
T. repens	30/06, 16/07, 22-24/07 and 27-28/07

In July measurements were carried out as before, but additionally an area of 50 cm x 50 cm was chosen close to the 75 ppb area. The height of the canopy did not exceed 30 cm; the highest percentage of grass species was found between 20 and 30 cm and the highest percentage of forb and legume species at 5 cm. Therefore O_3 was monitored at three different heights (top of the canopy (~30 cm), 20 cm and 5 cm) with three O_3 monitors (Model 202, 2B Technologies, Inc[10]), as was stomatal conductance and photosynthetically active radiation (PAR). The three 2B monitors were inter-calibrated before and at the end of the measuring period in July (Figure 43 and Figure 44). Whereas the O_3 monitors from Centre for Ecology & Hydrology (CEH) and Newcastle showed more or less the same O_3 concentration, the O_3 monitor from York measured higher O_3 concentrations. O_3 measurements were logged every minute. The higher readings of the O_3 monitor from York were taken into account when interpreting the O_3 profiles.

[10] http://www.twobtech.com/manuals/model_202_old.pdf

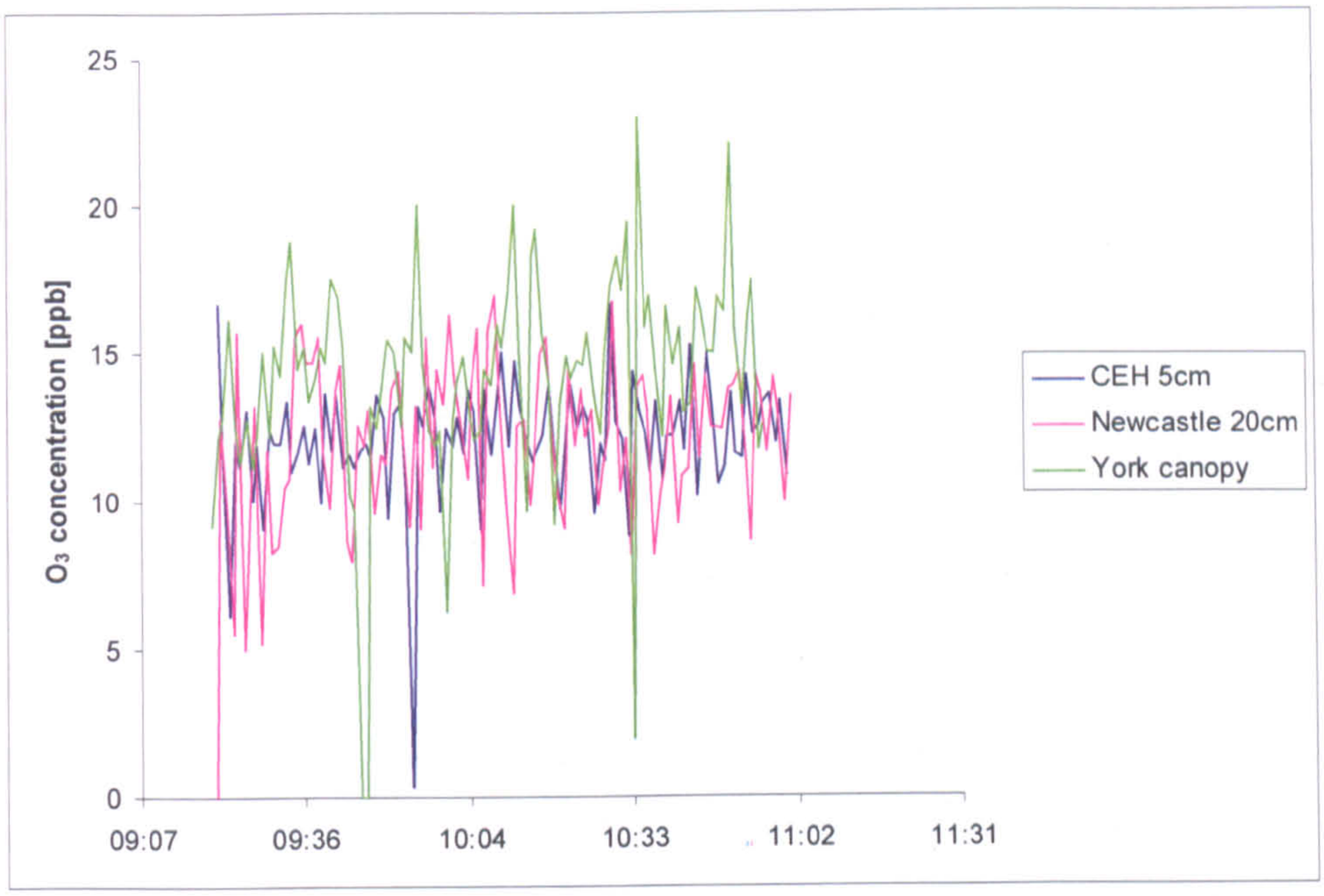


Figure 43: Inter- calibration of the three 2B O3 monitors before monitoring.

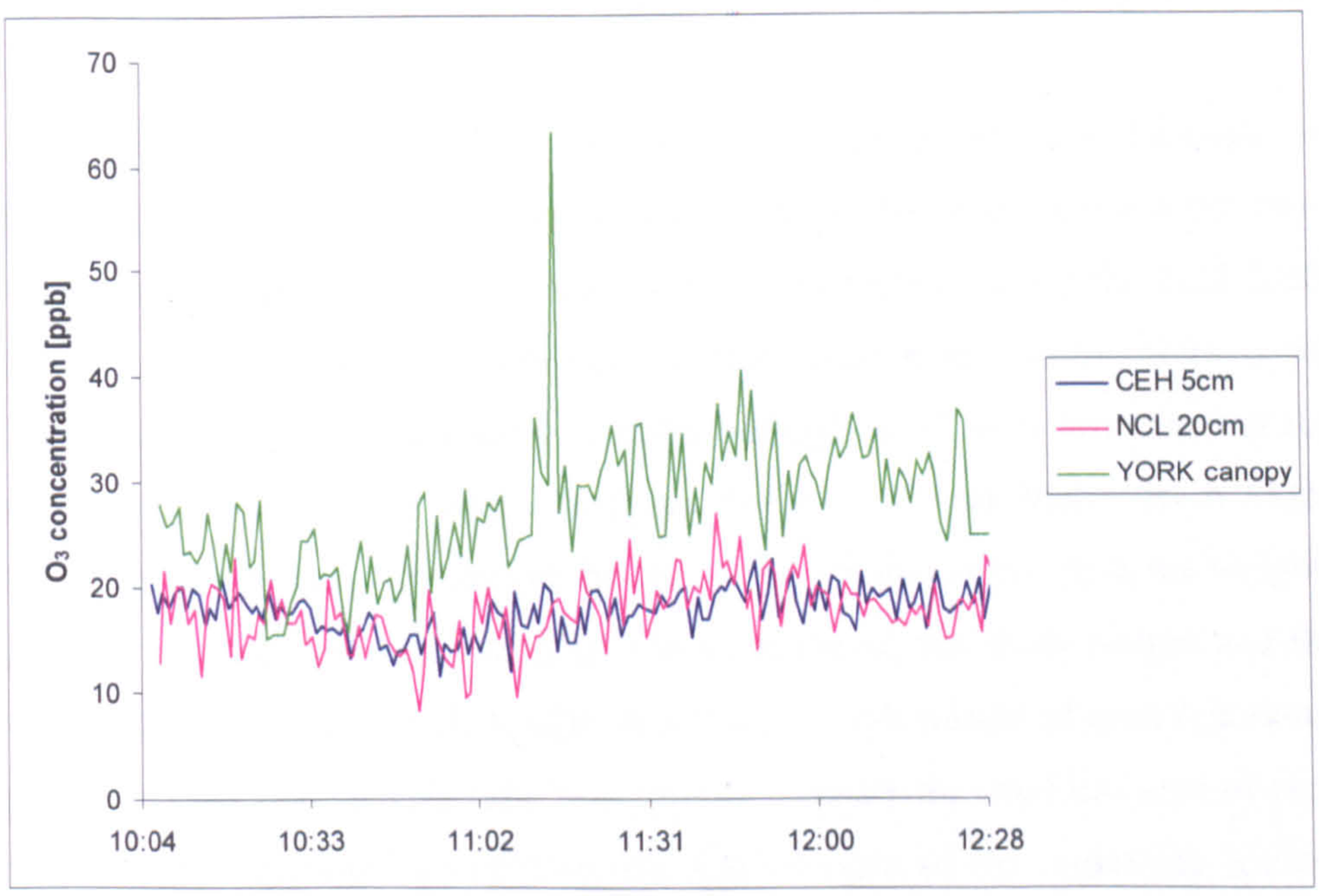


Figure 44: Inter- calibration of the three 2B O3 monitors at the end of monitoring.

For the stomatal conductance measurements, three different porometers were used, which were calibrated each day. Hardly any forbs and legumes were found at the height of 20 cm. Therefore forbs and legumes were measured only at 5 cm and grasses at 20 cm. Overall; about 500 gs measurements per species were collected. Due to the very wet and cold conditions at the site during the summer of 2008, very few days were suitable for these types of measurement and only a few measurements of gs (overall ~400 for six different species) were made in the profile area.

The leaf area index is an important variable in determining O_3 gradients, which alters during the seasonal cycle. The LAI of all plots was measured using a LAI-2000 (LI-COR, Inc., Cambridge) instrument. This instrument uses measurements of how radiation is attenuated as it passes through the canopy to estimate the LAI. The instrument measures the attenuation of diffuse sky radiation at five zenith angles simultaneously thereby also obtaining the foliage orientation[11] .

The fractional LAI was also determined destructively on six different days. On each day, a 50 cm x 50 cm plot was chosen from the areas between the three transects. First the total LAI of that plot was determined using the LAI-2000, and then the plot was cut at 5 cm and at 20 cm. Later these fractions were sorted into grasses, forbs and legumes. A small subsample of each functional group was taken and measured with a leaf area meter (Δ-T Area Meter; RCA M3M Video camera Hitachi Denshi) at the University of Newcastle; then the weights of these subsamples were recorded. On all material, the fresh weight and the dry weight was determined. A ratio of leaf area/ fresh weight of each functional group was calculated. This ratio was used to estimate the total leaf area of each fraction and functional group from the fresh weight of the remaining harvest plant material. The subsample was added in to get the total area of each fraction and group. The fresh weight and the leaf area were then set in relation to the dry

[11] ftp://ftp.licor.com/perm/env/LAI-2000/Manual/LAI-2000_Manual.pdf

weight. The calculated leaf area per g dry weight of each functional group was added to the sum of one fraction (>5 cm fraction or > 20 cm fraction), which was then related to the plot size (50 cm x 50 cm). For each fraction, the percentage of each functional group was calculated also.

3.2.2 Grassland mesocosms at Close House

Stomatal conductance measurements

Because weather conditions and system problems severely restricted the period of time when ozone fumigation occurred during measurements of stomatal conductance at High Keenley Fell, additional measurements were made in the grassland mesocosms in the Close House chambers, for which a contrast in ozone exposure was available. The study makes use of 64 MG3b [*Anthoxanthum odoratum* - *Geranium sylvaticum* (*Briza media* sub community) upland mesotrophic grassland] mesocosms established in 2000 with two levels of residual nutrient status ['high' (16-25 P_2O_5 mg l^{-1}) and 'low' (0-9 P_2O_5 mg l^{-1})] and two levels of *Rhinanthus minor* parasitism [RM absent or RM 60 plants m^{-2}]. These have been exposed, since March 2004, in duplicate open-top chambers (at Newcastle University's Field Station in rural Northumberland, 20 km west of the main city centre) to simulated present-day or 2050 upland ozone climates. The ozone exposure data for 2008 is listed in Table 24. The achieved O_3 concentrations were well below the target O_3 concentrations of 30 ppb and 50 ppb.

Table 24: Ozone exposure data in the Close House experiment for 2008 during periods when stomatal conductance measurements were made, showing the 9 h and the 24 h mean concentration of ambient and elevated treatments, including the standard error.

Time periods of fumigation	*9 h mean; elevated [ppb]*	*9 h mean; ambient [ppb]*	*24 h mean; elevated [ppb]*	*24 h mean; ambient [ppb]*
18/06/08 – 04/07/08	36.9±1.4	27.7±0.5	37±1.8	30.3±0.6
10/09/08-12/09/08	32.7±2.7	17.6 ±0.4	33.5±3.3	19.3±0.7
Total vegetation period	36.1±1.3	25.8± 1.1	36.7±1.5	28.1±1.2

The main focus in the grassland mesocosms was the dominant grass *Briza media*. *B. media* is one of the key species of MG3b grassland communities, and in the last four to five years of the experiment showed a gradual decrease in biomass in response to elevated O_3 concentrations (Peacock, pers. Com.). Stomatal conductance measurements were conducted with a porometer (type AP4, ΔT Devices Ltd), and were carried out in June (before the cut in early July) and September (after the cut), using four OTCs with two different target O_3 concentrations: ambient (+30 ppb = present day) and elevated (+50ppb = 2050). Measurements were made at two different nutrient levels, giving 12 mesocosms per OTC, i.e. 6 replicates of high and low nutrient concentrations. The mesocosms with *Rhinanthus* treatments were excluded because *R. minor* is a parasitic plant that might influence the stomatal conductance. Two stomatal conductance readings were taken from one leaf of two different plants in each mesocosm for each measurement period. Young and healthy looking plants were preferred and g_s measurements were taken from the middle of the leaf. Measurements followed a consistent order:

Measurements were started in one of the elevated treatments, beginning with the low nutrient treatments. After the last reading of the high nutrient treatments, the measurements were continued in one open top chamber (OTC) of the ambient treatments. This was continued in a rotating order. Each cycle took 2½ - 3 hours. PAR measurements were taken from the porometer while temperature and humidity readings were noted with a whirling hygrometer (ETI 817-001, Electronic Temperature Instruments Ltd), before the stomatal conductance measurements were started in each OTC and when the measurements finished. Measurement dates are listed in Table 25. Air temperature and humidity readings are given in Table 26; this shows that, overall, the air temperatures were lower in the 50 ppb treatment than in the 30 ppb treatment.

Table 25: Measurement dates of the stomatal conductance of *B. media* in 2008.

Species	*Measurement dates*
B. media	19th -20th, 26th June, 4th July and 10th -11th September.

Table 26: Average air temperature and humidity values in the open top chambers in the Close House experiment in 2008, during the periods of measurements.

Open top chambers		*Temperature [°C]*	*Humidity [%]*
OTC1 50ppb	Chamber 1	17.1±0.3	65.7±1.6
OTC5 30ppb	Chamber 2	18.1±0.4	65.8±1.9
OTC10 30ppb	Chamber 3	17.9±0.5	65.2±2.6
OTC15 50ppb	Chamber 4	17.4±0.5	65.8±1.8
Average elevated chambers	+50ppb	17.3±0.3	65.5±1.2
Average ambient chambers	+30ppb	17.9±0.3	65.5±1.7

Soil water samples of the grassland mesocosms

Previous studies from the grassland mesocosms had shown effects of ozone on the biomass of *B. media* and *Lotus corniculatus* (Peacock, pers. Com.) and in addition to that many authors point out the impact of ozone on the belowground part of the ecosystem (e.g. Andersen *et al.*, 2003), hence soil water samples were taken via rhizon samplers from the grassland mesocosms. Their pH was measured at 0- 10 cm and at 10- 20 cm with a pH meter (Thermo Orion, model 420) and they were then stored in a freezer until analysis. Later the NH_4^+ and NO_3^- concentrations of the soil water samples were analysed with an Autoanalyser (Autoanalyser 3, Bran + Luebbe). The analytical methods used are described in detail in Section 2.2.6.2.

3.2.3 Data analysis

Data summaries were carried out using Microsoft Excel (2003) and the statistical analysis was performed using SPSS 15.0. Before any statistics were applied, the dataset of the stomatal conductance to water vapour was cleared of any gs data which related to zero PAR values. After exploring the data, it was then adjusted to the 90th or 95th percentile. Values above 90th or 95th percentile were screened to avoid outliers depending on the scattering of the data and comparing it with data from other studies either the 90th or 95th percentile was chosen. While the grasses showed reasonable values and not much scatter below the 95th percentile, the forbs still showed much scatter and very high gs values between the 90th and 95th percentiles which were hardly found in the literature. Hence, before analysis, values above the 90th percentile were removed for forbs, and values above the 95th percentile were removed for grasses (Figure 45, Figure 46, Figure 47 and Figure 48).

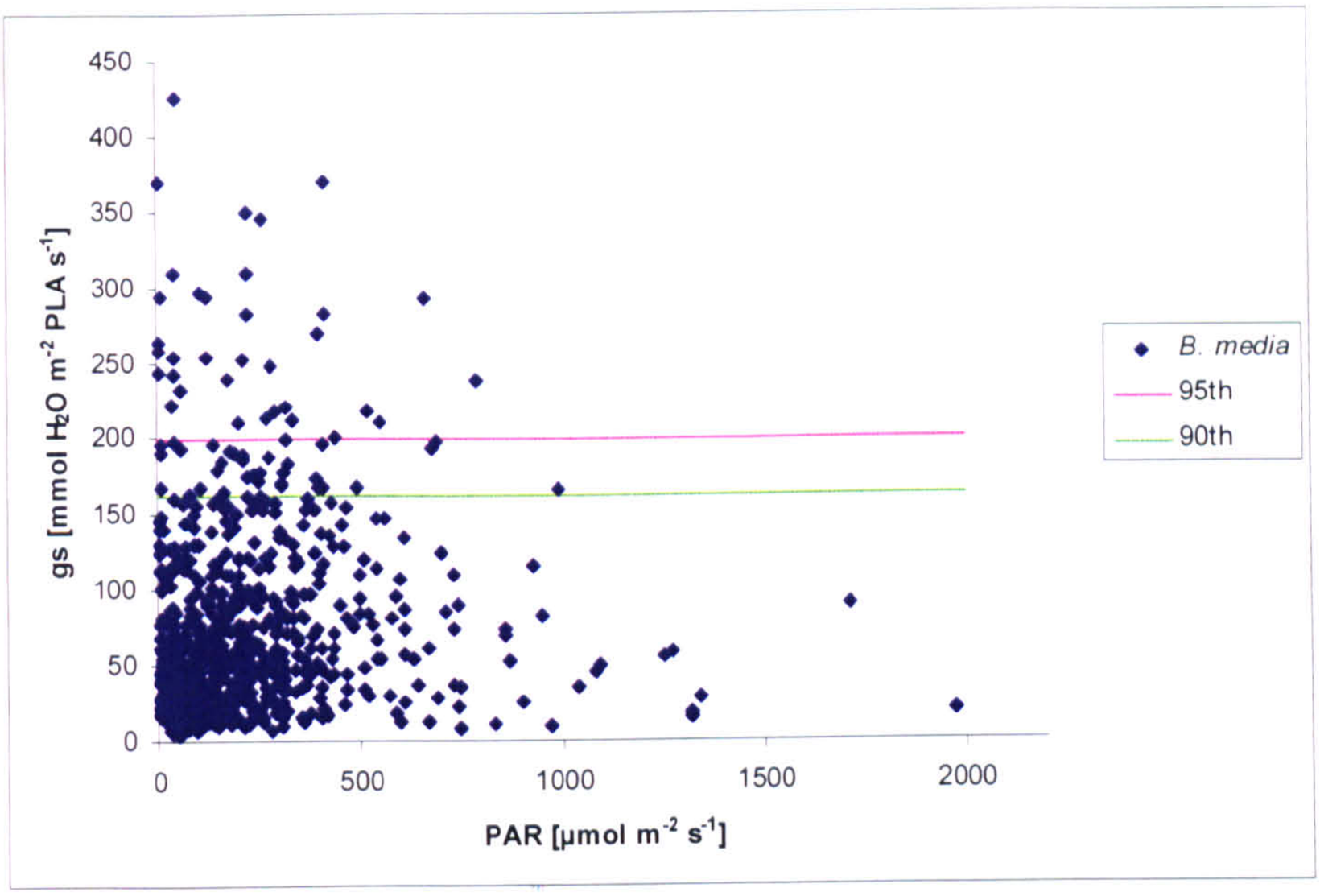


Figure 45: Stomatal conductance to water vapour of *B. media* (abaxial) plotted against PAR. The 90th and 95th percentiles are indicated.

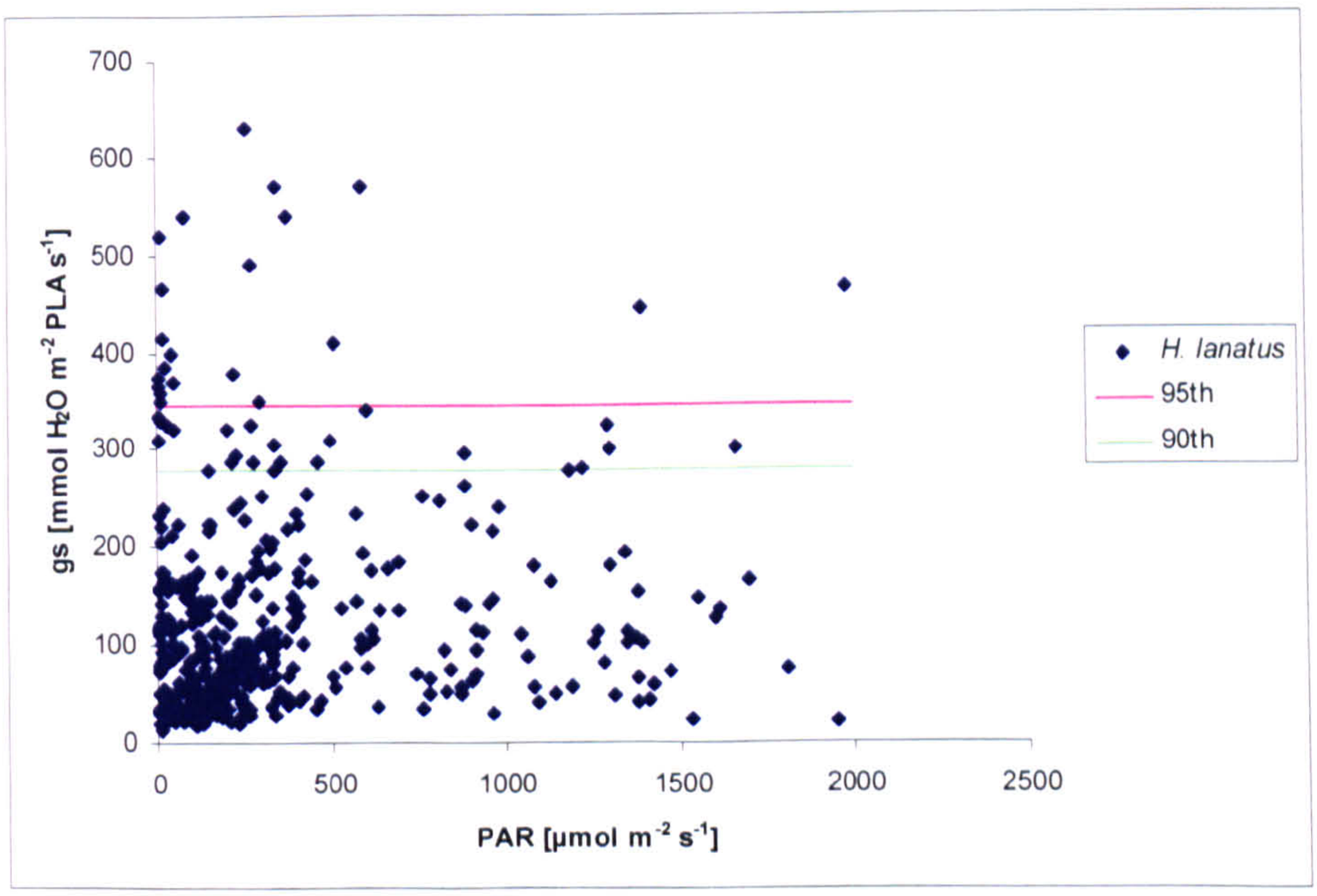


Figure 46: Stomatal conductance to water vapour of *H. lanatus* (abaxial) plotted against PAR. The 90th and 95th percentiles are indicated.

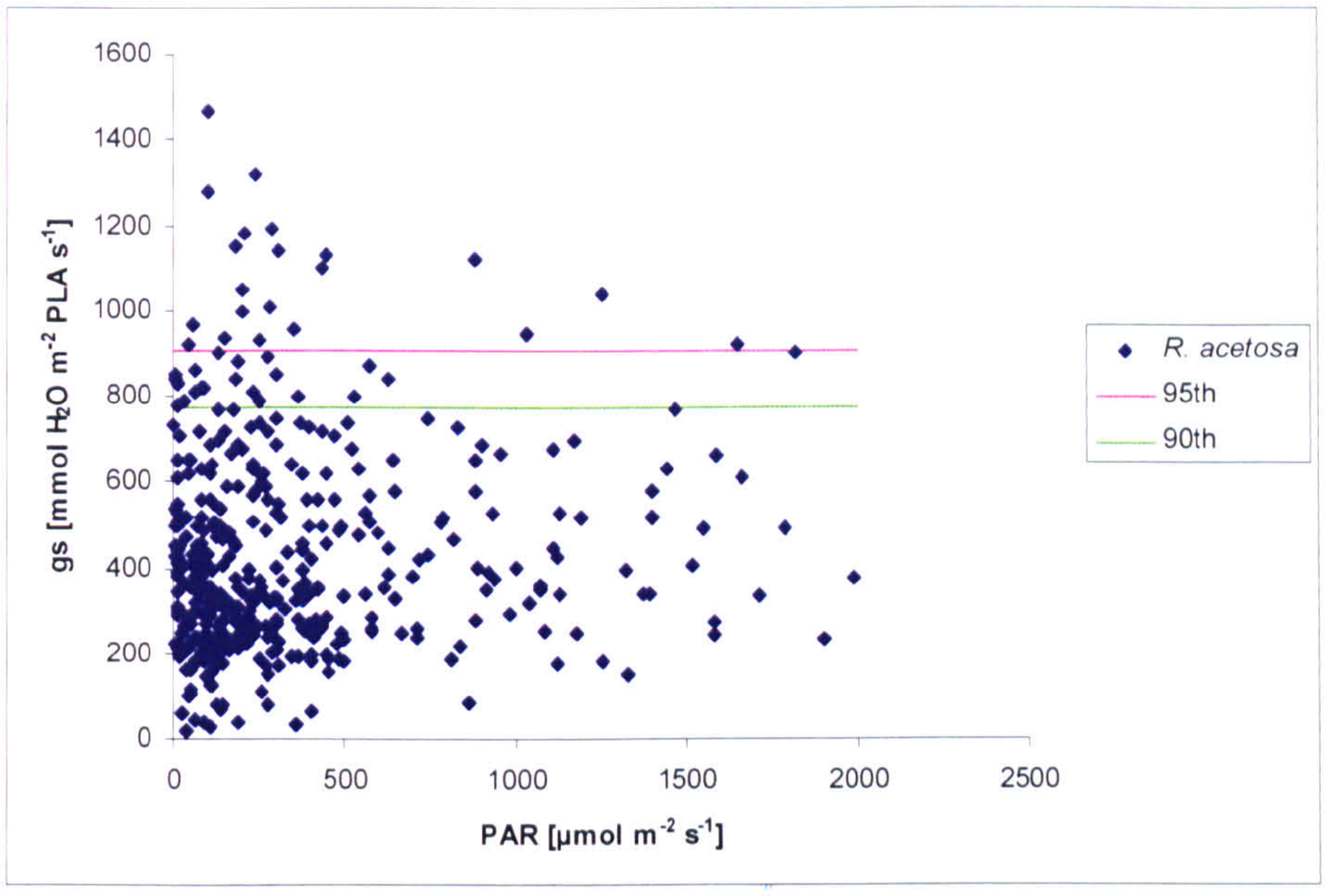


Figure 47: Stomatal conductance to water vapour of *R. acetosa* (abaxial) plotted against PAR. The 90th and 95th percentiles are indicated.

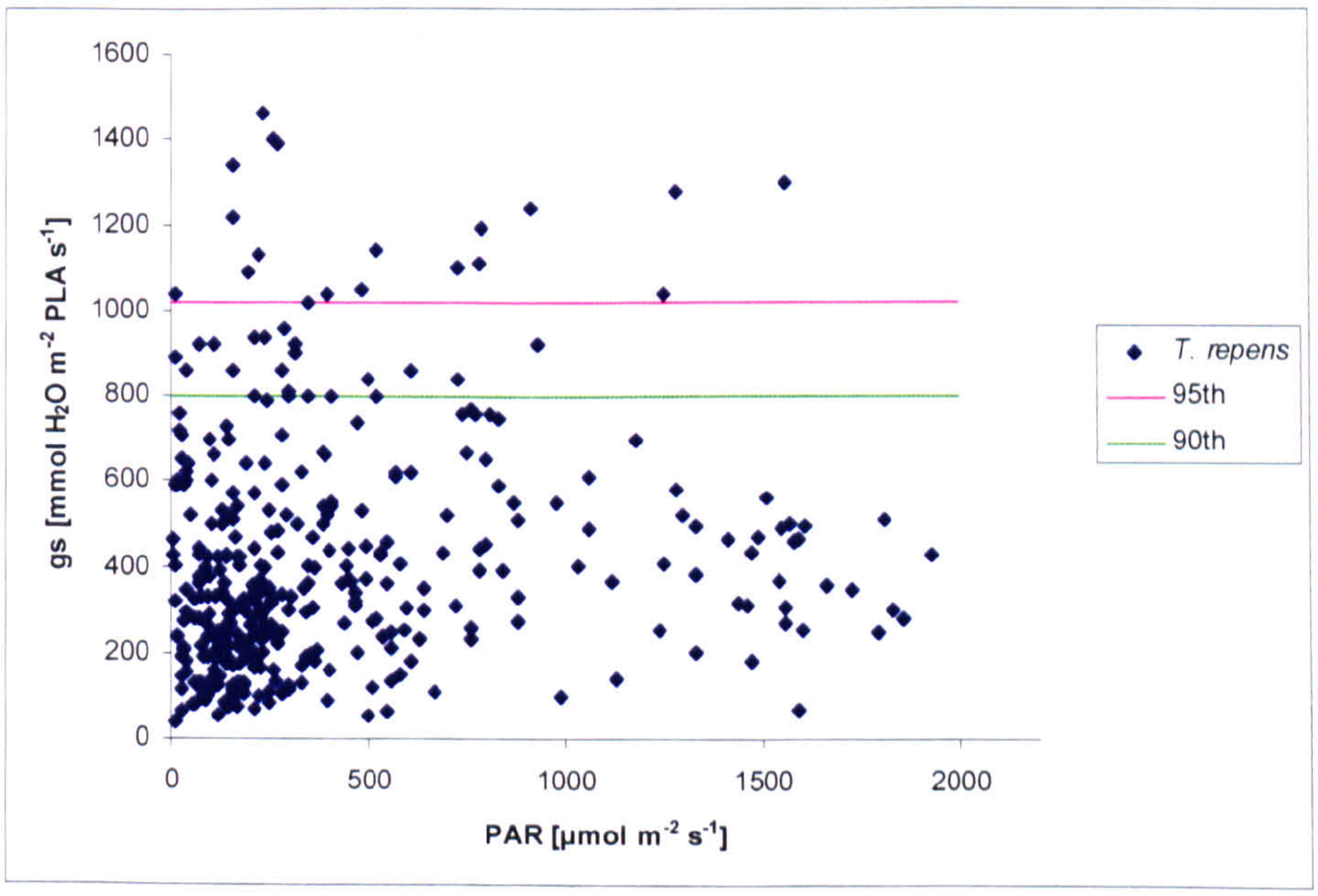


Figure 48: Stomatal conductance to water vapour of *T. repens* (abaxial) plotted against PAR. The 90th and 95th percentiles are indicated.

Each dataset was then explored via histograms, q-q-plots and boxplots, and examined for skewness and kurtosis, and for normality with the Kolmogorov-Smirnoff- test. Because the data were left skewed, the stomatal conductances were either log_{10} or square- root transformed before statistical analysis. The effects of the four O_3 treatments (Control, 75 ppb, 25 ppb and 10 ppb) on the species *Trifolium repens*, *Rumex acetosa* and *Holcus lanatus* were tested with mixed modelling. Fixed factors were transect and treatment, days were used as a random factor (model: gs ~ ozone transect ozone*transect). Homogeneity of variance was assessed via the Levene- test and the significance of specific contrasts was tested with Bonferroni adjustments. Differences were tested for significance at $P< 0.05$, unless the outcome of the Levene- test was inhomogeneous, in which case the P- level was set to $P< 0.01$. In order to estimate if there was an interaction between treatments, transects and days (days as a main factor) when stomatal conductance was measured, a univariate general linear model was applied, and the significance was tested in the same way as for the ANOVA. Additionally, time was tested as a main factor in the ANOVA for the stomatal conductance of all three species. The same P- values were used as above.

For *Briza media* from the grassland mesocosms, only two O_3 treatments and two N treatments were used. In order to test for significant differences, a mixed model was used. Fixed factors were ozone and nitrogen, chambers were used as a random factor (model: gs ~ ozone nitrogen ozone*nitrogen). The significance was tested in the same way as for the ANOVA. For the pH, ammonium and nitrate concentrations of the soil data, the same model was used.
In addition to this, chamber was tested as a main factor in the ANOVA for the stomatal conductance of *Briza media*, and for the pH, ammonium and nitrate concentrations of the soil data. The same P-values were used as above.

3.3 Results

3.3.1 Ozone gradient study

3.3.1.1 Weather conditions

During the 12 days of the High Keenley Fell ozone gradient study, PAR, air temperature and humidity showed a diurnal pattern (Figure 49 and Figure 50). There were colder periods around 16th to 21st July (especially 20th July), when PAR was also lower. Humidity was lower on the 20th /21st and 28th July, and PAR was lowest on 23rd July (max: ~1000 μmol m^{-2} s^{-1}). Overall, the mean air temperature was around 13.8°C, and the mean air humidity was around 87.4%. Precipitation was low overall and the most rain fell on 19th and 20th July.

Wind speed and friction velocity showed higher values during the day time (Figure 51) and the highest wind speed (6.7 m s^{-1}) was observed on 16th July. The wind speed and friction velocity (i.e. the square root of the negative covariance between instantaneous horizontal and vertical wind speed) were overall higher during the first half of the two weeks (16th to 21st July). The field site was fumigated with O_3 from 16th to 21st July when the wind came from the right direction (180 to 270°) (Figure 52). From 22nd-28th July, the field was not fumigated, and ambient O_3 concentration were especially low on 23rd July with ozone concentrations around 10-15 ppb (data for 24th July is missing), although on 26th July higher ambient O_3 concentration such as 64 ppb were reached (Figure 53). All data was collected from the met mast, which was provided by CEH Edinburgh. The weather conditions during the full years of 2007 and 2008 are described in Chapter 2.

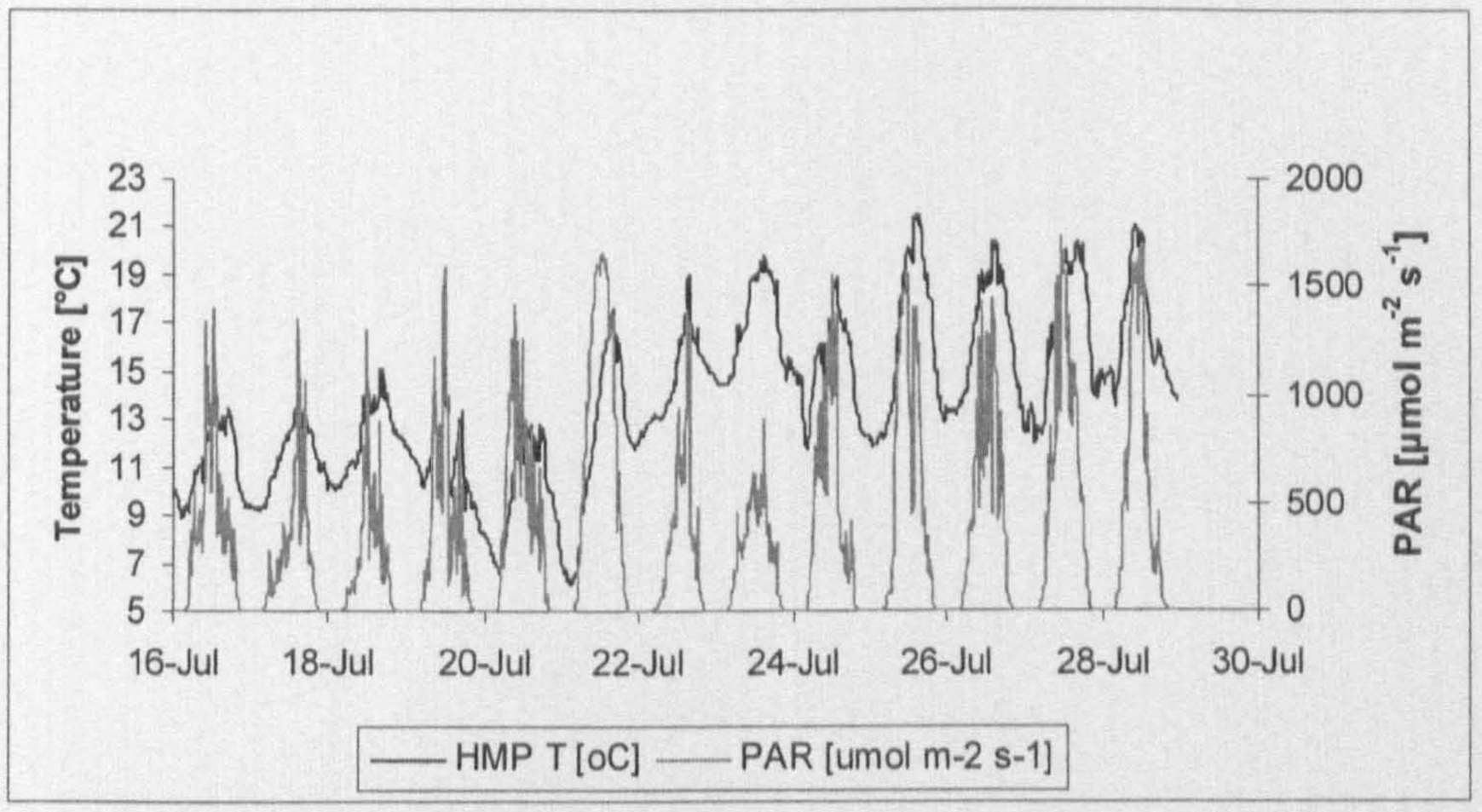


Figure 49: Time series of air temperature and PAR at High Keenley Fell during the period from 16th -28th July 2008 (measured at 1.5 m).

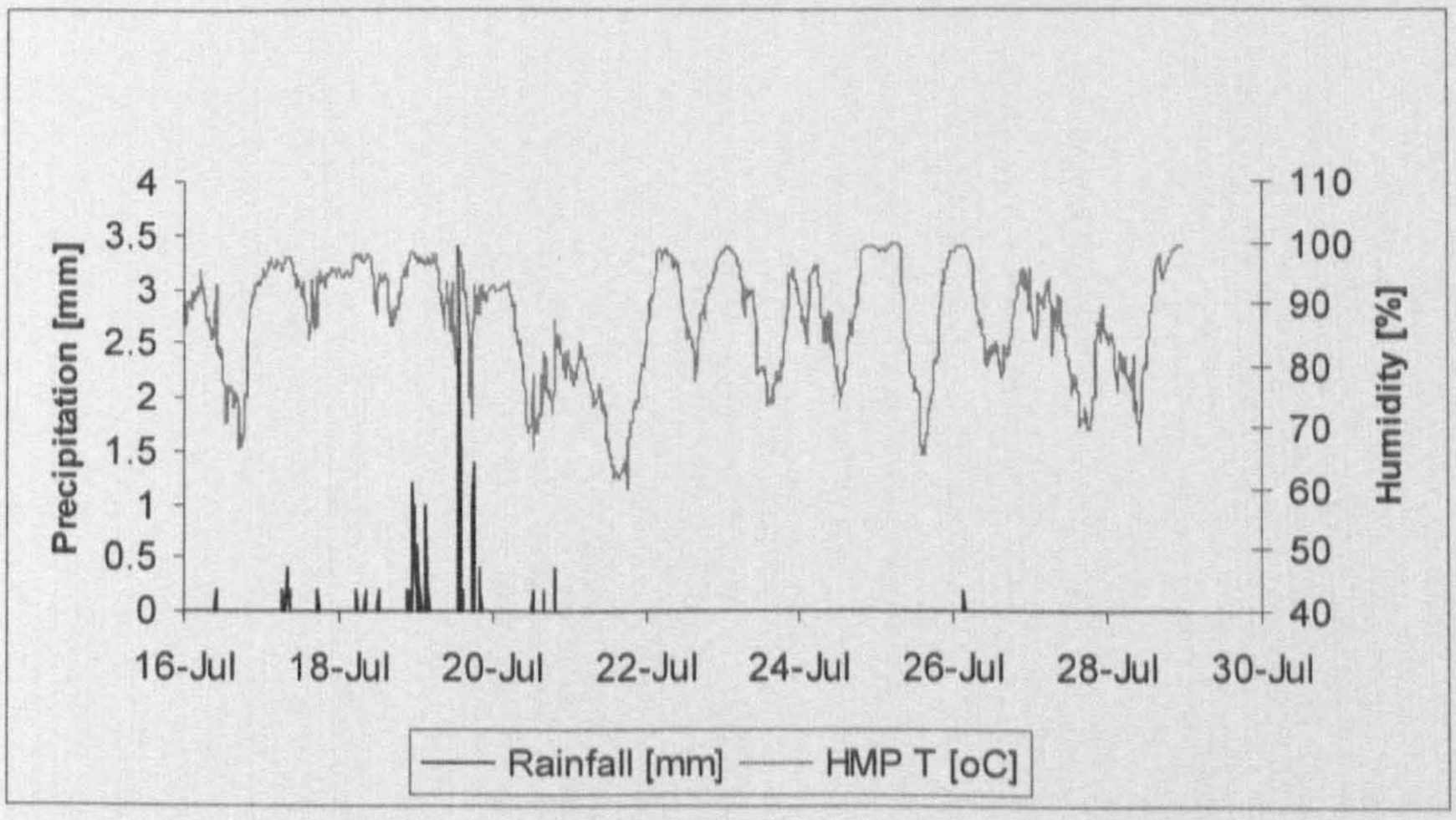


Figure 50: Time series of air humidity and precipitation at High Keenley Fell during the period from 16th -28th July 2008 (measured at 1.5 m).

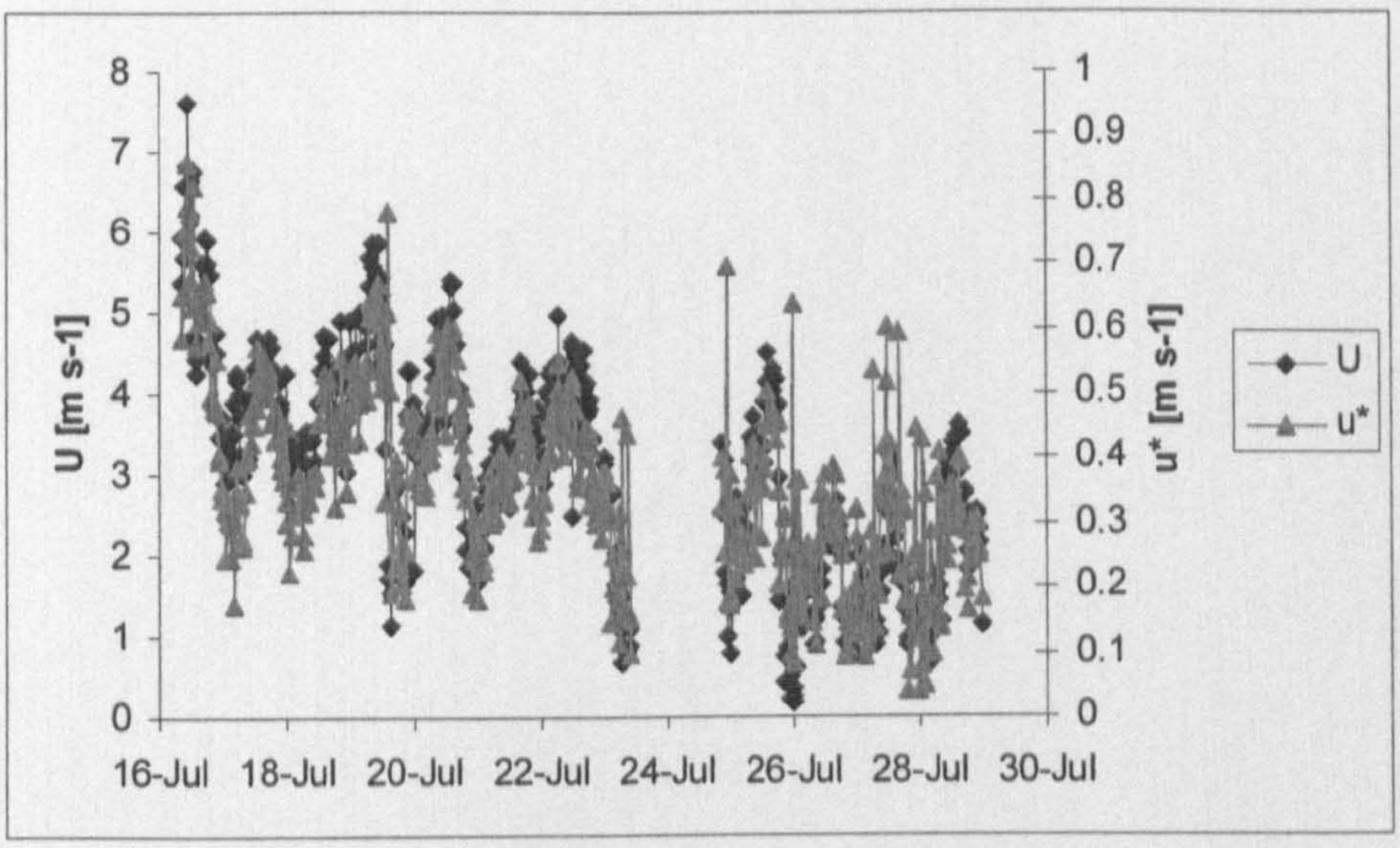


Figure 51: Time series of wind speed (U) and friction velocity (u*) at High Keenley Fell during the period from 16th -28th July 2008 (measured at 1.5 m).

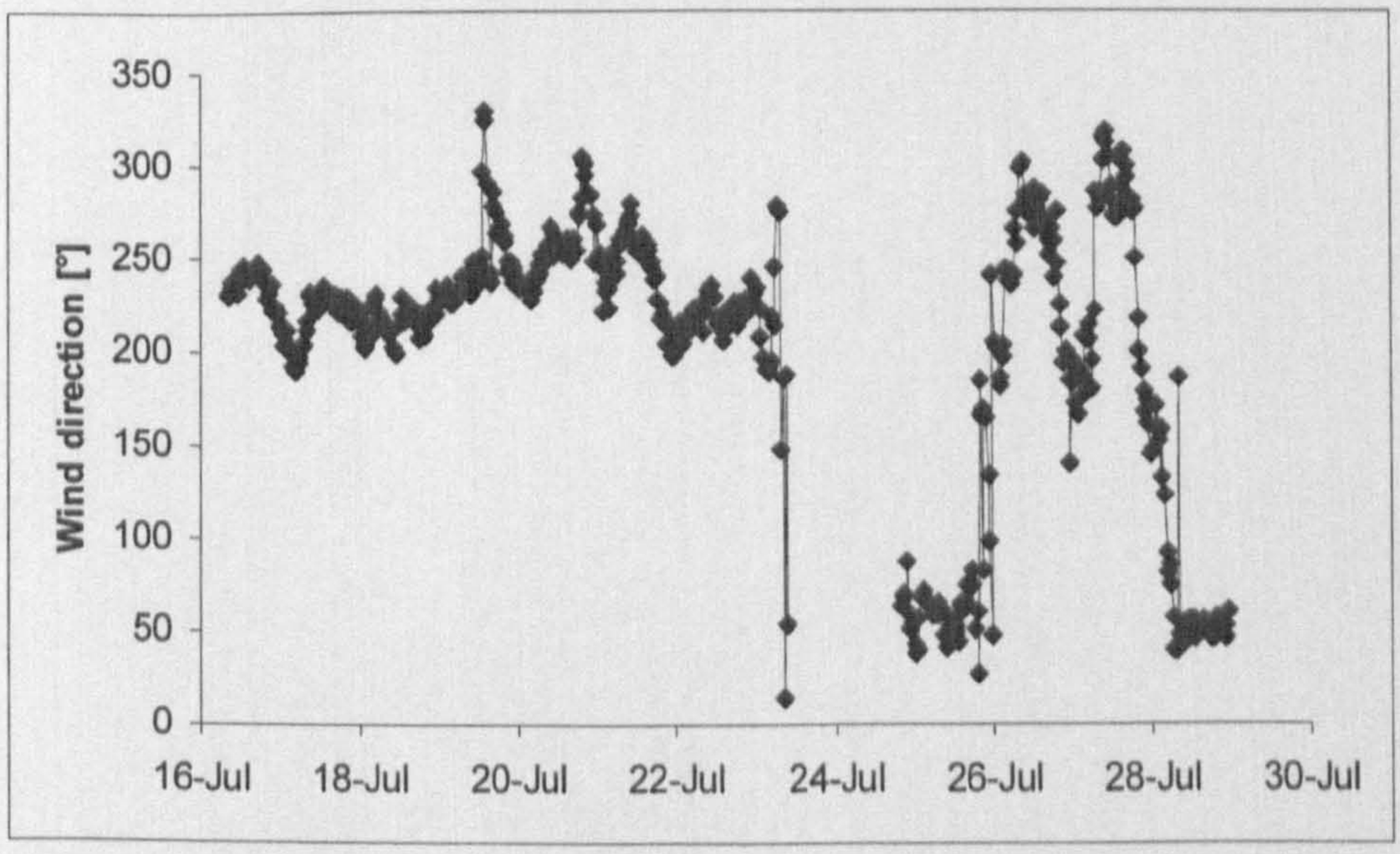


Figure 52: Time series of wind direction at High Keenley Fell during the period from 16th -28th July 2008 (measured at 1.5 m).

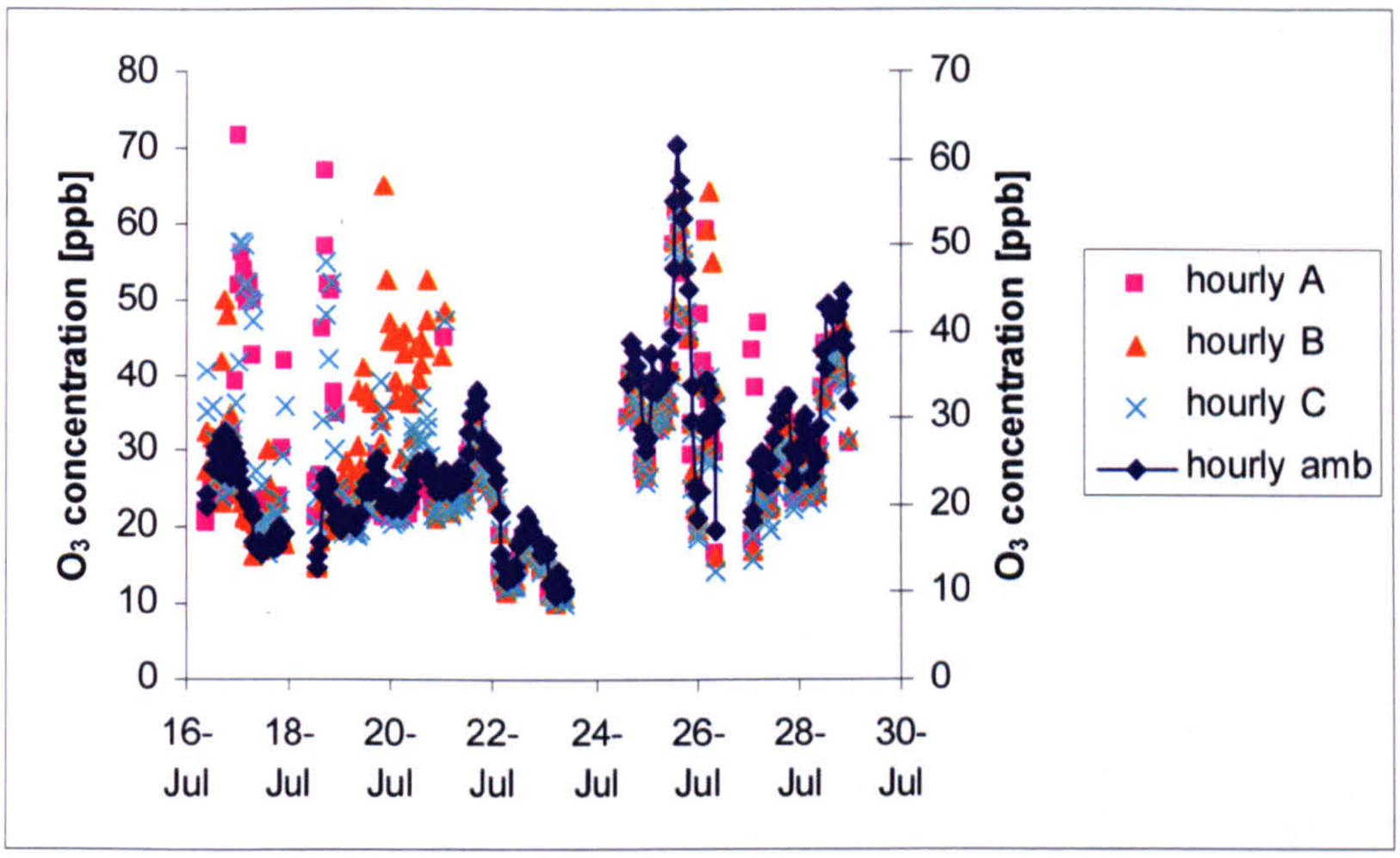


Figure 53: Ozone concentrations in Transects (A, B, C and the ambient control) recorded at 10 m from the release line during the period from 16th -28th July 2008.

Figure 54 shows the total LAI and the sward height measurements at the field site on 20th June to 6th August. The LAI increased from 3.11 m^2 m^{-2} to 5.8 m^2 m^{-2}, and the height of the canopy increased from 13 to 26 cm. Figure 55 shows the fractional leaf area index (FLAI) for the canopy fraction >5 cm, >20 cm and the total LAI measured between the three transects during the period 16th -28th July. The fractional LAI above 20 cm canopy height was lower than between 5 cm and 20 cm. The total LAI was around 4 m^2 m^{-2}. Although the total LAI was not much higher than the fractional LAI at 5-20 cm, it needs to be considered that the total LAI was measured with a different instrument that takes account of the attenuation of diffuse sky radiation at five zenith angles thereby taking into account the foliage orientation.

In Figure 56 the percentage of the functional groups in the two fractions is presented. At both heights, the legumes and grasses constitute about 50% of the biomass, whereas forbs accounted to 70% of the biomass at 5- 20 cm and to only 30% above 20 cm.

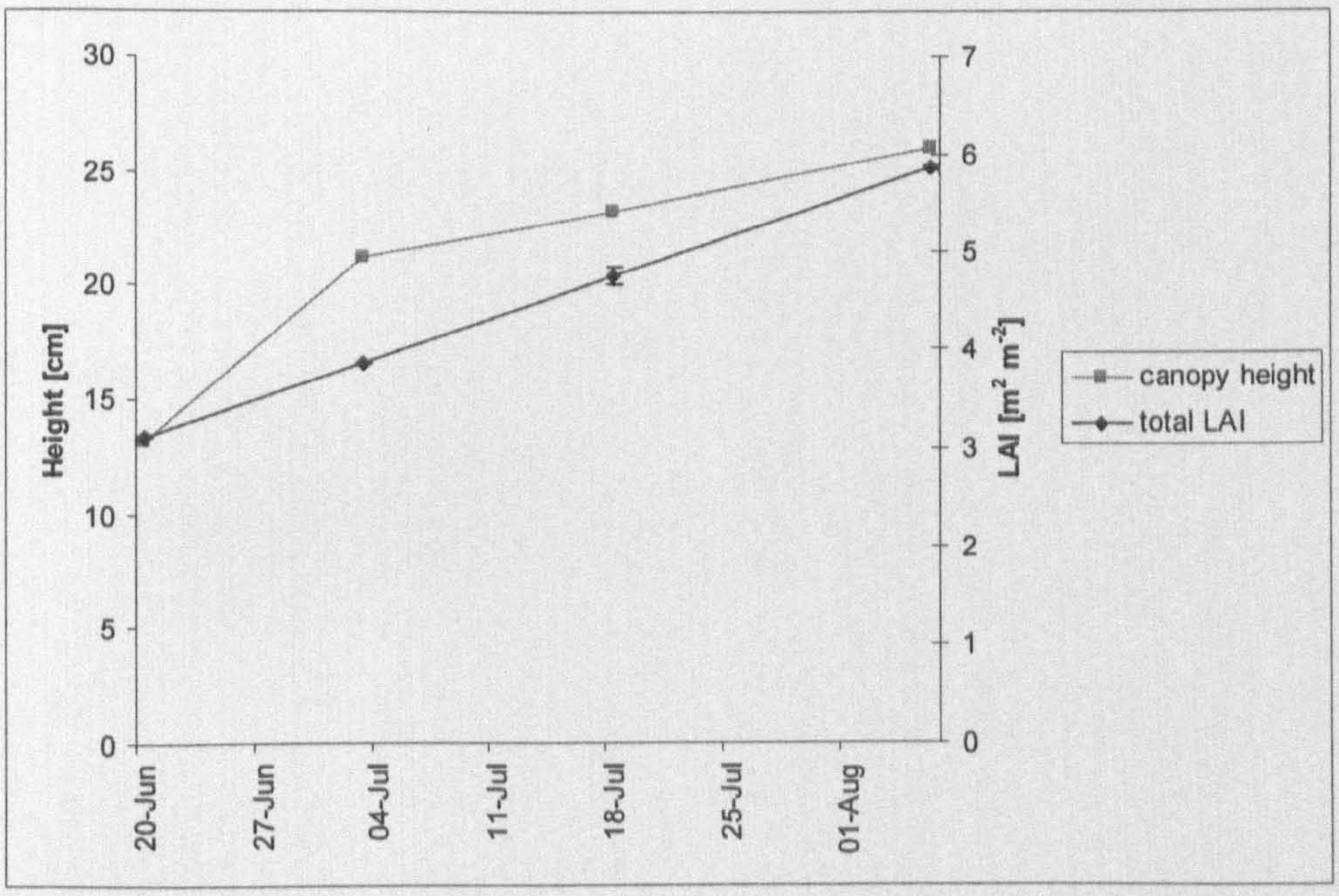


Figure 54: Mean total LAI and sward height in all plots measured at High Keenley Fell on 20th June to 6th August 2008.

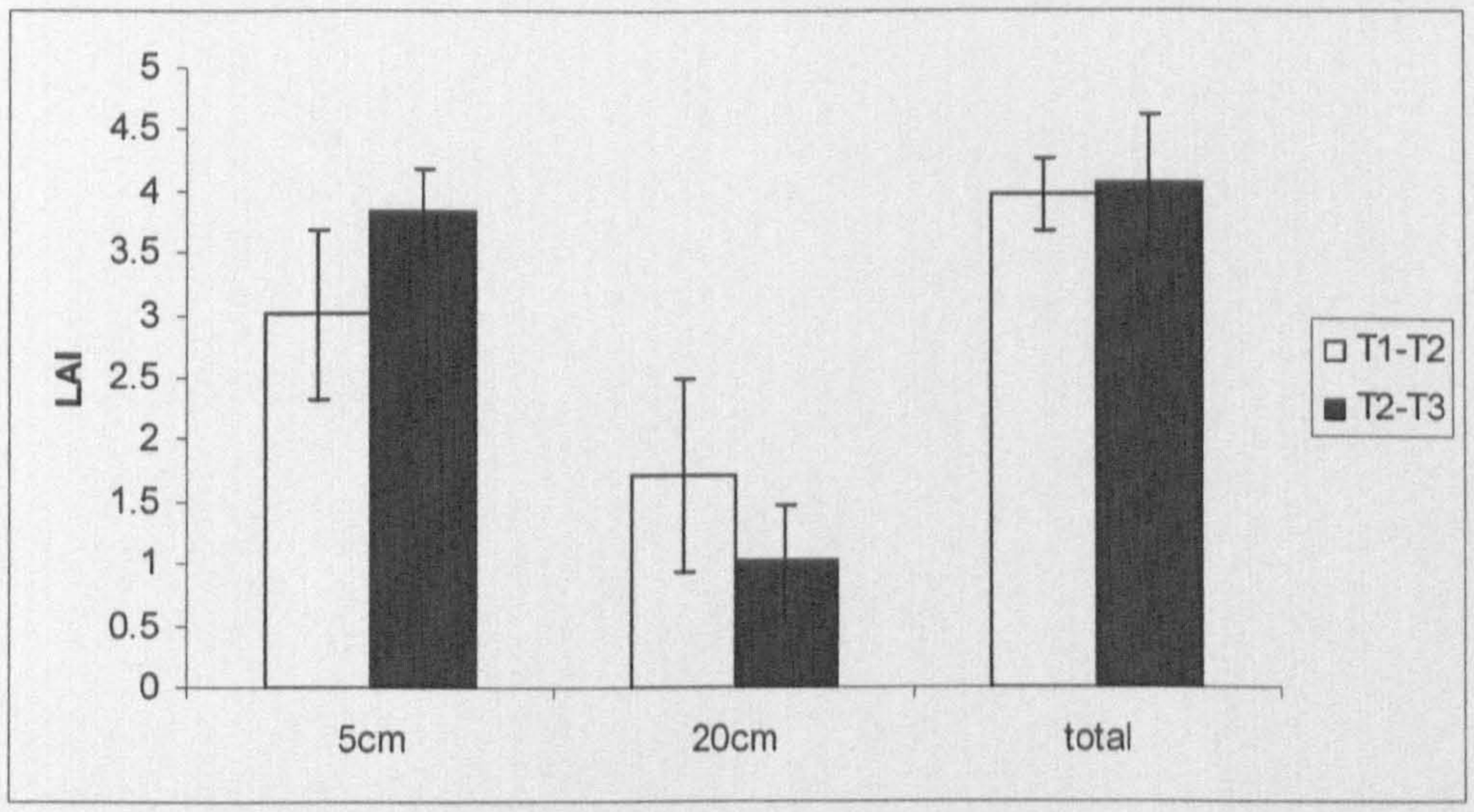


Figure 55: Fractional LAI at 5- 20 cm and above 20cm height, and the total LAI, measured between transect 1 (T1) and 2 (T2) and between transect 2 and 3 (T3) at High Keenley Fell during the period 16th -28th July in 2008. The fractional LAI was measured with a leaf area meter, the total LAI with Licor 2000 instrument. The error bars present the standard error of the measurements.

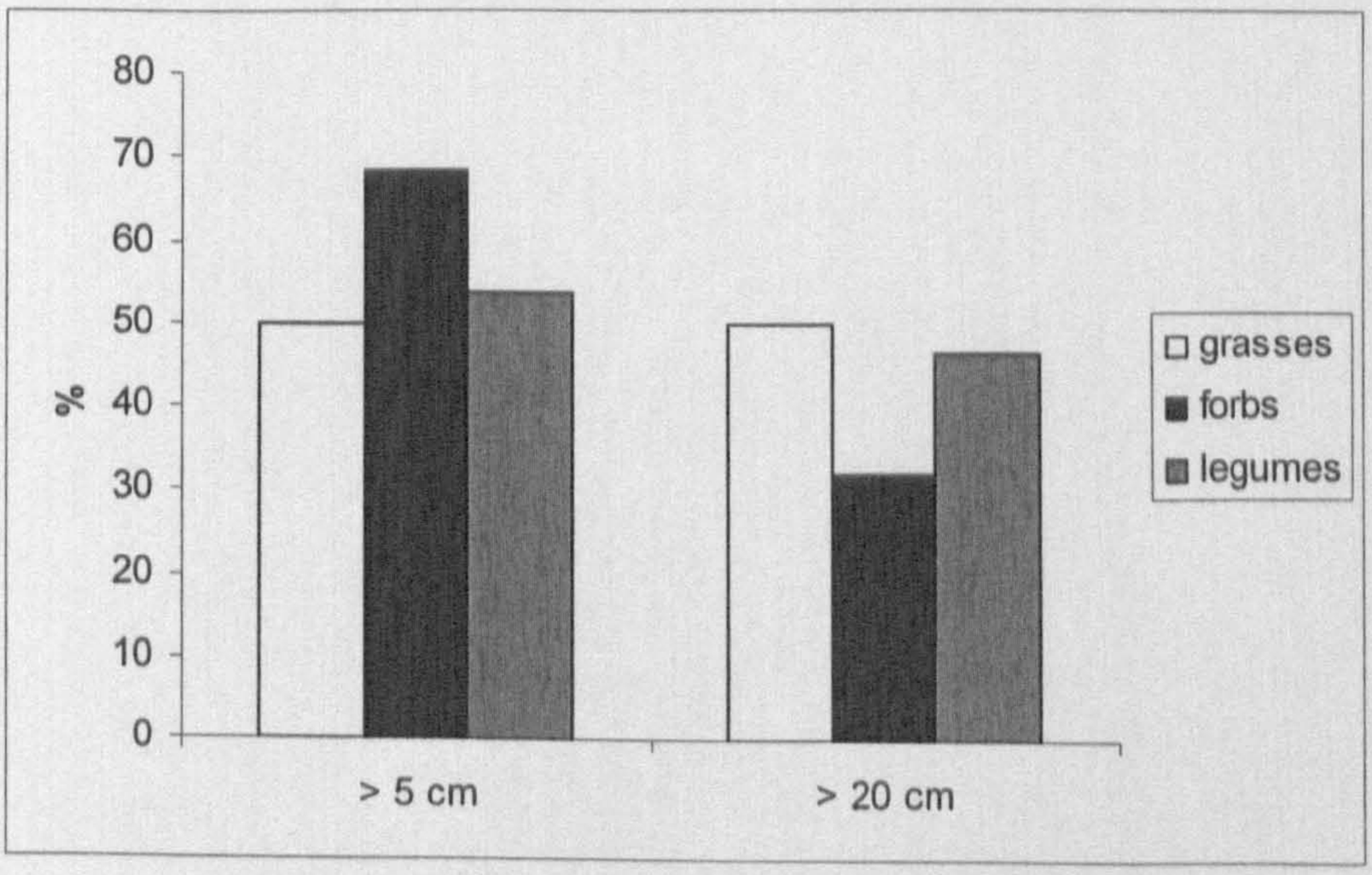


Figure 56: Percentage of forbs, legumes and grasses in the fractional LAI at 5-20 cm and above 20cm height, measured at High Keenley Fell during the period 16th -28th July 2008.

3.3.1.2 In canopy ozone profiles

The O_3 concentrations were measured at three different heights with three 2B monitors. The mean O_3 concentrations during the measurement campaign above the canopy (~30 cm) were consistently higher than O_3 concentrations measured at 20 cm and 5 cm, and also the O_3 concentrations at 20 cm were higher than at 5 cm (Figure 57). Overall the mean O_3 concentrations during this period were around 42.1- 49.1 ppb above the canopy (taking the differences of the monitors into account), 37.8 ppb at 20 cm and 19.4 ppb at 5 cm. The gradient in ozone concentrations was evident on each individual day, although the concentrations above the canopy varied greatly between days.

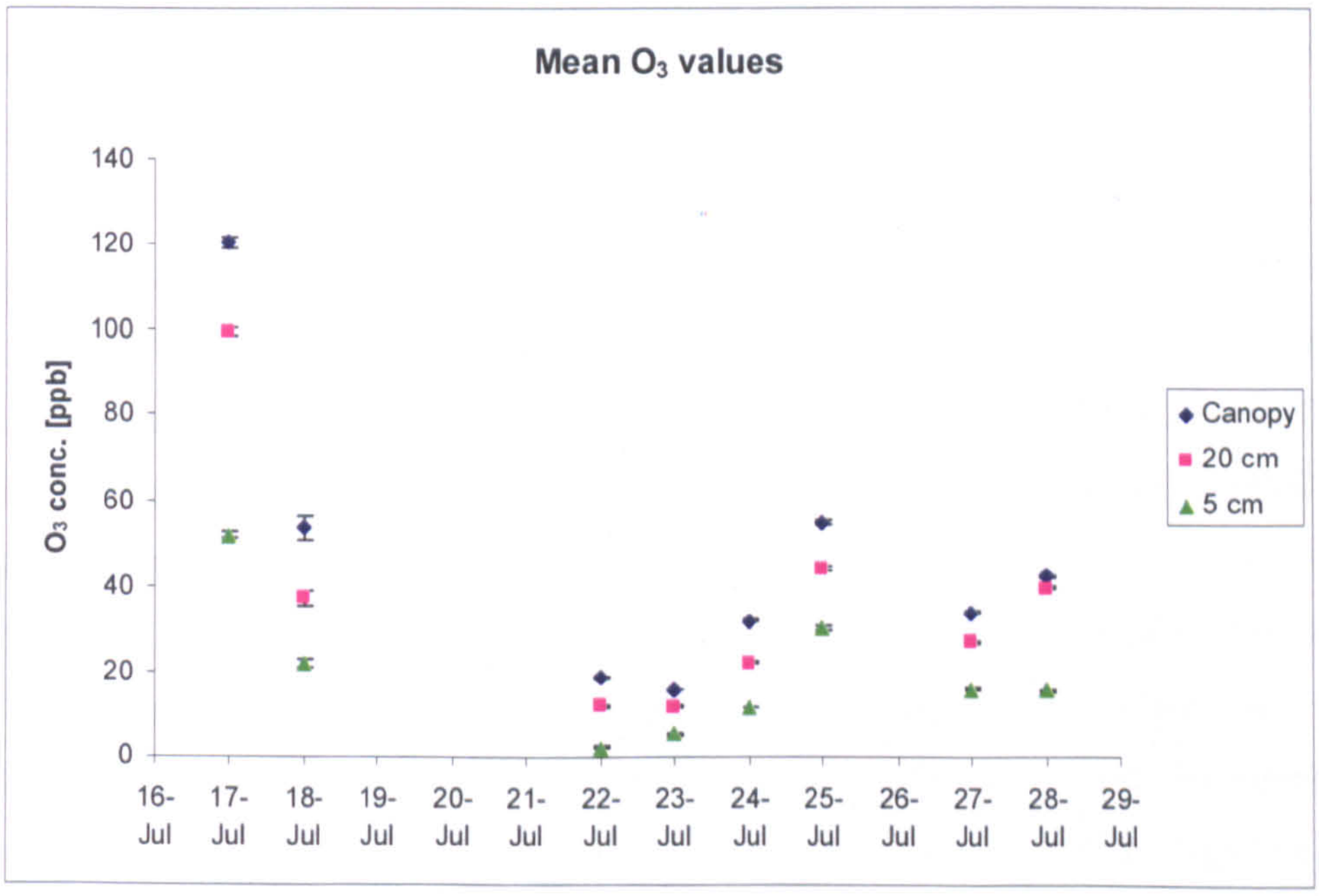


Figure 57: The daily mean O3 concentration during the measurement periods at three different heights, for the period 16th -28th July 2008. Error bars present the standard error of the measurements.

In Figure 58, the O_3 concentrations at 5 cm and 20 cm height are plotted against the O_3 concentrations at the top of the canopy. There was a strong linear relationship between the O_3 concentrations above the canopy and the O_3 concentrations within the canopy at 20 cm and/or at 5 cm. A threshold line of 40 ppb indicates that the O_3 concentrations have to reach 95 ppb above the canopy to reach a concentration of 40 ppb at a height of 5 cm within the canopy. Similarly, the O_3 concentration has to get to 50 ppb above the canopy in order to reach concentrations of 40 ppb at a height of 20 cm within the canopy. By exploring the relationship between the O_3 concentrations within the canopy at 20 cm and at 5 cm, it was found that O_3 concentrations had to attain $\geq$ 75 ppb at a height of 20 cm to reach a concentration of 40 ppb at a height of 5 cm (Figure 59). All of the regression lines show a strong correlation of $R^2 > 0.85$. The group of high values in Figure 58 is due to the O_3 concentrations of 17th, 18th and 25th July, when the O_3 fumigation system was on and concentrations were elevated above background.

In order to assess the effect of environmental variables on the ozone concentration, the O_3 concentration at 5 cm was expressed as a percentage of that at 20 cm and that above the canopy, while the O_3 concentration at 20 cm was expressed as a percentage of that above the canopy. Figure 60, Figure 61 and Figure 62 show these values plotted against the air temperature, humidity and the friction velocity. All figures show a similar pattern. The ratios at the 5 cm level are related to lower air temperature and very high humidity values. There seems to be a difference between the three heights. While the ratio at 20 cm shows a small increase with increasing temperature and decreasing humidity, the ratio at 5cm decreases. A similar pattern is found in Figure 62. Whereas the ratio above the at 20 cm stays more or less the same, the ratio at 5 cm decreases slightly with increasing friction velocity. However, the figures of these relationships were not significant.

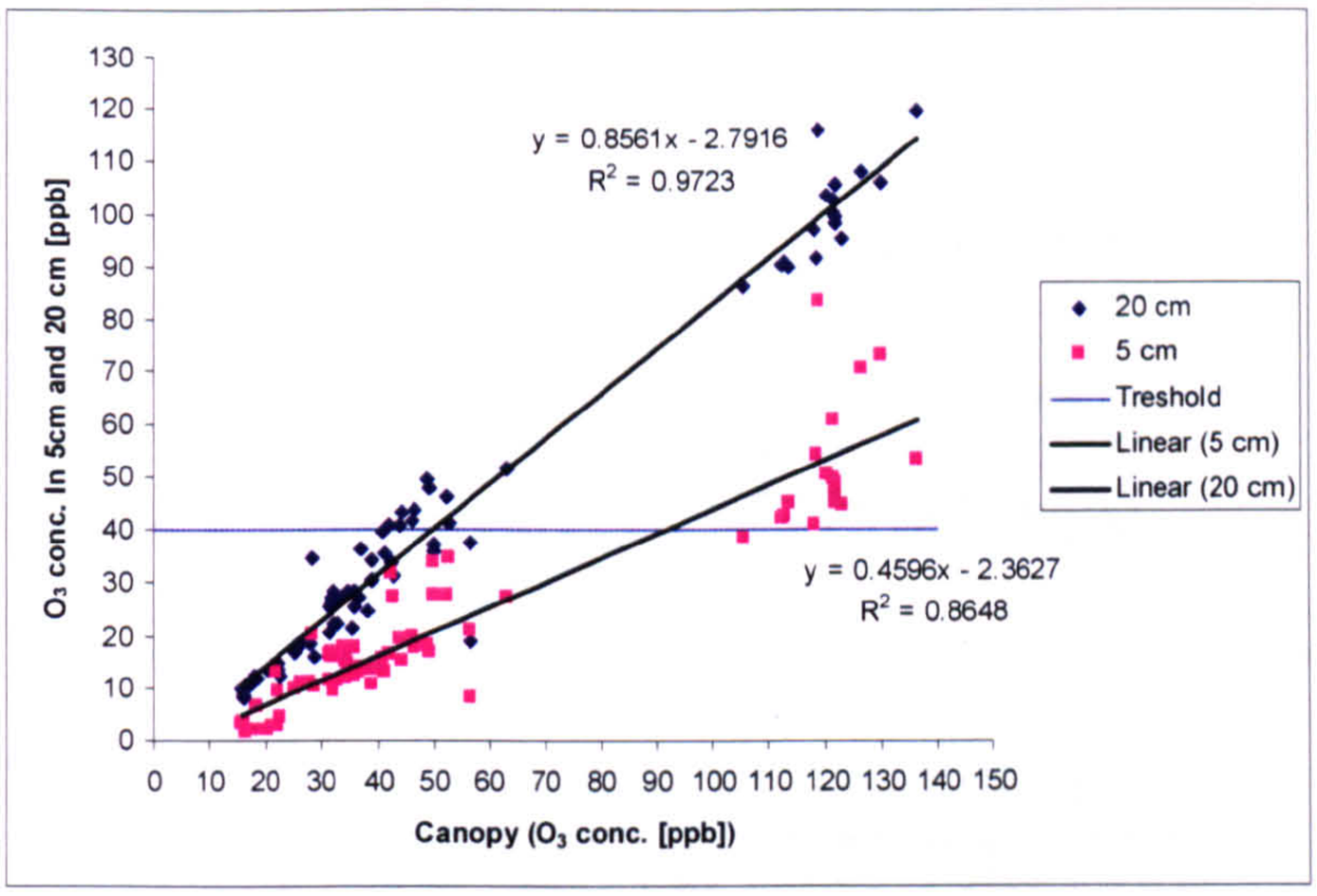


Figure 58: The relationship between O_3 concentrations at the 5 cm level (■) and 20 cm level (♦) and the concentrations at the canopy level (30 cm) for the period 16th -28th July 2008. Each data point shown represents a 30 minute measuring interval. The linear regression lines are also shown. A threshold of 40 ppb is indicated.

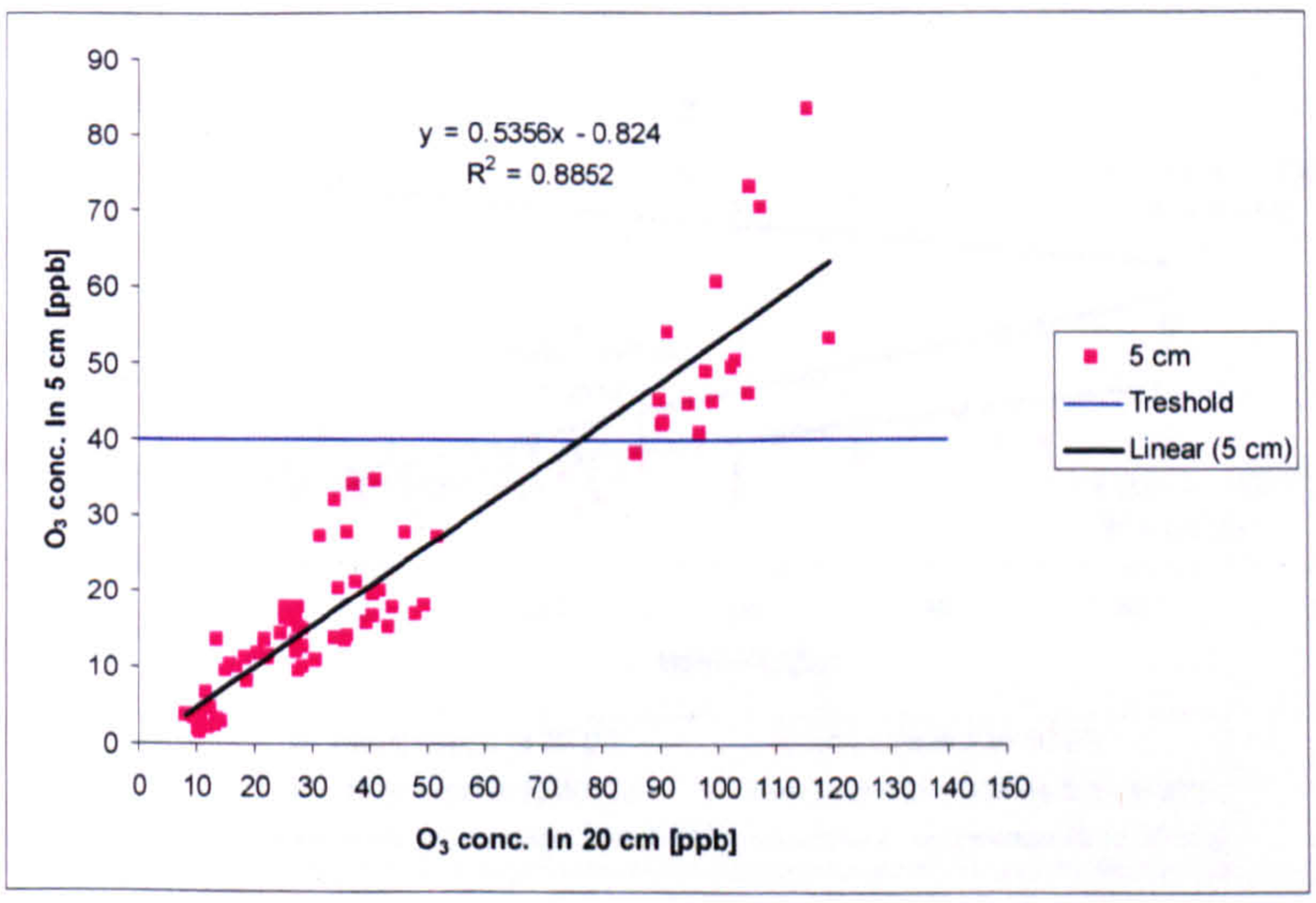


Figure 59: The relationship between O_3 concentrations at the 5 cm level (■) and the concentrations at the 20 cm level for the period 16th -28th July 2008. Each data point shown represents a 30 minute measuring interval. The linear regression line is also shown. A threshold of 40 ppb is indicated.

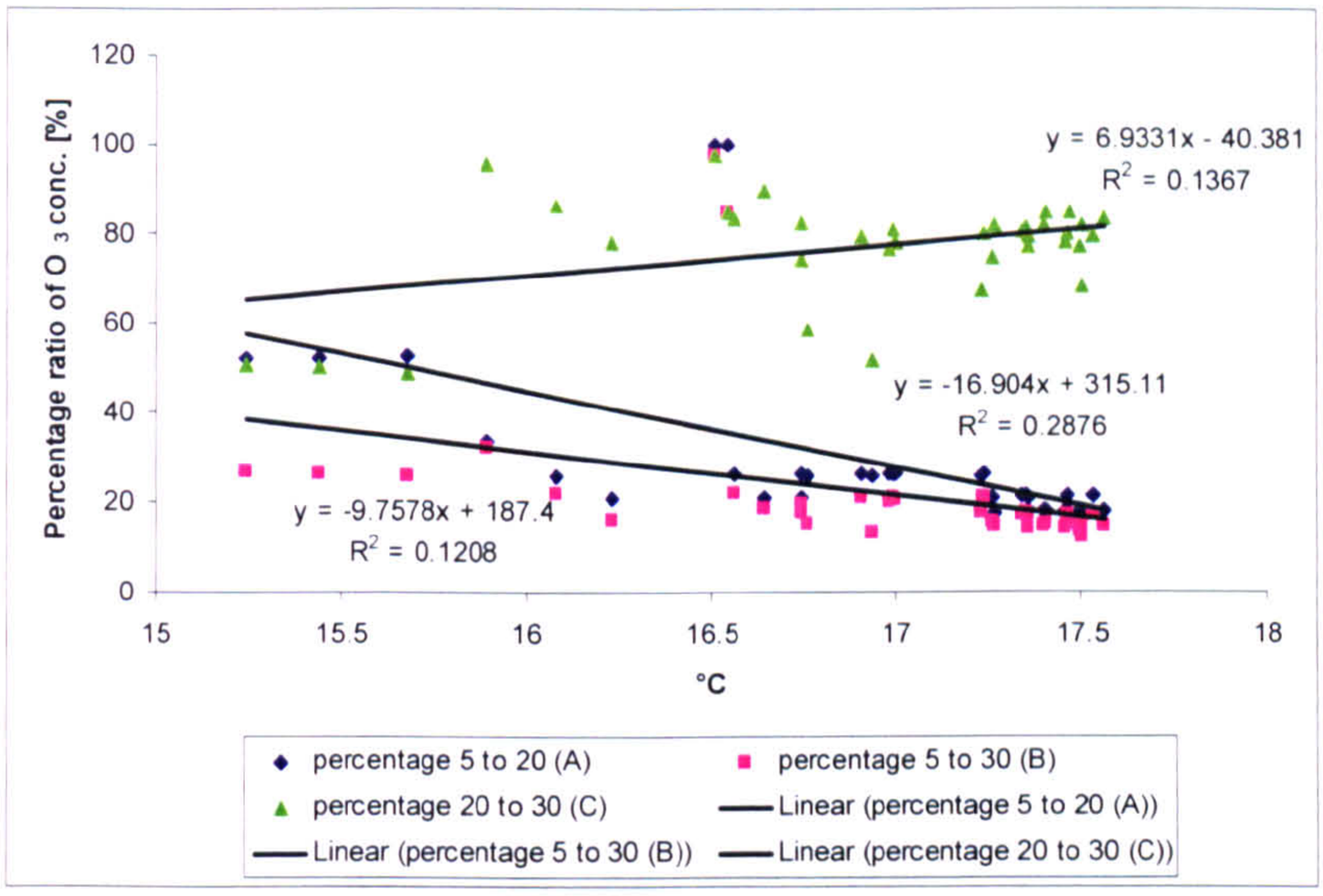


Figure 60: The relationship between mean O3 concentrations at the 5 cm level, the 20 cm level and the canopy level and the air temperature during the period from 16th -28th July in 2008. The data points are the means of 15 minute measuring intervals. The regression lines are indicated.

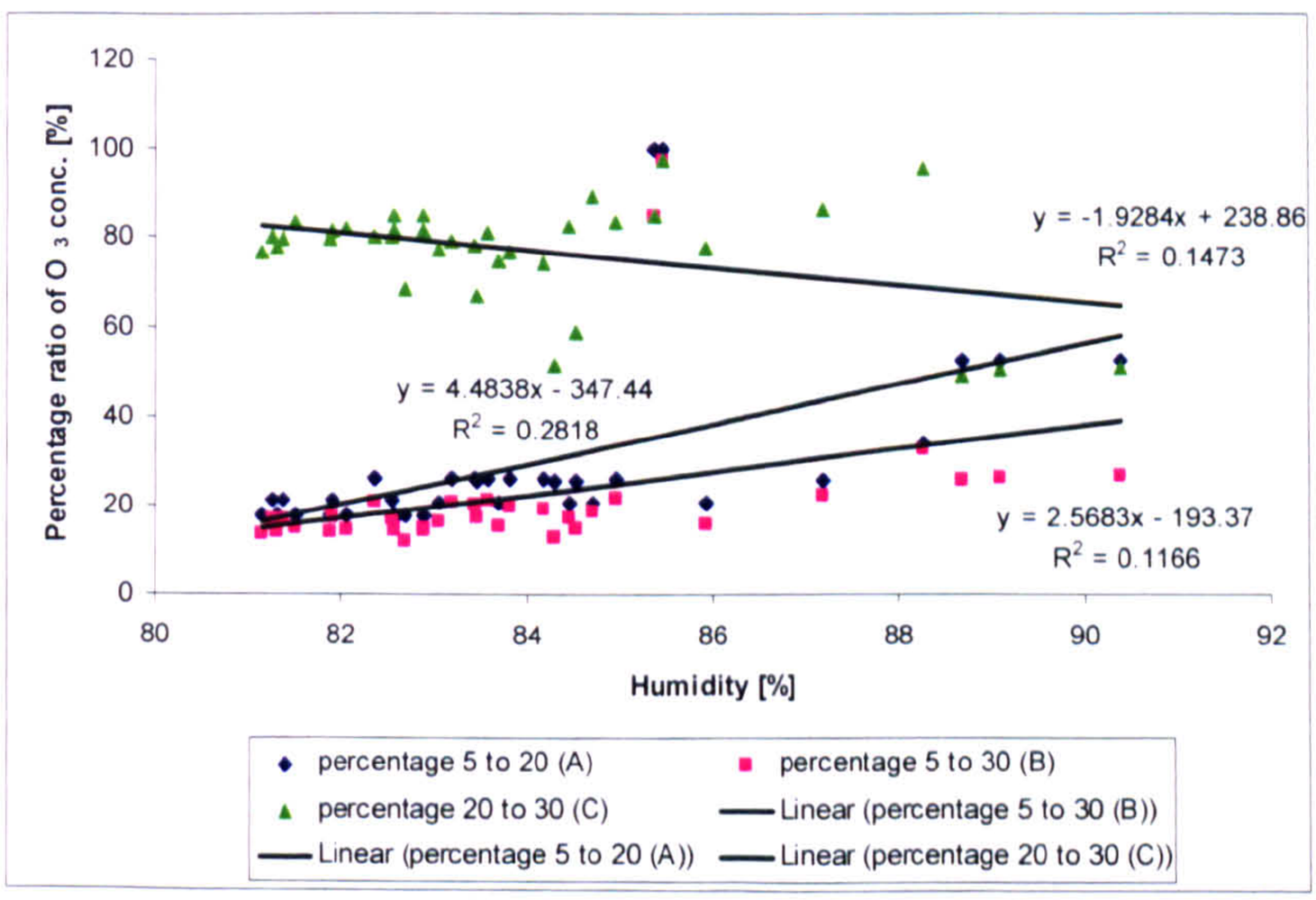


Figure 61: The relationship between mean O_3 concentrations at the 5 cm level, the 20 cm level and the canopy level and the air humidity during the period from 16^{th} -28^{th} July in 2008. The data points are the means of the 15 minute measuring intervals. The regression lines are indicated.

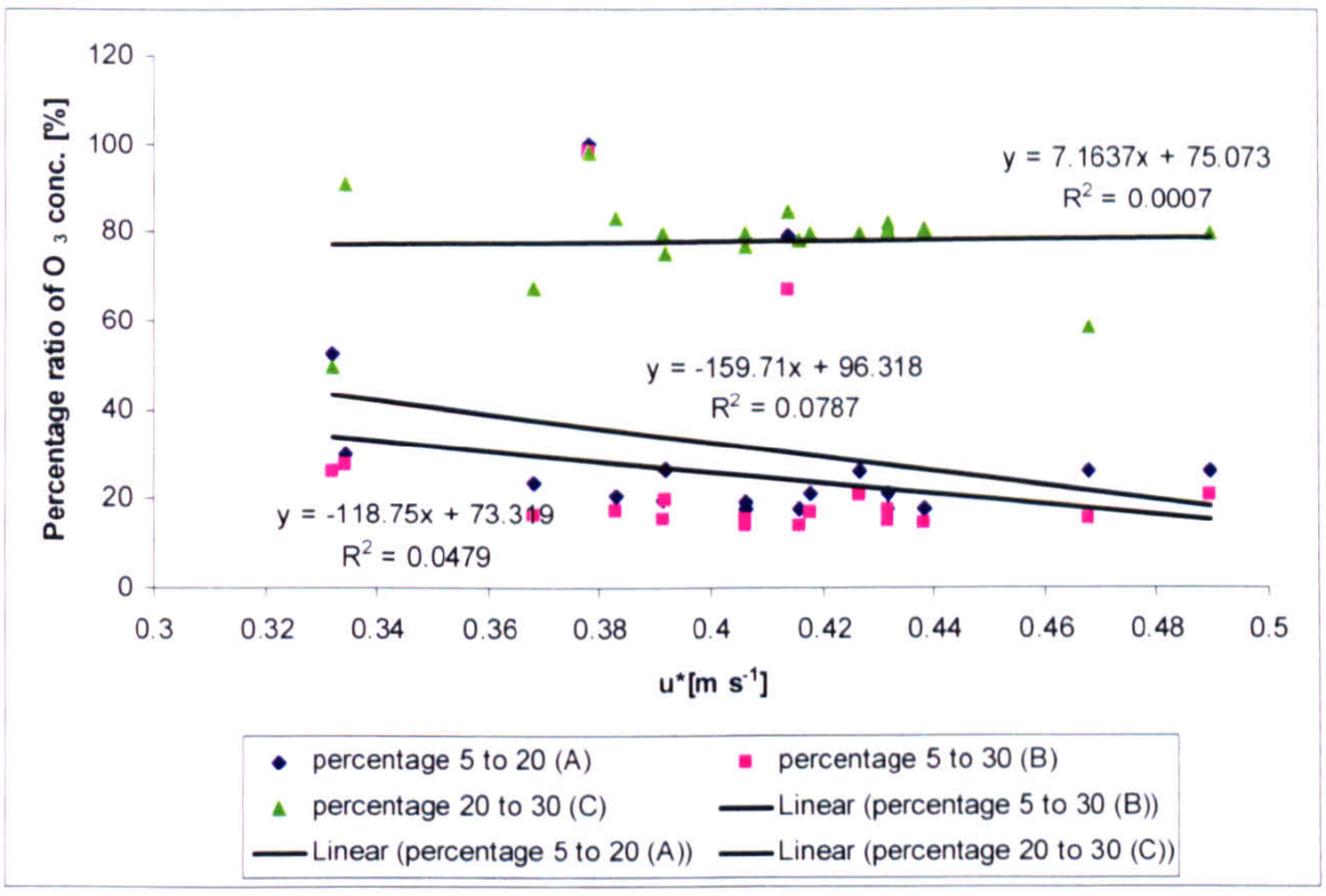


Figure 62: The relationship between mean O_3 concentrations at the 5 cm level, the 20 cm level and the canopy level and the friction velocity (u*) during the period from 16th -28th July in 2008. The data points report the means of the 30 minute measuring intervals (friction velocity was measured every 30 minutes). The regression lines are indicated.

The PAR data for the period 16th -28th July 2008 showed a distinctive difference between 5 cm and 20cm (Figure 63). The PAR at 5cm was lower than at 20 cm, and both were also lower than the PAR measured above the canopy. All three lines followed a diurnal pattern, with its highest point around noon, which is more pronounced at 20cm and above the canopy. When compared to PAR values at the top of the canopy, PAR was reduced on average by 27.4% at 20 cm, and by 73.6% at 5 cm.

Figure 63: The photo-synthetically radiation (PAR) at 5 cm, 20 cm, and above the canopy for the period 16th -28th July in 2008. The data points show the means of 5 minute measurement intervals.

3.3.2 Stomatal conductance of three functional groups

The grass *Holcus lanatus*, the forb *Rumex acetosa* and the legume *Trifolium repens* were measured in four different O_3 treatments and three different transects. *R. acetosa* and *H. lanatus* showed no significant differences between treatments and transects (Table 27, Figure 64 and Figure 65). However, a significant interaction of treatment and transect was observed for *H. lanatus* (P= 0.006). This effect is illustrated in Figure 66. The stomatal conductance shows an increase with increasing O_3 concentration (C, 10 ppb < 25 ppb, 75 ppb) in T3, but not in the other two transects. *T. repens* on the other hand showed significant effects of O_3 (Figure 67). Abaxial gs was significantly higher in the control treatment than in the 75 ppb treatment (P= 0.011), while adaxial stomatal conductance was significantly lower in the 25 ppb treatment than in the control treatment (P= 0.005), and the 10 ppb treatment (P= 0.043). Figure 68 shows the combined stomatal conductance of both leaf surfaces (abaxial and adaxial) of *T. repens*. Also in this case the stomatal conductance was significantly higher in the control treatment than in the 25 ppb and the 75 ppb treatment (P= 0).

Table 27: The effects of ozone concentration and transect (separate and in combination) on stomatal conductance of *Holcus lanatus, Rumex acetosa* and *Trifolium repens* in 2008. Post-hoc differences are significant at P< 0.05 (ns= non significant)

	Effects of ozone	*Effects of transect*	*Ozone*transect*
Holcus lanatus (abaxial)	0.186	0.266	**0.006**
Rumex acetosa (abaxial)	0.681	0.253	0.371
Trifolium repens (abaxial)	**0.011**	0.371	0.727
Trifolium repens (adaxial)	**0.005**	0.522	0.201
Trifolium repens (all)	**0**	0.676	0.892
Post-hoc			
Holcus lanatus (abaxial)	ns	ns	Control, 10ppb < 25ppb, 75ppb in T3
Rumex acetosa (abaxial)	ns	ns	ns
Trifolium repens (abaxial)	Control > 75ppb	ns	ns
Trifolium repens (adaxial)	Control > 25ppb, 10ppb > 25ppb	ns	ns
Trifolium repens (all)	Control > 25ppb, 75ppb		

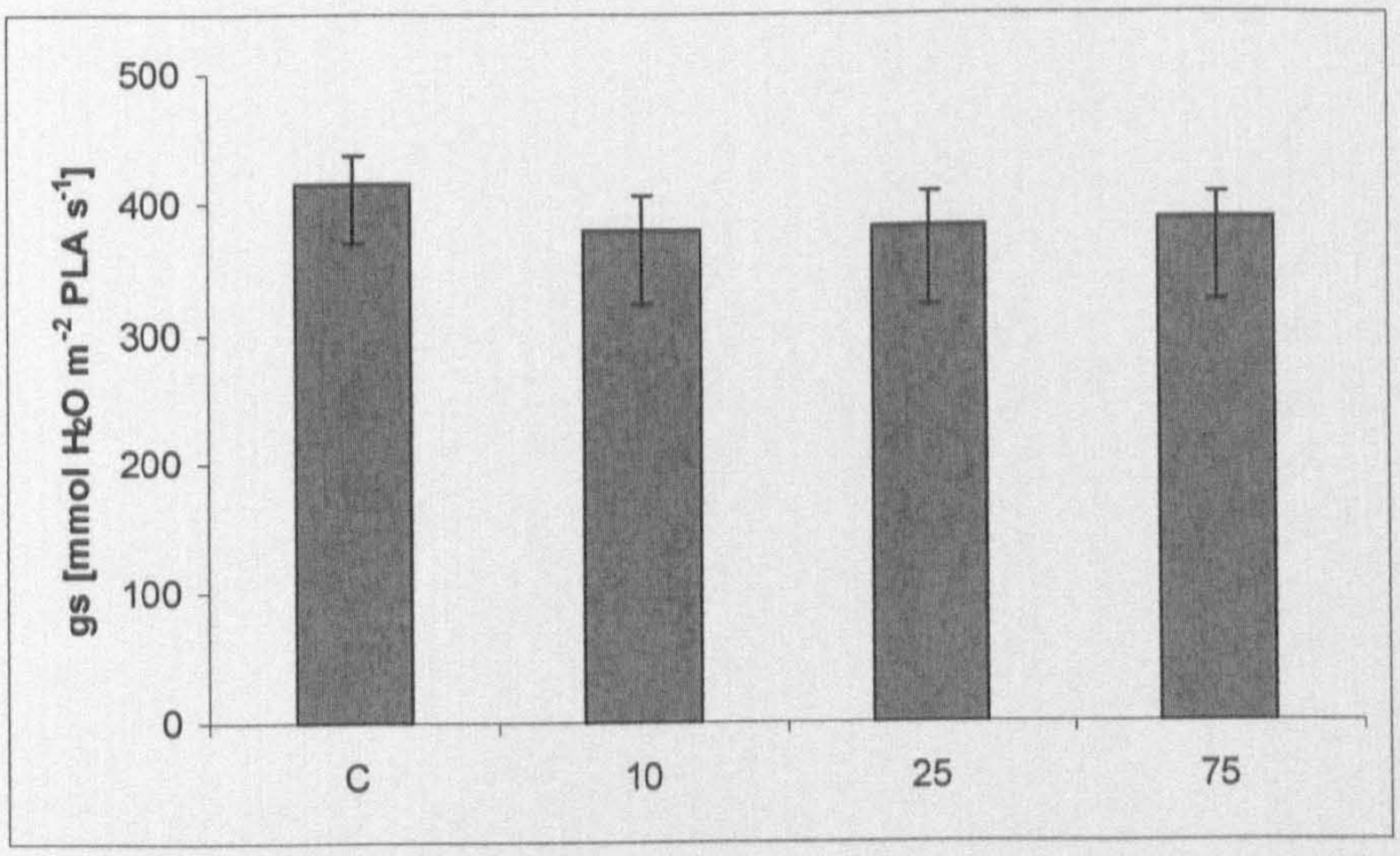


Figure 64: Mean stomatal conductance (g_s) to water vapour of Rumex acetosa at four different ozone treatments (Control (C), 10ppb, 25ppb and 75ppb) during 2008. The error bars represent standard errors.

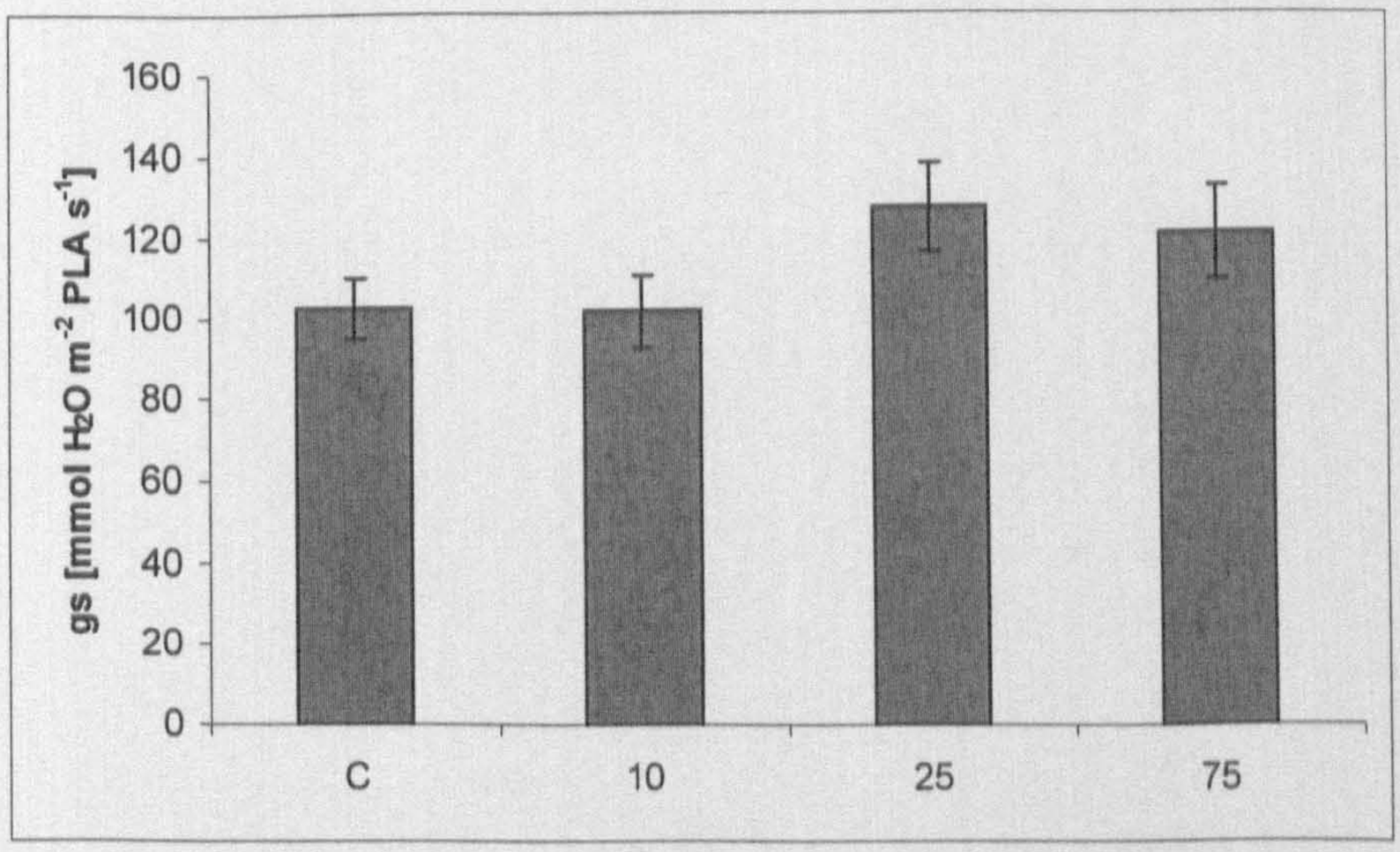


Figure 65: Mean stomatal conductance (g_s) to water vapour of *Holcus lanatus* at four different ozone treatments (Control (C), 10 ppb, 25 ppb and 75 ppb) during 2008. The error bars represent standard errors.

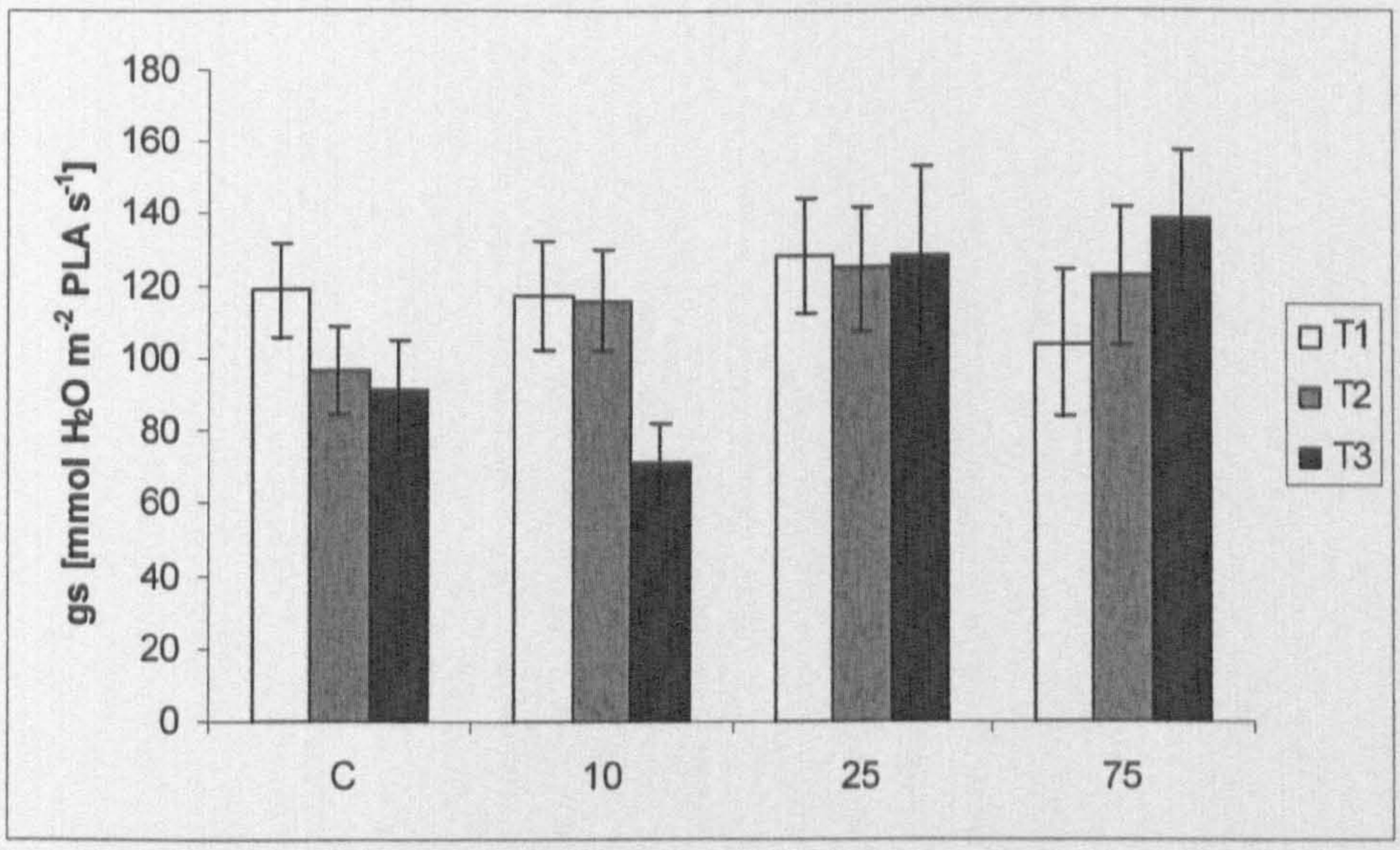


Figure 66: Mean stomatal conductance (g_s) to water vapour of *Holcus lanatus* in all three transects (T1, T2 and T3) at four different ozone treatments (Control (C), 10 ppb, 25 ppb and 75 ppb) during 2008. The error bars represent standard errors.

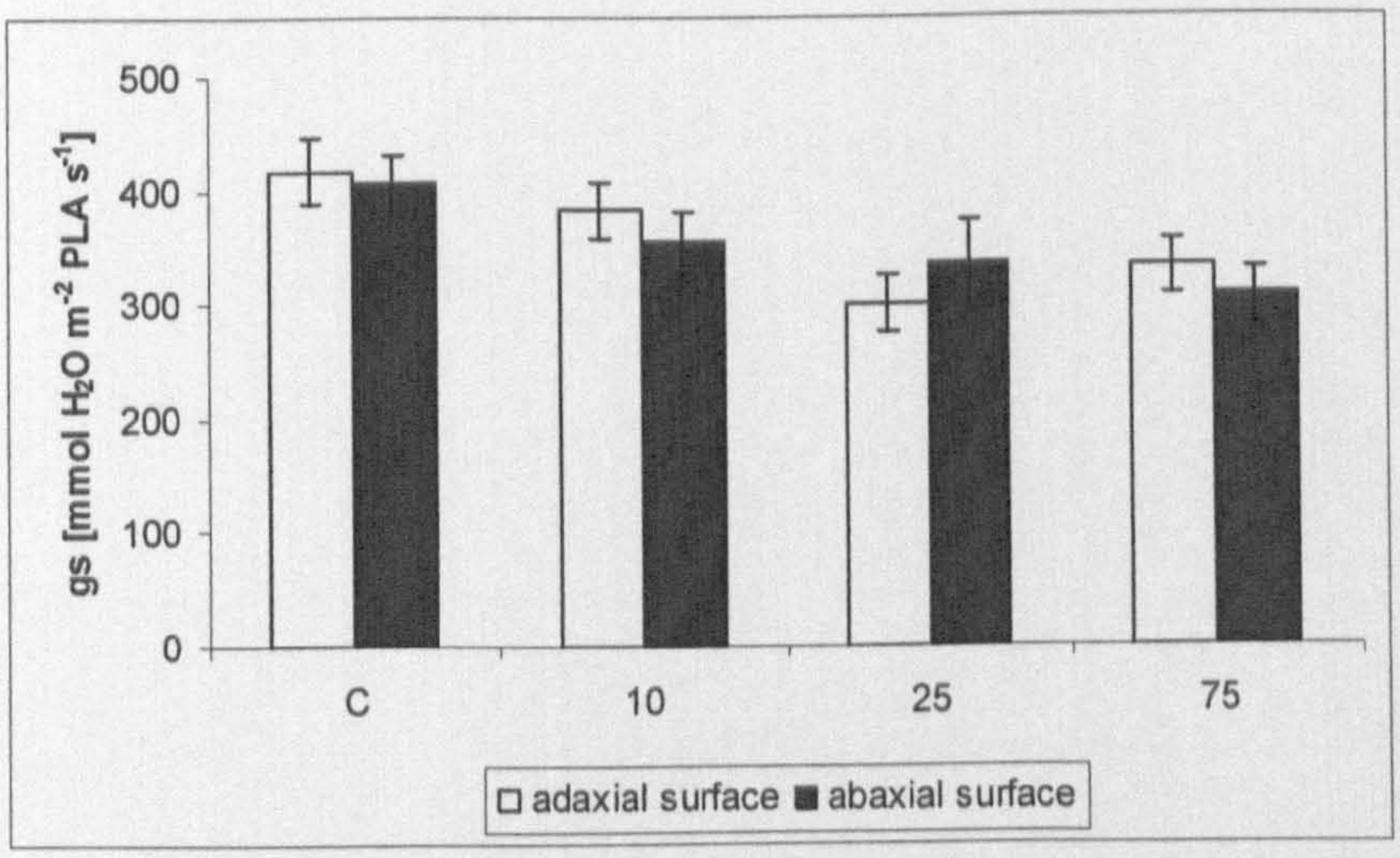


Figure 67: Mean stomatal conductance (g_s) to water vapour of *Trifolium repens* showing the difference of gs at the abaxial and adaxial surface at four different ozone treatments (Control (C), 10 ppb, 25 ppb and 75 ppb) 2008. The error bars represent standard errors.

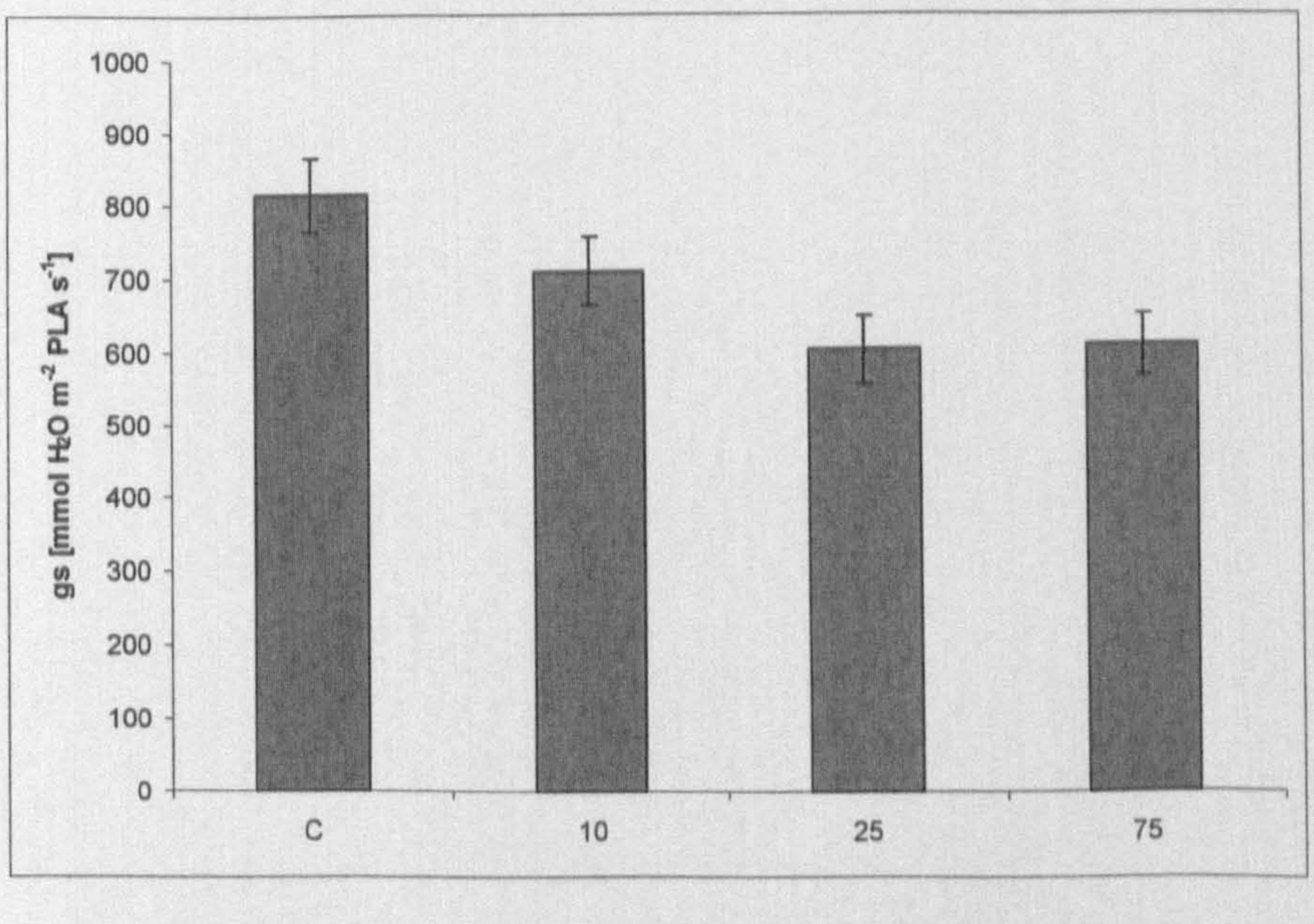


Figure 68: Mean projected (adaxial + abaxial) stomatal conductance (g_s) to water vapour of *Trifolium repens* at four different ozone treatments (Control (C), 10 ppb, 25 ppb and 75 ppb) 2008. The error bars represent standard errors.

Table 28 compares the effects of ozone and transect on *T. repens*, and the ozone*transect interaction, on days when there was fumigation and when there was no fumigation. In both cases, no significant differences were found between the treatments in post-hoc tests. However, on the days with fumigation there seemed to be trend of higher stomatal conductance in the control plots than in the 75 ppb plots (Figure 69). Interestingly, on 16th July, when O_3 concentrations were highest, reaching 120 ppb, the O_3 treatment affected *T. repens* significantly. The stomatal conductance was significantly higher in the control treatment than in the 75 ppb treatment (P= 0.02).

Table 28: Effects of ozone and transect on the stomatal conductance of *Trifolium repens* in 2008. Effects of ozone, transect and the interaction of ozone and transect are shown for days with high and low O_3 concentrations. Post-hoc differences are significant at P< 0.05 (ns = non significant)

Trifolium repens	*Effects of ozone*	*Effects of transect*	*Ozone*transect*
No fumigation	0.088	0.625	0.511
Fumigation	**0.049**	0.886	0.929
16th July	**0.02**	0.673	0.9
Post-hoc			
No fumigation	ns	ns	ns
Fumigation	ns[12]	ns	ns
16th July	Control > 75ppb	ns	ns

[12] Trend detected between control and 75 ppb treatment, P< 0.1

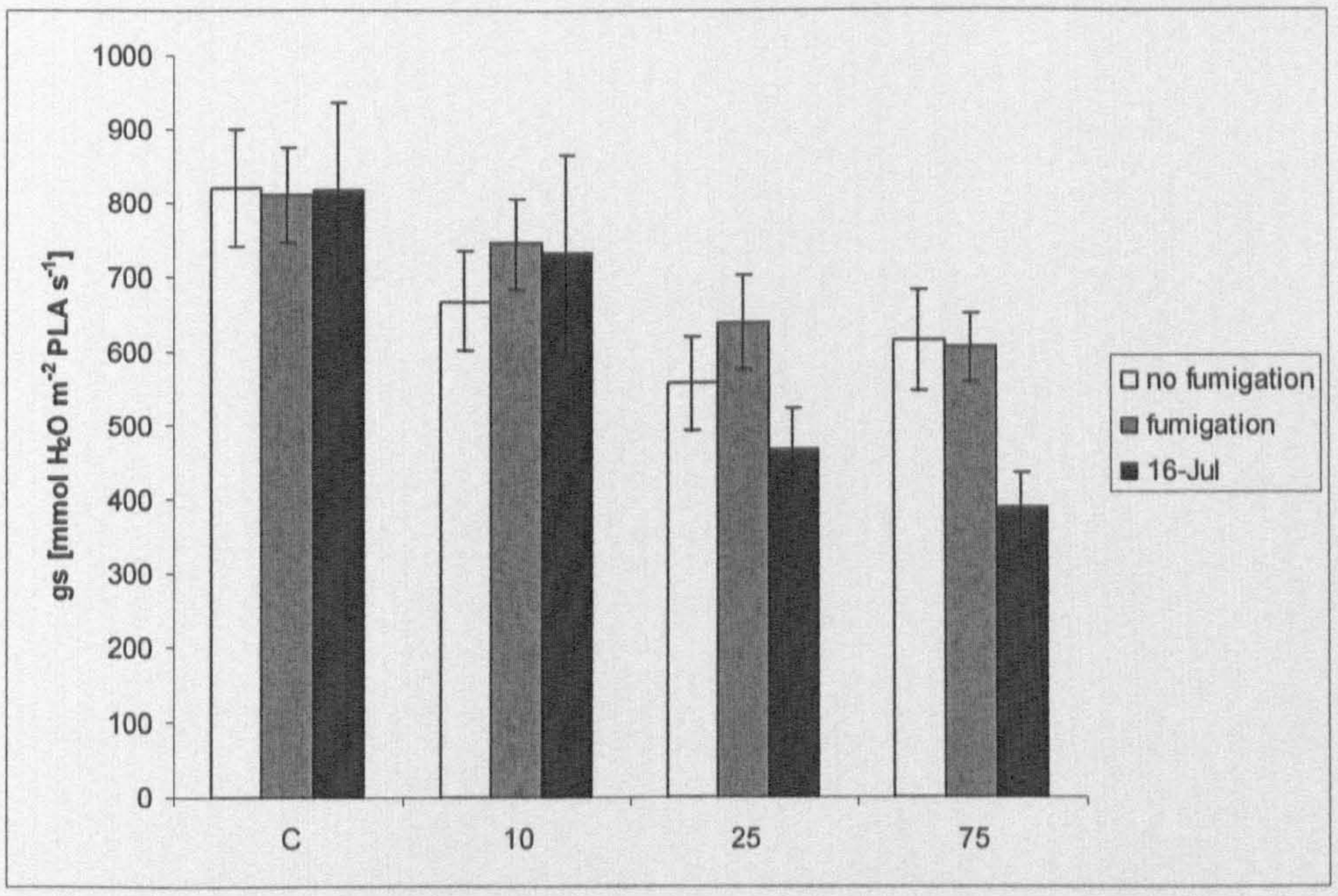


Figure 69: The mean stomatal conductance (g_s) to water vapour of *Trifolium repens* in four different treatments (Control (C), 10 ppb, 25 ppb and 75 ppb) on days of fumigation vs. days of non fumigation in 2008. The day with highest O_3 concentrations (16th July) is also illustrated. The error bars represent standard errors.

3.3.3 Grassland mesocosms

Briza media was measured in two different O_3 treatments and in two different nutrient treatments. Significant differences in stomatal conductance were found between the 50 ppb and the 30 ppb treatment, with values being significantly higher in the latter (P= 0). No significant effects of nutrient treatment were detected, and no significant interaction of nutrient and ozone was found (Table 29, Figure 70). When comparing stomatal conductance measured before the cut (June/ July) to after the cut (September), significant effects of ozone were only found in the latter period (Table 30 and Table 31). The stomatal conductance

was significantly higher in the 30 ppb treatment than in the 50 ppb treatment in the September period only (P= 0.014) (Figure 71 and Figure 72).

Table 29: Effects of ozone and nutrients on stomatal conductance of *Briza media* during June, July and September 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant).

	P	***Post-Hoc***
Ozone	**0**	30ppb>50ppb
Nutrient	0.117	ns
Ozone*Nutrient	0.478	ns

Table 30: Effects of ozone and nutrients on stomatal conductance of *Briza media* before the cut (June/July) in 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant).

	P	***Post-Hoc***
Ozone	0.277	ns
Nutrient	0.422	ns
Ozone*Nutrient	0.231	ns

Table 31: Effects of ozone and nutrients on stomatal conductance of *Briza media* after the cut (September) in 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant).

	P	***Post-Hoc***
Ozone	**0.014**	30 ppb> 50 ppb
Nutrient	1.0	ns
Ozone*Nutrient	0.641	ns

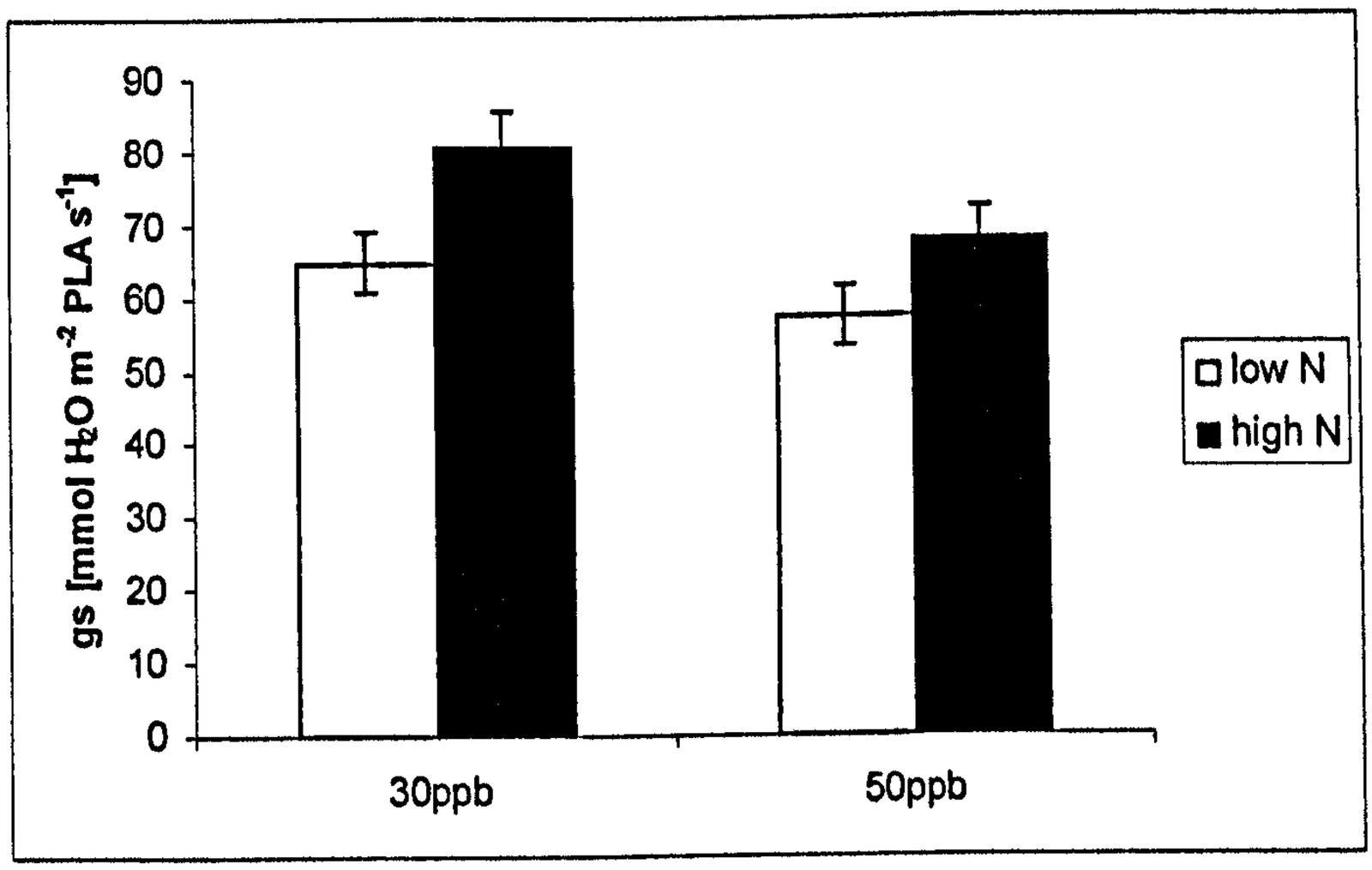


Figure 70: The mean stomatal conductance (g_s) to water vapour of *Briza media* in two different O_3-treatments and two nutrient treatments (50ppb, 30ppb; low N, high N) during the vegetation period in 2008. The error bars represent standard errors.

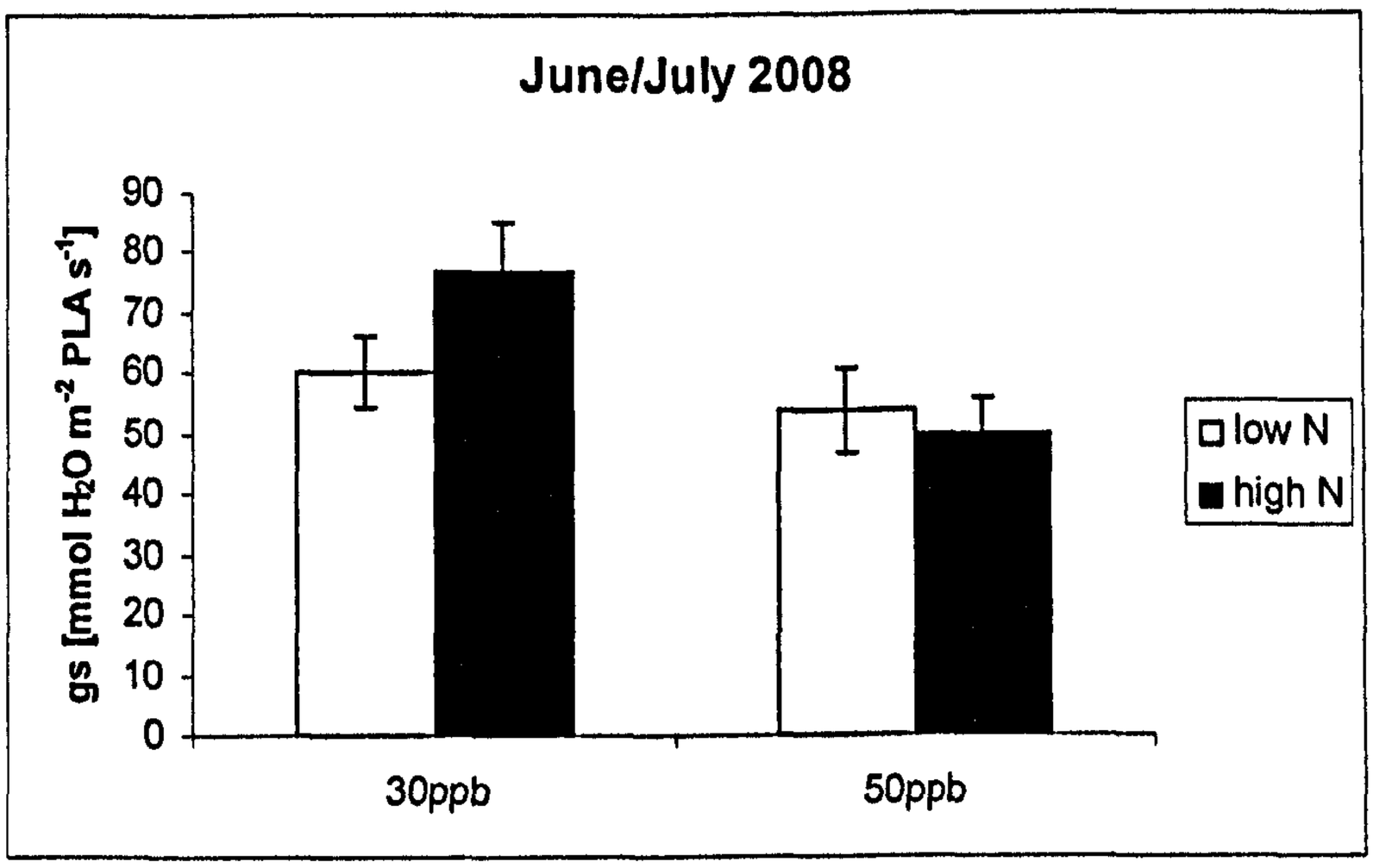


Figure 71: The mean stomatal conductance (g_s) to water vapour of *Briza media* in two different O_3-treatments and two nutrient treatments (50 ppb, 30 ppb; low N, high N) before the cut in 2008. The error bars represent standard errors.

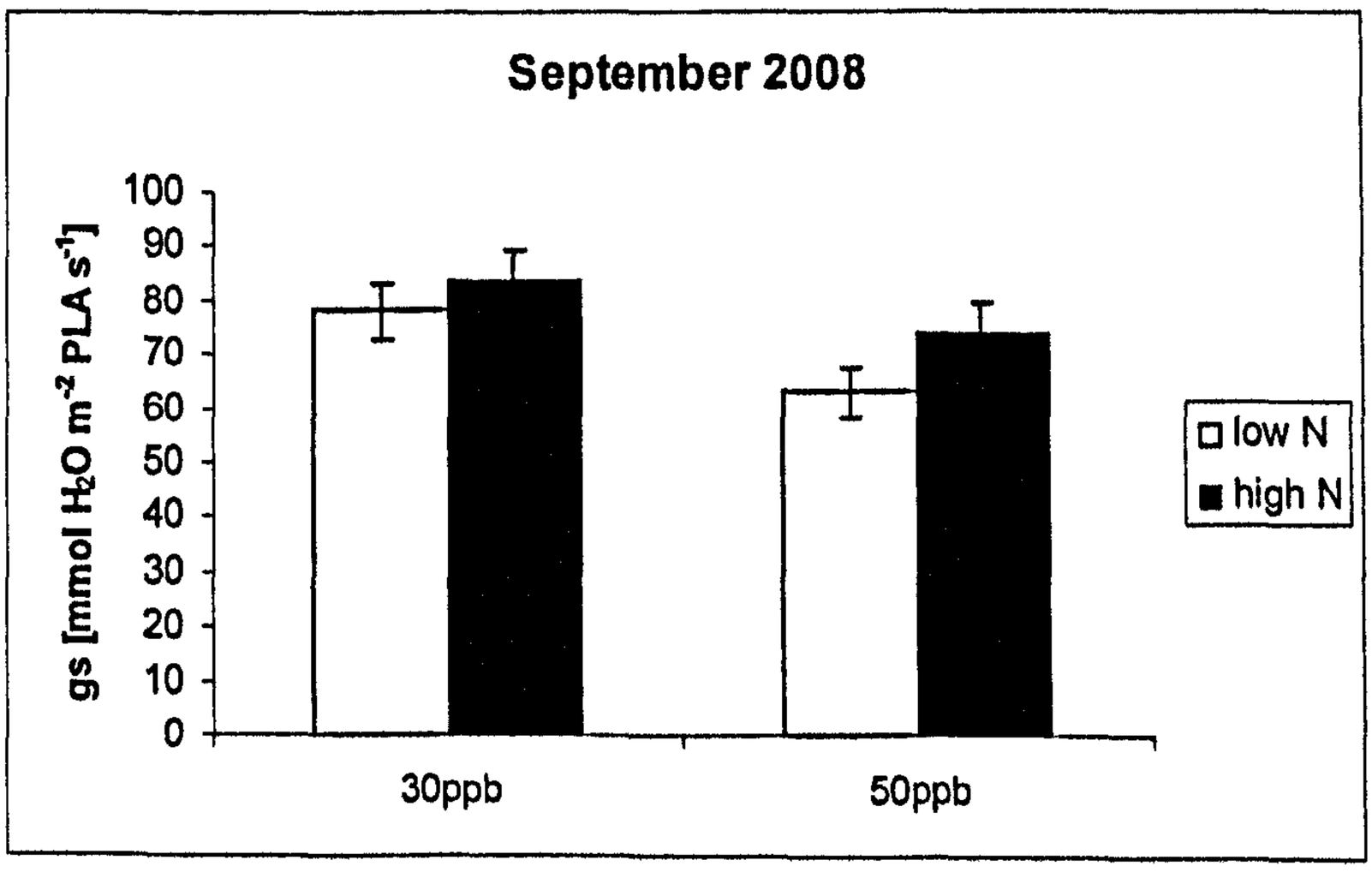


Figure 72: The mean stomatal conductance (g_s) to water vapour of *Briza media* in two different O_3-treatments and two nutrient treatments (50 ppb, 30 ppb; low N, high N) after the cut in 2008. The error bars represent standard errors.

However, significant differences were found between chambers within the ozone treatment before the cut. The stomatal conductance was significantly higher in Chamber 2 than in Chamber 3, which also received ambient air, as well as in the two chambers 1 and 4 receiving elevated ozone (Table 32, Figure 73). After the cut there were no significant differences observed between the chambers to confound the significant effect of ozone.

Table 32: Results of the ANOVA of chamber effects on stomatal conductance of *Briza media* overall the whole season, and before (June/July) and after (September) the cut in 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant); Chambers 1, 4 = elevated, Chambers 2, 3 = ambient.

	P	***Post-Hoc***
Overall	**0**	2 > 1
June/July	**0.017**	2 > 1, 3, 4
September	0.066	ns

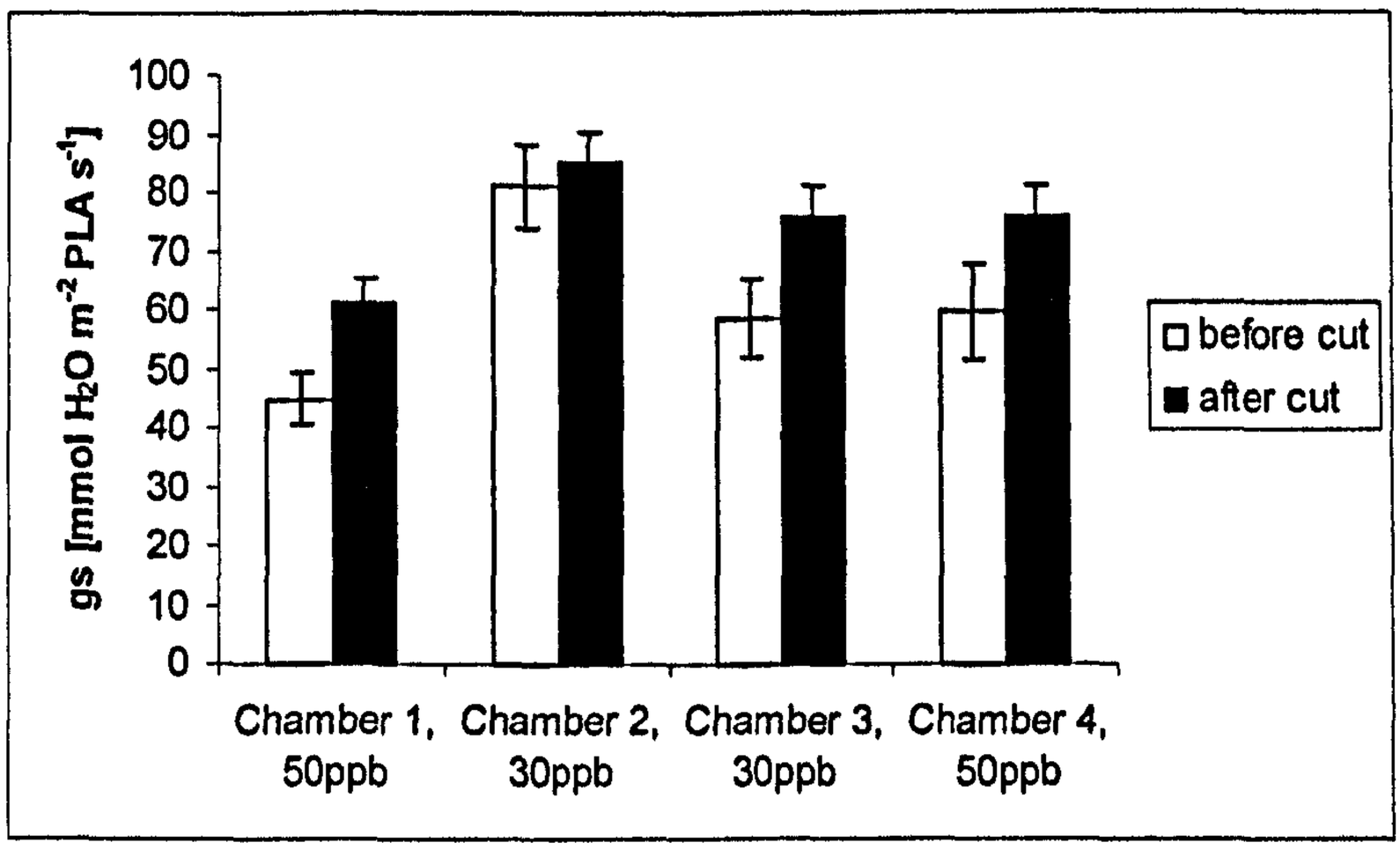


Figure 73: The mean stomatal conductance (g_s) to water vapour of *Briza media* in four different chambers (Chamber 1, 4 = elevated; Chamber 2, 3 = ambient) before and after the cut in 2008. The error bars represent standard errors.

Additional results of a soil water analysis of the grassland mesocosms, which showed only significant effects of ozone for the pH, are not shown here but can be found in Appendix 7.3.

3.4 Discussion

Ozone gradient within the canopy

The within canopy measurements of O_3 concentrations during the period from 16th -28th July revealed that O_3 concentrations at two different heights within the canopy were distinctively different from the O_3 concentrations above the canopy. The average O_3 concentrations were 52.1 ppb at the canopy level (30 cm), 37.8 ppb in the higher vegetation layer (20 cm) and 19.4 ppb in the lower vegetation layer (5 cm). Comparing the two layers with the canopy according to the currently used threshold of 40 ppb showed that O_3 concentrations have to reach more than 95 ppb above the canopy in order to exceed 40 ppb at 5cm, and more than 50 ppb to exceed 40 ppb at 20 cm. Taking into account that the ozone concentrations at the canopy were about 3-10% lower than the measured O_3 concentrations at the canopy, the thresholds would probably be higher.

Two other studies have measured O_3 concentrations within a grassland canopy. Veit & Henning-Müller (2001) found that whereas at 50 cm 90% of O_3 concentrations of those above the canopy were measured, at 1 cm they were reduced to 53%. They also found a drop in O_3 concentrations already at a level between 10 cm and 5 cm within the canopy (top of canopy 250 cm). In the study of Jaeggi *et al.* (2006), in which they studied profiles of O_3 concentrations in a grassland canopy, they discovered that at 0.5 m the ozone concentrations were reduced to 92% and to 64% at 0.25 m. In this study the measured ozone concentrations were overall lower but also similar to the ozone profiles of Jaeggi *et al.* (2006).

Differences between the three studies may be related to the structure of the leaf canopy or to the meteorological conditions. In this study, the leaf area index showed an increase from 3.1 m^2 m^{-2} to 5.8 m^2 m^{-2} within the two week period, and the height of the canopy showed an increase from 13 cm to 26 cm. In the study of Jaeggi *et al.* (2006) LAI values increased from 4.7 m^2 m^{-2} to 6.8 m^2 m^{-2} and during the same time, the canopy height increased from about 40 cm to 76 cm height.

The bulk stomatal conductance of the canopy remains almost constant for LAI values of more than 4 m^2 m^{-2} because the area of sunlit leaves does not change much (Saugier & Katerji, 1991, Menzel 1997). Other studies (e.g. Jaeggi *et al.*; 2006) have confirmed this. Stronger influence on the deposition and profile of O_3 is expected at low LAI values such as 0.5 m^2 m^{-2} (Saugier & Katerji, 1991, Menzel 1997). Although the fractional LAI revealed low LAI values at 20 cm which were below 3 m^2 m^{-2}, these values were probably still too high to affect the ozone profiles. The percentage of functional groups showed that while forbs accounted for 70% of the biomass at > 5 cm and for only 30% at > 20 cm, the legumes and grasses constituted about 50% of the biomass at both heights. This also revealed that forbs play a major role within the lower layers of the canopy, but are less present in the upper layers of the canopy. The light transmittance was much lower at 5 cm (27.4%) than at 20 cm (73.6%). This is in contrast to the study of Jaeggi *et al.*, (2006) in which the reduction of PAR was only between 20% and 40% in the middle of the canopy, but similar to the study of Davison *et al.* (2003). They reported results for a monoculture stand of coneflower with a total LAI of 5.5 m^2 m^{-2}, and a reduction of PAR by 90% in the upper 50% of the canopy. Overall, in their study, the reductions were greater in PAR than in ozone. At 50 cm, ozone varied from 15-90% of ambient, whereas PAR was consistently lower. They concluded that the ozone profile was variable due to differences in canopy structure, stomatal conductance and friction velocity. Both studies showed a constant decrease of ozone per unit LAI. Davison *et al.* (2003) found a decrease of 15.7% and Jaeggi *et al.* (2006) observed a decline by 13%. Jaeggi *et al.* (2006) also found that a general relationship between LAI and ozone profiles does not exist, similar to forests; the main reduction in forests is below the crown region or the main LAI contribution (Jaeggi *et al.*, 2006). However, the display, as well as the amount and the distribution of the leaves, also affects light interception, and thus another important property of the canopy is the angular presentation of the leaves. The distribution of leaf area within the canopy defines where most o the light will be absorbed, and is defined by the leaf area density, the area leaf per unit volume of canopy space (Russel

et al., 1989). At 5-20 cm the denser canopy with the majority of planophile leaves only 30% of the light penetrated through the canopy, whereas at 20-30% with the majority of electrophile leaves 70% of the light transmited. Light plays a vital part in controlling stomatal conductance, hence this study points out how important measurements such as LAI, fractional LAI, PAR are in understanding the ozone exposure and the ozone flux within the canopy.

However, as plants develop from spring onwards, increasing in height and leaf area index, the degree of ozone penetration into the canopy changes so that successive leaves are exposed to different combinations of ozone, light, temperature and humidity. Such environmental changes alter stomatal conductance and therefore ozone flux. Stomata respond to environmental factors such as VPD and water stress; i.e. conductance and flux alter over time scales of hours or days independent of the ambient ozone above the canopy. Hence, it would be important to determine the relationship between ozone above the canopy and at each leaf surface (Finkelstein *et al.*, 2004).

Veit & Henning-Müller (2001) looked at the ratio of O_3 concentrations at 1 cm compared with those at 250 cm, and their results can be compared with those in this study. Both studies found that ozone levels at the base of the canopy, expressed as a percentage of those above the canopy, increased with humidity and decreased with temperature. However, while Veit & Henning-Müller (2001) found that the ratio increased with wind speed, this study found a decrease with atmospheric turbulence. In addition, Jaeggi *et al.* (2006) found that the turbulence intensity was the main environmental factor controlling in-canopy O_3 profiles. The relative O_3 concentration also showed a systematic dependence on the measured friction velocity for most of the canopy but the lowest part. In this study, the O_3 concentration of the lowest part of the canopy was neither much influenced by the friction velocity, the temperature nor the humidity. Besides higher air temperature, air humidity and friction velocity only had a small effect on the O_3 concentrations at the 20 cm level. In the current study the ozone profiles were only measured for a short term period (2 weeks), and during that period there was not much turbulence. Also, Jaeggi *et al.* (2006) measured

ozone concentrations and friction velocity during day and night, which showed predominantly medium to high values with more than 70% above 0.14 m s^{-1} during the night.

Effects of elevated ozone on the stomatal conductance of functional groups

Neither the stomatal conductance of *Rumex acetosa,* nor the stomatal conductance of *Holcus lanatus,* was significantly affected by the ozone treatment, although an effect of O_3 was found in Transect 3 for *H. lanatus.* However, a significant effect was observed for the stomatal conductance of *Trifolium repens*, both abaxial and adaxial. When both surfaces were combined, gs were significantly lower in the 25 ppb and the 75 ppb treatment than in the control treatment. When the same analysis was carried out comparing days with and without fumigation, no significant differences were found between the treatments. However, on the days with fumigation there was a trend of lower stomatal conductance in the elevated ozone plots, which was significant on the day with the highest measured O_3 concentration, i.e. when ozone concentrations reached 120 ppb.

In the OTC mesocosm experiment with *Briza media,* significantly lower values of stomatal conductance were found in the elevated ozone treatment. Comparing results before the cut (June/ July) and after the cut (September), significant effects of ozone were only found in the latter period, showing a reduced stomatal conductance in the 50 ppb treatment. Mean O_3 concentration in the elevated treatment before the cut was about 37 ppb, compared to 28 ppb in the ambient treatment, whereas after the cut mean O_3 concentration were 33 ppb in the elevated treatment and 18 ppb in the ambient treatment. Although the mean O_3 concentrations after the cut were relatively low, especially compared with the intermittently high concentrations at High Keenley Fell, they had a significant effect on the stomatal conductance of *B. media.* Wilbourn *et al.* (1995) found that cutting and ozone exposure affected yield and species composition of a two species mix of *T. repens* and *Lolium perenne*. As a result of cutting, canopy structure and related exposure along gradients of ozone were removed and species occupying lower canopy layers became exposed to higher ozone

levels and more light during the initial phase of regrowth. As *B. media* was now exposed completely to the full ozone exposure, this might have led to an increased stomatal closure trying to limit water loss. In contrast to other studies (e.g. Mills *et al.*, 2009; Reiling & Davison, 1995), the ozone concentrations used in this study probably were to low in order to affect the the stomatal apparatus of the plant negatively. However, significantly higher g_s values were observed before the cut in the 2nd chamber than in the other three chambers, but this chamber did not have an unusually high air temperature for instance. Furthermore, the difference in temperature is unlikely to be large enough to have such an effect, and also the the chamber results are not relevant for the findings from this study as they not confound the significant results.

Although there is an extensive literature on the effects of ozone on stomatal function, relatively few studies have considered the grassland species examined in this study. Mills *et al.* (2009) tested *L. hispidus*, *Ranunculus acris* and *Rumex acetosa* on the response of their stomata to a range of abscisic acid concentrations when exposed to elevated O_3. They all showed a less reduced transpiration with ABA in the O_3 treatment. This reduced ability of leaves of *R. acetosa* to limit water loss when exposed to higher O_3 concentrations. This observation was not consistent with the results in the current study, but this is also not relevant for the findings from this study as there was no water stress due to a very cool and wet summer. Higher O_3 concentrations have been reported to influence the stomatal closure apparatus negatively (e.g. Reiling & Davison, 1995; Mills *et al.*, 2009). Recent studies by Mills *et al.* (2009) showed that by even small increases (15- 40 ppb above ambient) in background O_3 concentrations, the stomatal conductance of *Leontodon hipidus* and *Dactylis glomerata* increased. The grassland mesocosms in the present study were already in their fifth year of treatment, whereas the species in the study by Mills *et al.* (2009) had been exposed to higher O_3 concentrations (range of 21.4-102.5 ppb) for a short term period (May-September in 2007), and their study was related to soil water stress. Another study, assessing the leaf conductance of semi-natural grassland species was done by Jaeggi *et al.* (2005). They

conducted a study on the effects of O_3 and soil water stress on leaf $\delta^{13}C$ and leaf conductance on plants in a semi- natural grassland in a free-air fumigation system for two years (2002-2003). They found that elevated O_3 led to reduced gs and increased $\delta^{13}C$ in *Holcus lanatus, Plantago lanceolata* and *Trifolium pratense* (but not in *Ranunculus friesianus*) from non-irrigated plots. The use of a ring-based free-air fumigation system instead of the gradient system used in this study meant that vegetation was exposed to ozone for a much greater proportion of the time, and the semi-natural grassland they used in their study had received high O_3 concentrations for about 5 years (1998-2003). In contrast, in this study the plants were exposed to higher O_3 concentrations for only two years, with the first year receiving O_3 for less than 50% of the time. Hence, the greater effects found by Jaeggi *et al.* (2005) may be explained by the higher and longer exposure in that study.

The lack of response to *H. lanatus* and *R. acetosa* in this study was thus not consistent with published literature, while no published studies could be found for *B. media*. The sensitive legume *Trifolium repens,* on the other hand, has already been shown to react to elevated O_3 concentrations by reducing stomatal conductance. Francini *et al.* (2007) exposed resistant and sensitive clones of clover to 200 ppb O_3 for 5 hours. The stomatal conductance of the resistant clones and of the controls increased by 73% whereas, that of sensitive clones decreased by 38% during the recovery period of 24 hours. Crous *et al.* (2006) undertook a similar study. They also exposed resistant and sensitive clones of *T. repens* to O_3 concentrations of 0- 95 ppb for 7 hours day^{-1} for five days. They found that the stomatal conductance of the sensitive clone decreased significantly in the elevated treatments after visible injury occurred. Degl'Innocenti *et al.* (2003) studied the elevated effects of O_3 (150 nl l^{-1} for three hours) on photosynthesis of *T. repens.* They observed an inhibition of the photosynthetic activity, an enhanced stomatal closure and an increase of the reduced state of the photosystem II primary acceptor. Although these three studies were only short-term studies and the O_3 concentrations were much higher than in this study, they are consistent with the impact of O_3 on *T. repens* found in this

study, which was also found on the release day when ozone concentrations were highest.

The elevated O_3 treatment and the nutrient treatment in the OTC's of the grassland mesocosm did reveal effects on the pH on 22nd July and 12th September (Appendix 3). While shortly after the cut there was a negative correlation between high ozone treatment and low nutrient regime and pH at 0- 10 cm, 2 ½ months later the pH was still influenced by the low nutrient treatment but not by the ozone treatment. But the pH showed a decrease in the ozone treatment and in the high nutrient treatment. Effects of ammonium concentration were only found for the nutrient treatment which was higher in the low nutrient treatment at 0- 10 cm in July, in September no effects on ozone and nutrients were observed. This may point to a change within the soil chemistry, although a significant decrease in pH could be due to other causes such as higher rainfall for instance. However, Holmes *et al.* (2006) observed a blockage of nitrogen mineralization in their study of a Northern temperate forest ecosystem under elevated carbon dioxide and O_3 concentrations in the Aspen FACE experiment for about four years. The reduced N- availability was due to an altered rate of litter production and not related to changes in the litter chemistry. As the mesocosm in this study were exposed for about five years to higher O_3 concentrations, and a reduced biomass of the key species *B. media* was observed, the soil chemistry might have changed indeed. But the soil conditions would need to be monitored in more detail in order to identify the real cause.

3.5 Conclusion

The results of this study suggest that any assessment of ozone damage based on modelled stomatal flux would need to take into account:

1. The gradient of ozone within the canopy. Current models are based on leaves at top of the canopy but for more sensitive species much of leaf material is found deeper in the canopy where ozone levels are significantly reduced. This study confirms two other studies in demonstrating the large gradients within dense grassland canopies in mid-summer.
2. The gradient of the light penetration within the canopy. Large differences between two heights were observed in this study, which has also been confirmed by two other studies.
3. The LAI gradient within the canopy. Lower LAI values were found rather close to the top of the canopy than lower within the canopy, as most of this area was occupied by forbs. Irradiance and spectral quality also affect stomatal conductance, which vary with canopy height, density and leaf area index (Davison *et al.* 2003).
4. The direct effect of ozone on stomatal conductance (g_s). This is considered in models for crops but only in terms of cumulative exposure, this study suggests short-term peak exposures could also be important.

However, the current study was limited in time and scale. If the results were to be used for detailed modelling, more within canopy monitoring of the most influencing parameters such as temperature, PAR, light, wind speed, LAI and O_3 concentration would be needed for a longer duration of time, since all measurements in this study were made two weeks before the harvest; i.e. earlier in the season, when the canopy was more open, the flux situation was probably different. Also the stomatal conductance studies would probably benefit from a longer experimental period. For instance, another year of elevated ozone treatments could show if the shown effects of this study would be consistent. Hence, ongoing studies will be necessary, particularly long- term studies.

4. Effects of ozone on a lowland mire

4.1 Introduction

All ecosystems described in English as a swamp, bog, fen, moor, muskeg and peatland are defined as mires (Gore, 1983), but are often used synonymously with peatlands (Heathwaite *et al.*, 1993). The major characteristics of natural peatlands include permanent water logging, development of specific vegetation, the consequent formation and storage of peat and the continuous (upward) growth of the surface. Peatland distribution, peat formation and storage are primarily a function of climate, which determines water conditions, vegetation productivity and the decomposition rate of dead organic material (Mitsch & Gosselink, 2000).

Mires are of special importance for the atmospheric trace gas composition as they play a dual role: a long-term sink for CO_2 and a significant source of CH_4 (Gorham 1991; Bartlett & Harris, 1993). The strength of peatlands as a carbon sink is determined by CO_2- fixation in photosynthesis and decomposition, and the carbon balance varies according to environmental conditions; e.g. drought decreases plant assimilation and may accelerate decomposition, and reduces CH_4 emissions, which could turn a peatland from a sink into a source of atmospheric CO_2 (Alm *et al.*, 1999).

Plants play a key role in the exchange of these gases, as the cover of vascular plants which contain aerenchyma. Aerenchyma is an airy tissue found in roots of plants, which allows exchange of gases between the shoot and the root. It contains large air- filled cavities, which provide a low-resistance internal pathway for the exchange of gases such as oxygen and ethylene between the plant parts above the water and the submerged tissues (Evans, 2004). It also controls the transport rate of CH_4 from peat to the atmosphere; otherwise the

CH_4 oxidation rate within the peat mainly determines the CH_4 efflux from sites with no transport in aerenchymatous plants (Schimel, 1995; King *et al.*, 1998). As background ozone concentrations can decrease plant photosynthesis and biomass production, and alter carbon allocation (Fuhrer *et al.*, 1997; Davison & Barnes, 1998; Andersen 2003), adverse effects of O_3 on peatland plants and ecosystems (e.g. on plant physiology and anatomy and hence indirectly affect related processes) could indirectly influence greenhouse gas exchange in peatlands with possible feedbacks on atmospheric gas composition (Rinnan *et al.*, 2004). This threat has significantly increased due to the doubling of tropospheric O_3 concentrations during the last century and is likely to further increase in the future because background concentrations of O_3 are expected to continue to rise (Volz & Kley, 1988; Vingarzan, 2004; West & Fiore, 2005; West *et al.*, 2006).

So far despite its global importance only a few studies have considered effects of O_3 on peatland vegetation (Gagnon & Karnosky, 1992; Potter *et al.*, 1996a,b; Rinnan & Holopainen, 2004), and even fewer have examined the effects of ozone on trace gas fluxes (Niemi *et al.*, 2002; Rinnan *et al.*, 2003, Mörsky *et al.*, 2008). In addition, the effects of O_3 on CO_2 and CH_4 flux and its underlying processes (e.g. CH_4 production and consumption), have not yet been thoroughly investigated, and there is very little data available on O_3 effects on wetland communities such as mires. It has been suggested that long- term ozone exposure may have an impact on carbon and nitrogen cycling, for instance through effects on carbon storage, litter quantity and microbial activity and through increased dark ecosystem respiration and methane emissions (for more detail review Section 1.8). Most studies reported to date on mire ecosystems have only been performed on a short- term basis (8- 10 weeks), and have not considered the consequences of long- term O_3 impacts which could be quite different.

Hypotheses

This study on the effects of O_3 on a mire mesocosms aimed to determine whether long-term O_3 exposure has a significant effect on carbon cycling. In a 3-year experiment, a mire community from a lowland-raised mire was exposed to ambient and elevated ozone concentrations in open-top chambers. Overall this experiment was set up to test whether:

i. Carbon cycling is altered through effects of O_3 on methane emission and dark ecosystem respiration;
ii. O_3 has an effect on gas fluxes through changes in plant species composition and plant carbon allocation; and
iii. Long- term O_3 exposure has an impact on carbon cycling through effects on carbon storage, litter quantity and microbial activity.

In order to obtain more in- depth information, this study focused on effects observed during the final summer of exposure (2007) and on a harvest carried out at the end of it. The work described in this chapter addressed the following specific hypotheses:

- Long- term O_3 exposure alters carbon cycling through effects of O_3 on dark ecosystem respiration and methane emission.
- O_3 exposure affects the above- ground biomass of the main species such as sedges and mosses.
- O_3 exposure has an impact on carbon cycling through changes in soil chemistry.
- Litter decomposition, litter quality and the associated microbial activity are affected by O_3.

4.2 Experimental design

4.2.1 Experimental site and establishment of mesocosms

The mire mesocosm study was set up in May 2005 at the University of Newcastle field station at Close House, Heddon-on-the-Wall, Northumberland, (National Grid reference NZ128659, latitude 54°59'N, longitude 1°48'W; elevation 30 m). This is the same site, and same OTCs used for the grassland experiment that was studied in Chapter 3.

The peat cores originated from the lowland-raised mire at Roudsea Wood and Mosses, Cumbria, UK. The plant community represented a M18 *Erica tetralix* mire community (NVC) which was dominated by *Sphagnum papillosum* and *Eriophorum vaginatum.* It included *Andromeda palifolia, Erica tetralix* and *Drosera rotundifolia.*

On 9th May 2005, cores form this site were transferred into PVC tubes (diameter= 24 cm, length= 35 cm) that were plugged watertight at the bottom (through plastic bags up to 15- 20 cm high, taped to the cores with duck tape). The tubes were provided with two layers of holes to maintain the selected water table level. These cores were placed in buckets (diameter 30 cm at bottom and 34 cm at top, 26 cm deep) which were filled with deionised water. The water table was maintained at 5 cm below the moss surface during the winter and at 10 cm during the summer growing season.

The cores were paired both in terms of the vegetation composition (i.e. even distribution of *E. vaginatum and S. papillosum* between treatments) and the soil conditions (i.e. same water table and same state of degraded *Sphagnum*), and equally distributed between two different ozone treatments in open- top chambers (OTCs). Overall there were eight replicate cores per treatment (2 replicates per chamber), with one treatment exposed to ambient ozone and the other to elevated ozone. The mesocosms were distributed among eight OTC chambers and placed in an area of the OTCs which ensured that they received rain water (Figure 74).

Figure 74: Lowland mire mesocosm, showing a peat core placed in a bucket filled with dionised water (Source: Dr. Toet).

4.2.2 Ozone fumigation

Initially, all OTCs (3.5 m max diameter by 3.3 m in height) were ventilated with non- filtered air (NFA) at a rate sufficient to achieve two air changes per minute in each chamber. From 10th May 2005, ozone was injected into the NFA supply of four of the eight chambers to attain the target ozone treatments. Ozone was generated by electric discharge through pure oxygen (model OZ2000 Biofresh Ltd., Ripponden, West Yorks, UK which incorporates a Sequal oxygen concentrator). Ozone concentrations were measured at canopy height in the centre of each OTC with a photometric analyser (Dasibi, Model 1008 UV Photometric Ozone Analyzer). The analyser was serviced weekly and calibrated monthly against a Dasibi 1008PC unit which in turn was calibrated against National Physics Laboratory standards every 6 months. The concentrations of ozone in individual chambers were checked against target concentrations daily by an on-site instrumentation technician, and where minor adjustments were required, the introduction of ozone to each chamber was

manually regulated *via* stainless steel needle- valved gap flow meters (Pontailler *et al.*, 1998; Barnes *et al.*, 1995).

The ambient ozone treatment consisted of non- filtered ambient air (control) and the elevated ozone treatment of targets of ambient +40 ppb from April – September 2005 and 2007, or +60 ppb from April – September 2006, and ambient +10 ppb from October – March 2006 and 2007. Ozone treatments were randomly assigned to chambers, and mire mesocosms were moved between duplicate treatment chambers every 4- 6 weeks (Figure 75). The ozone fumigation was carried out between 9 am and 5 pm seven days a week; for the remaining time ambient ozone concentrations levels were maintained. Target concentrations were met. The values of O_3 treatment during the different time periods are listed in Table 33.

Figure 75: Lowland mire mesocosms placed in open top chambers at the field station Close House, New Castle (Source: Dr. Toet).

Table 33: Ozone exposure data for all years (2005-2007); showing the 8 h and the 24 h mean, and the accumulated exposures over a threshold of 40 (AOT 40) of ambient and elevated treatments The standard error represents the variation between replicate chambers.

Time periods of fumigation	*12/05/2005 to 15/10/2005*	*16/10/2005 to 30/03/2006*	*31/03/2006 to 15/10/2006*	*16/10/2006 to 12/04/2007*	*13/04/2007 to 30/09/2007*
9 h mean [ppb]					
ambient	25 ± 0.5	24 ± 1.1	28 ± 0.7	30 ± 0.5	27 ± 0.7
elevated	64 ± 2	46 ± 8	80 ± 13	51 ± 13	66 ± 1
24 h mean [ppb]					
ambient	28 ± 1	24 ± 2	32 ± 1	30 ± 0	29 ± 1
elevated	44 ± 0.9	30 ± 0.3	50 ± 0.8	36 ± 0.5	39 ± 1.
AOT 40 [ppb h]					
ambient	646 ± 497	1270 ± 1190	3779 ± 265	290 ± 51	2412 ± 1948
elevated	54577 ± 2896	7116 ± 1173	93946 ± 3456	8856 ±652	66389 ± 17154

4.3 Materials and Methods

4.3.1 Overview of measurements

During the vegetative growth period from May to September 2007, a survey of the mire mesocoms at Close House was carried out. On five dates, at intervals of approximately four weeks (22/05, 27/06, 19/07, 20/08 and 18/09) the following measurements were undertaken.

1. Methane and carbon dioxide fluxes (dark ecosystem respiration) were measured;
2. Plant measurements, e.g. the length increment of *S. papillosum* and the shoot density of *E. vaginatum,* were carried out;
3. Soil water samples were taken at two different depths (0- 10 cm, 10- 20 cm) and analysed for pH, NH_4^+/NO_3^- and DOC, while the soil temperature was measured at - 9 cm and - 17.5 cm.

In addition to the monthly measurements, samples of *S. papillosum* were taken in August 2007 in order to determine capitulum dry weight, capitulum density, and membrane permeability was measured. For *E. vaginatum*, leaves were collected at the same time for assessing the leaf length, the leaf dry weight and the C/N content. Most of the stated methods were chosen from consistency with measurements carried out in previous years (2005 and 2006), which focused on changes in plant parameters, dark ecosystem respiration and methane emissions.

A litter experiment was carried out on 2nd October 2007 with litter of *E vaginatum* in order to study the effects of soil water and litter quality from ambient and elevated treatments on decomposition rates. Litter was collected from ambient and elevated treatments and was then infused with soil water, which also derived from ambient and elevated treatments and was then incubated in a dark chamber.

A final harvest was carried out at the end of the growing season (25th September); above-ground vascular plant species and soil samples were also

taken for NH_4^+/NO_3^-, and pH determination. The measurements made during the summer of 2007 are summarised in Table 34.

Table 34: List of measurements and sampling frequencies carried out in 2007

Measurements carried out monthly	*Additional measurements and their sampling days*	*Measurements at the end of growing season*
CH_4 and CO_2 fluxes (dark ecosystem respiration). *E. vaginatum* shoot density. *S. papillosum* length increment	Membrane permeability of *S. papillosum* (20/08)	Harvest of *A. palifolia, E. tetralix, E. vaginatum*
Soil temperature (2 depths (-9 cm and -17.5 cm)). Soil water samples taken at 2 depths (0-10 cm, 10-20 cm) for pH , NH_4^+/NO_3^- and DOC	Capitulum dry weight, capitulum density, C/N content of *S. papillosum* (20/08)	Soil samples for NH_4^+/NO_3^- and pH determination (soil extractions at 0-10 cm)
	Leaf length, leaf dry weight, C/N content of *E. vaginatum* (20/08))	
	Soil water sampled for litter experiment (18/09)	

4.3.2 Methane and carbon dioxide flux measurements

To evaluate the effect of ozone on CH_4 and CO_2 (dark ecosystem respiration) fluxes, gas samples were taken from the mesocosms after they had been enclosed for a short time period in cylindrical chambers (according to Niemi *et al.*, 2002). Gas samples were taken at five time intervals (t0, t30, t60, t90, t120), approximately every 25- 30 minutes. The chamber (OTC) air temperature and the sampling time were recorded every 30 minutes.

To begin the actual gas sampling, the peat cores were first enclosed in cylindrical chambers (diameter = 24 cm, length = 30 cm; grey PVC plate on top: 3 mm thick, with a vent at the top). These were sealed onto the mesocosms with a rubber band (inner tyre tube). In order to minimise the temperature increases during the 2 h period, each chamber was covered with aluminum foil. Through the vent at the top of the chamber, a Tygon tube with a septum was inserted. This consisted of: 1.5 m Tygon (Saint-Gobain, Akron, Ohio) tubing with i.d.: 1.6 mm and o.d.: 3.2 mm, through a rubber stopper (suba-seal, 14 mm neck, VWR Limited International LTD, Lutterworth, UK) (Figure 76).

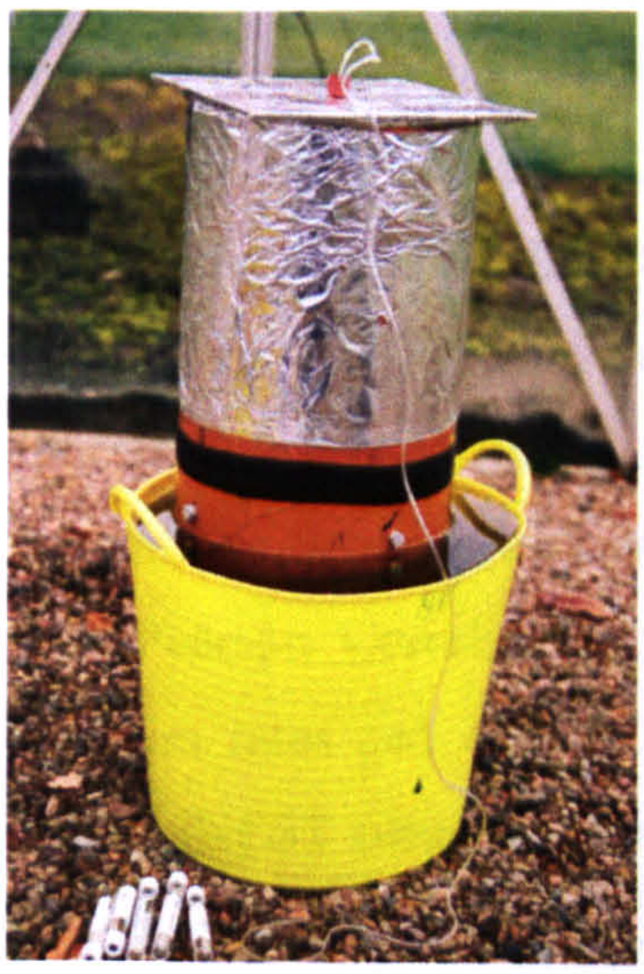


Figure 76: Gas flux measurements using cylindrical chambers (Source: Dr. Toet).

Before taking the first gas sample at t0, plastic stoppers were put in the core holes (which were there for water table adjustment). The tubing was then closed at the other end using a clamp, and a syringe was attached by inserting a needle into the tubing.

After attaching the syringe, a piston was pumped three times before withdrawing the actual sample. 20 ml of gas was sampled from the headspace. The sample from the 20 ml plastic syringe was then injected into a flushed and evacuated exetainer (flushed with N_2, 40 s per exetainer and evacuated 20 s per exetainer, 12 ml volume) from. The syringe was flushed three times with air before another gas sample was taken from a different mesocosm.

At the end of the measurements, core stoppers and chambers were removed again. Samples were transported back to York, and methane and CO_2 concentrations of the gas samples were measured within 4 days on a PerkinElmer-Arnel gas chromatograph (AutoSystem XL, PerkinElmer Instruments, Shelton, CT, USA) equipped with a flame ionisation detector and a 3.7 m Porapak Q 60/80 mesh column (N_2 carrier gas flow of 30 ml min-1; and injector, column and detector temperatures of 120, 40 and 350°C, respectively). Carbon dioxide was converted to CH_4 before detection by use of a Ni reduction catalyst. For each run, six reference standards (reference gas: 9.40 ppm N_2O, 103 ppm CH_4, 523 ppm CO_2 in N_2) were used, and during each day of measurement three blanks (N_2) were also included.

The CH_4 and CO_2 fluxes were calculated from the linear change in CH_4 and CO_2 concentrations over the 2h measurement period (slope of regression). Linear correlations between concentrations and time were first calculated. Samples with Pearson Correlation coefficients with an r^2 <0.9 were rejected. CH_4 emission rates and dark CO_2 respiration rates in ppm min^{-1} were calculated using LINEST (uses the "least squares" method to calculate a straight line that best fits the data, and then returns an array that describes the line). The CH_4 emission rates and dark CO_2 respiration rates were then converted to units of mg m^{-2} h^{-1} as follows:

CH_4/CO_2 flux [mg m^{-2} h^{-1}] = (CH_4 or CO_2 flux [ppm min^{-1}]*(1/molecular volume t°C [L mol^{-1}]*1000000)*(MW CH_4 or CO_2 *1000)*($volume_{cuvette}/area_{cuvette}$)*60

Where

$volume_{cuvette}$ = 13.123 L = volume of measurement chamber

$area_{cuvette}$ = 0.044 m2 = surface area of base of chamber

$MWCH_4$ = 16.04288 = molecular weight of methane

$MWCO_2$ = 44.098 = molecular weight of carbon dioxide

t°C = temperature at degree Celsius

4.3.3 Plant structural assessments

Eriophorum vaginatum green shoot density

To determine the *Eriophorum* shoot density, all live shoots of *Eriophorum* were counted and recorded at each measurement interval. At the end of the active growth period, the length of *Eriophorum* shoots was surveyed as well. Ten *Eriophorum* shoots per core were collected in paper bags, and their length was measured and recorded. Then they were dried at 80°C for 48 hours and their weight was recorded.

Sphagnum papillosum length increment

To assess the length increment of *Sphagnum*, special cranked stainless steel wires (diameter: 1.5 mm, length of total crank: 30 cm; 8 cm up vertical, 3 cm horizontal, placed 19 cm down into soil core) were used.

One end of the wire was pushed into the *Sphagnum* vertically (or parallel to the stems if these were not vertical). The horizontal section was level with the capitula, whilst a free end projected into the air (Clymo, 1970). The free ends, which were of known length, were used for growth measurements (Figure 77). Five crank wires were placed into each mesocosm at the start of the growing season.

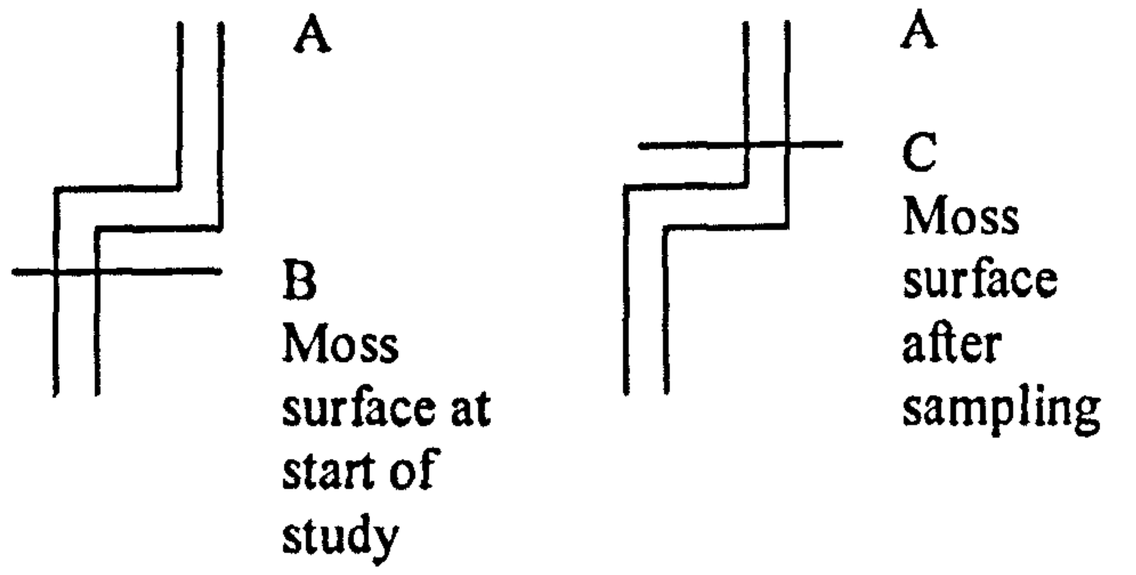


Figure 77: Use of crank wires as an estimation of linear growth of moss. A to B= top vertical portion of known length; A to C= top of wire that remains visible; difference AB and AC gives the growth rate.

On five measuring dates, the length increment of *S. papillosum* was measured using a precise glass tube which fitted exactly over the crank wires. Their length was recorded for each mesocosm. If the moss was about to overgrow the crank wires, the crank wires were partly pulled out and a new t0 length was noted.

In order to determine the total length increment over time, length increments were added to the sum of length increments from former measurements. Values were then averaged over the five crank wires used in each mesocosm on each date. For each date the mean values for each treatment were calculated, as well as the standard error.

S. papillosum capitulum biomass and grown shoot biomass

On the last day of measurements, the biomass of .*S papillosum* was estimated as follows. All (live) capitula of *S. papillosum* were counted and the numbers recorded. Additionally, ten capitula (0- 1 cm) and ten stems (1- 3 cm) per mesocosm were placed in pre- weighed aluminium foil pieces (1 per core) and

stored at -20°C. The samples were then freeze-dried and put in desiccators until their weight was determined.

To calculate the change in capitulum biomass, the capitula dry weight (dwt) was proportioned to 10 mm length of capitula, and the stem dry weight proportioned to 20 mm of length of stem.

The shoot biomass increase was calculated as the dwt of the stem multiplied by the length increment (proportioned to 20 mm length of the stem). The shoot biomass increase was corrected by a possible change in capitulum biomass, by subtracting capitulum dwt from the year before from the dwt capitula of this year.

Sphagnum biomass was calculated by multiplying the calculations above with the capitula density, which was expressed relative to the area of the cores (0.044 m^2) (Gehrke, 1998).

The Sphagnum production was calculated as follows:

Sphagnum production [g/ m^2] =

((dwt stem*(length increment/20) + (dwt capitula 2007 [mg]-dwt capitula 2006 [mg]))/1000) * capitula density

4.3.4 *S. papillosum* membrane permeability

The membrane permeability of *S. papillosum* was surveyed by taking one subsample of each mesocosm (according to methods of Niemi *et al.* (2002)). Four *S. papillosum* shoots (2 cm) per core were collected in small plastic bag and stored overnight in a fridge at 4°C.

A humidity chamber (a large transparent plastic box with wet tissues at the bottom and on the wall, soaked with deionised water, covered with cling film) was prepared and put in a controlled environment cabinet at 20°C. The *S. papillosum* shoots were put in the humidity chamber for two hours at 20°C in light conditions, with each sample placed in a different weighing boat.

Afterwards, the capitula (top 1 cm) were cut and quickly rinsed in deionised water to remove adhered particles and ions, and blotted on tissue paper. Then, three capitula from each sample were transferred to McCartney bottles filled with 9 ml deionised water and kept at 20°C for two hours. Three controls with only 9 ml deionised water were also prepared. The leachates were then transferred to storage tubes, and the conductivity in leachates and controls was measured. In order to avoid cross- contamination the conductivity of the deionised water was checked before and the lowest conductivity reference was used, two different beakers for the syringe and the meter were used while measuring, and the water was replaced after ten samples. Finally 45 µl concentrated HNO_3 (aristar) was added to each leachate and they were stored at 4°C until analysis.

To determine the total ionic contents of the capitula, 9 ml deionised water was again added to the bottles containing capitula, and they were autoclaved at 80°C for 1 hour in order to destroy cell membranes. The capitula were removed from the bottles when they cooled down to room temperature, and put in paper bags. The conductivity was measured in leachates and controls after autoclaving, as before 45 µl concentrated HNO_3 (aristar) was then added to each leachate and the leachates were stored in a fridge. The capitula were dried at 80°C for at least 48 hours and their dry weight was determined.

Potassium concentrations in the leachates were measured by atomic absorption spectroscopy (AA- 6300, Shimadzu). The leachates were diluted 1:1 with 10,000 ppm CsCl, to give final concentrations of 5,000 ppm CsCl in the diluted leachates. The leachate concentrations were expressed on the basis of the dry weight of the capitula samples.

4.3.5 %C, %N contents of plant tissue

For *E. vaginatum* eight leaves per core were collected. Samples were dried at 80°C for at least 48 hours and weighed. Afterwards the samples were ground with a mortar and pestle. These samples were then dried in at 80°C for at least 1 h and stored in a dessicator. Then the samples were weighed into tin capsules

(weight: 20-50mg) and analysed with a C/N analyser (Macro elementar, Analysensysteme). 50 mg of glutamic acid were used as a reference standard.

4.3.6 Plant harvest

On 25th September in 2007 the mesocosms were harvested. The vascular plants were cut just above the moss surface, then separated into live and senescent material and put in paper bags. Plant species were dried at 80° C for about 2 days and weighed.

4.3.7 Soil water sampling and soil temperature

The soil temperature was measured with a thermometer at -9 cm and -17.5 cm below the moss surface and recorded.

For soil water sampling, rhizon samplers were placed vertically in the soil of each mesocosm at 0-10 cm and at 10-20 cm below the moss surface (from the summer water table downwards). At each sampling date, the rhizon samplers were connected to 20 ml syringes. These were evacuated at the end of the day and the syringe contents were emptied into 30 ml plastic tubes for storage. Before taking the actual sample, 5 ml of soil water were discarded and the pH of the soil water samples (pH meter, Thermo Orion, model 420) was then measured and recorded. Afterwards, each sample was filtered (0.45 µm) and stored in a freezer (-20°C). Later, they were analysed for ammonium and nitrate concentration at an Autoanalyser (Autoanalyzer 3, Bran + Luebbe) and for dissolved organic carbon (DOC) content using a TOC -Analyser (Liqui TOC, Elementar Analysensysteme). All analyses were carried out in duplicates.

4.3.8 Soil harvest

On 25th September in 2007, soil cores were removed from the mesocosms. The soil cores were sliced in four parts and compared visually with each other in order to take a representative sample of each horizon. Soil samples were taken

for ammonium and nitrate- extraction from 0-10 cm. All samples were collected using a 100 ml beaker and placed in plastic bags. Samples for ammonium and nitrate- extraction were placed a fridge at 4°C until their extraction.

The roots were removed from the fresh soil samples. One sub- sample was spiked with water for pH determination. A second sub- sample was extracted with a 0.5 M solution of sodium chloride (17.5 g moist soil to 50 ml of solution). All samples were shaken for 1 hour at 120 rpm, and then extracted overnight using rhizon samplers which were connected to 60 ml syringes. An extra soil sample (10 ± 0.01 g) of each mesocosm was weighed into a pre-weighed crucible and placed in an oven at 105°C overnight in order to determine the soil water content. The next day, after cooling, they were put in desiccators and reweighed. The pH of the water sample extracts was measured and recorded on the same day as the soil solutions were extracted. All extracts were placed in a freezer at -20°C for storage. Later they were analysed for ammonium and nitrate using an Autoanalyser (Autoanalyzer 3, Bran + Luebbe) and for potassium concentration using an Atomic Absorption Spectrometer (AA-6300, Shimadzu). Samples for potassium analysis were diluted 1:1 with 10,000 ppm CsCl to give a final concentration of 5,000 ppm CsCl in the diluted samples (Rowell, 1994).

The ammonium, nitrate and potassium concentrations were expressed on a soil dry weight basis, calculated from the soil moisture contents and the fresh weights.

4.3.9 Litter experiment

At the end of September 2007 a respiration experiment was set up in order to assess the potential decomposition rate of plant litter. This was measured as the CO_2 emitted by micro-organisms associated with the organic material (Stumm & Morgan, 1981; Farina, 1998; Scheffer, 2001).

This experiment was conducted in a controlled environmental cabinet at York University. It used a factorial design, in which litter from the two treatments was incubated with soil water from each treatment. Litter samples of *E. vaginatum* and soil water samples were collected from the eight mesocosms per treatment. About 200 mg of litter was kept and dried at 80°C, and later analyzed for C/N ratio determination as described above. 500 mg of litter sub-samples were cut into 2.5 cm long pieces and remoistened (soaked for 24 h at 20°C in darkness), using 30 ml of pooled and filtered soil water (Whatman GF/C filter, pore size 1.2 µm) from the two different treatments. For each core, one part of the litter was soaked with soil water derived from the ambient treatment and the other with soil water from the elevated O_3 treatment. The soil water from each treatment was collected from each mesocosm and was pooled for each treatment.

The litter samples were then incubated in glass jars. To each 100ml glass jar, 8 ml of K_2SO_4 buffer solution was added in order to keep relative humidity (RH) constant at 97% at 20°C (120 g K_2SO_4 in 1L, buffer was kept at a pH of 4.3 using sodium acetate and HCl), and three layers of glass beads (8 mm) was added. In the end two gauzes were placed above the buffer solution, on top of the glass beads. The litter was put on top of the gauze and the jars were then placed in a dark climate room at 20°C and 97% RH. Three blank jars were prepared, containing everything but the soaked litter (Figure 78).

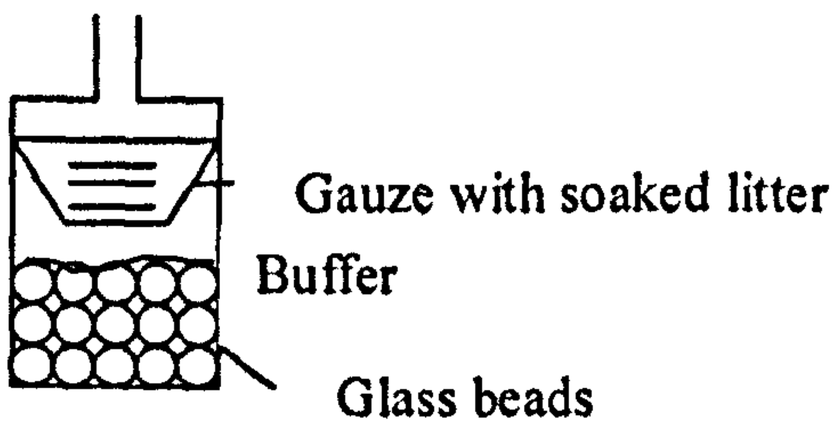


Figure 78: Glass jar used in the litter experiment.

The day before the measurements, the litter was remoistened with about 15 drops of deionised water. The next day, the incubation jars were closed by a silicon septum before taking samples. At first, 10 ml of air was injected into the glass jar with a 10 ml syringe, and then 3 ml of gas were sampled and the time (t0) was recorded. The jars were kept closed for 4 h (which was later extended to 6 h), and then another sample of 3 ml of gas was taken and the time was recorded (t4 or t6). Afterwards the jars were opened, and put back in the dark chamber. All samples were measured with an Infra Red Gas Analyser (IRGA), with three reference gas samples (375 ppm CO_2) every ten samples. The data output from a paper recorder which was connected to the IRGA during the analysis was converted into vpm (volume per minute), based on the output data from the reference gas.

The overall carbon dioxide production rate ($\mu g g^{-1} h\text{-}1\ CO_2$) was calculated as the difference in the absolute amount of CO_2 in the jars between t0 and t4 or t6, whereby both the amount of CO_2 in the headspace and in the buffer of the jar had to be calculated, as follows:

1. CO_2 ppm calculation of the headspace with removal correction (at t0 10 ml of air were added and one sample of 3.5 ml of the diluted head space were removed; at t4 another sample of 3.5 ml of the diluted head space were taken away).

Removed at t0 (Rt0): (0.0035/Vhs +0.0035) * Ct0
Removed at t4 (Rt4): Ct4+Rt0

Whereby
Ct0 [vpm] = concentration of CO2 in the headspace at t0
Ct4 or Ct6 [vpm] = concentration of CO2 in the headspace at t4 or t6
Rt0 = 3.5 ml gas sample removed at t0 (added 10 ml of air before)
Rt4 or Rt6 = 3.5 ml gas sample removed at t4 or t6
Vhs [L] = volume of the headspace of the jars

2. Conversion of CO_2 ppm to µg CO_2 g^{-1} DW litter of the headspace (Mhs)

Mhs = (Rt4-
Rt0)*((MWCO_2*100000*(Vhs+0.0035)/ ((1000000*(0.08207*(273.15 +t°C))*
DW litter))

Whereby
Mhs = conversion of CO_2 ppm to microgram CO2 per g dw litter in headspace
MWCO_2 = 44.0098 g mol^{-1}

3. Calculation of µg CO_2 g^{-1} DW litter in K_2SO_4 buffer solution

M_{K2SO4} = (Rt4-Rt0)*((MWCO_2*100000*V_{K2SO4}*(p*(KH/α°))/ ((1000000*DW
litter))

Whereby
α° = the fraction of total inorganic carbon dissolved in the buffer ($[H^+]^2$/(K1*
$[H^+]+[H^+]^2$))
M_{K2SO4} = calculation of microgram per g dw litter in K_2SO_4 solution/buffer
KH at 18 = 0.028841458 (Henry's constant (0.0223 mol l-1 atm-1 @20°C -
ionic strength 2,353 M))
pH = 4.3
Pt = atmospheric pressure [1 atm] =1
α° = 1

4. Calculation of CO_2 flux [µg $g^{-1}h^{-1}$]: (Mhs +M_{K2SO4})/T [h]

Whereby
CO_2 flux = µgram per gram per hour
T = time [h]

4.3.10 Statistical analysis

Statistical analysis just focused on 2007 data. Data compilation was carried out using Microsoft Excel (2003) and the statistical analysis was performed using SPSS 15.0. Each dataset was first explored via histograms, q-q-plots and boxplots. Examining the datasets for skewness, kurtosis and normality with the Shapiro-Wilk-test showed that most of the data needed to be transformed. The data was log10 or square-root transformed in order to gain normality. The transformed data were then tested with parametric tests. A generalized linear model with repeated measurements was applied to compare the means and to determine significant differences between the two treatments along five or six different time periods. Homogeneity of variance was assessed via the Levene-test, and the significance of contrasts was tested with the Bonferoni- test. Differences were tested for significance at $P< 0.05$, unless the outcome of the Levene- test was inhomogeneous, in which case the P-level was set to $P< 0.01$.

In the special case of *Eriophorum vaginatum* biomass, a univariate model (UNIANOVA) was employed, using the green leaf density of *E. vaginatum* as a covariate with the significance tested in the same way as for the RM- ANOVA. To compare the means, and to determine significant differences between the two treatments on specific dates, a simple independent t-test was used. The significance was tested in the same way as listed above.

The data for membrane leakage, soil water temperature, nitrate, and ammonium and potassium concentrations of the soil extracts did not reach normality despite transformation; therefore these datasets were tested using non-parametric tests. Treatment effects were tested via the Kruskal- Wallis test, and in order to test the significance of specific contrasts the Mann-Whitney-test was applied. For correcting for Type 1 errors, and for determining significant differences, a Bonferoni adjustment of $P< 0.025$ (two observations) or $P< 0.0125$ (four observations) was used.

4.4 Results

4.4.1 Trace gas fluxes

Figure 79 summarises the results for CH_4 emissions over a 3-year period (2005-2007). Overall there was a significant effect of time, showing that effects differed between measurement dates, but no significant effect of the O_3 treatment was found during the summer of 2007 (Table 35).

A significant decrease in CH_4 fluxes due to ozone was found in the summers of 2005 and 2006, but not during the winter (Toet, pers. Comm.). The CH_4 fluxes were in general lower throughout the summer of 2007 compared with previous summers, but the difference between high and low ozone treatment was less pronounced in this year compared to the two previous years. Whereas the methane emission increased by 33.8% (elevated) and 24.2% (ambient) between the years of 2005 and 2006, there was an decrease of 53% (elevated) and 53.9% (ambient) in the methane emissions between the years of 2006 and 2007.

The summer of 2007 was cool and wet, and additionally the level of ozone exposure was about 15% lower than in 2006 but about similar to the ozone exposure in 2005 (see Section 4.2).

Table 35: Results of the RM- ANOVA of effects of ozone and time on methane and carbon dioxide fluxes. The effects of ozone, time and the interaction of ozone and time are shown. Post-hoc differences are significant at $P < 0.05$.

	Effect of time	*Effects of treatment*	*Time*treatment*
CH_4	**0**	0.86	0.352
CO_2	**0**	0.059	0.193

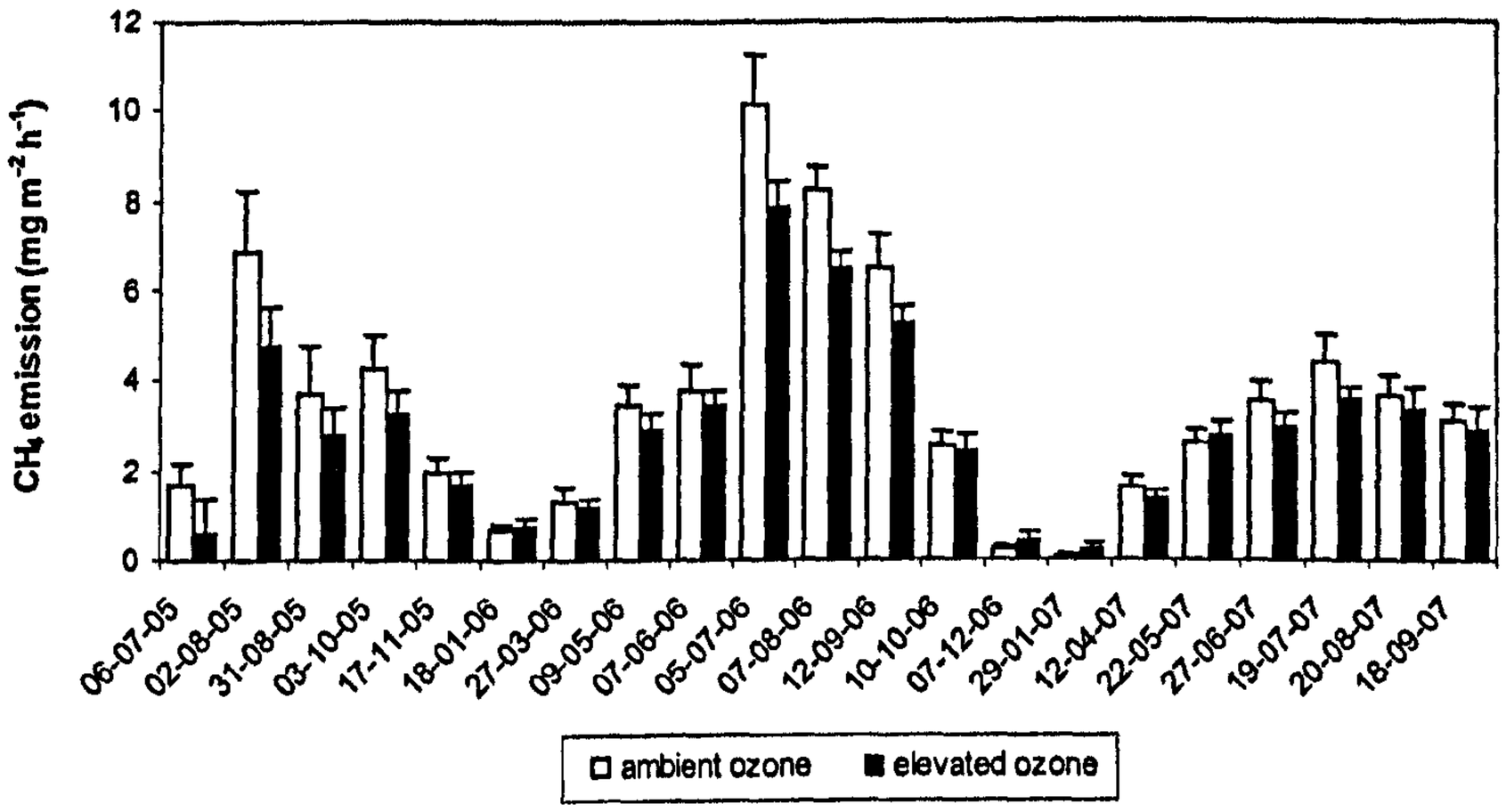


Figure 79: Changes over time in CH_4 emissions from mesocosms treated with ambient and elevated ozone. The error bars represent the standard error of 8 mesocosms.

In general no significant ozone effects on CO_2 fluxes were found during the summer of 2007 (Figure 80, Table 35). The CO_2 fluxes were higher in the summer of 2006 than in the previous year, and the following summer, and they stayed low during the winter months. The dark respiration increased by 38.3% (elevated) and 44.4% (ambient) between the years of 2005 and 2006, whereas it decreased by 19.2% (elevated) and 16.9% (ambient) between the years of 2006 and 2007.

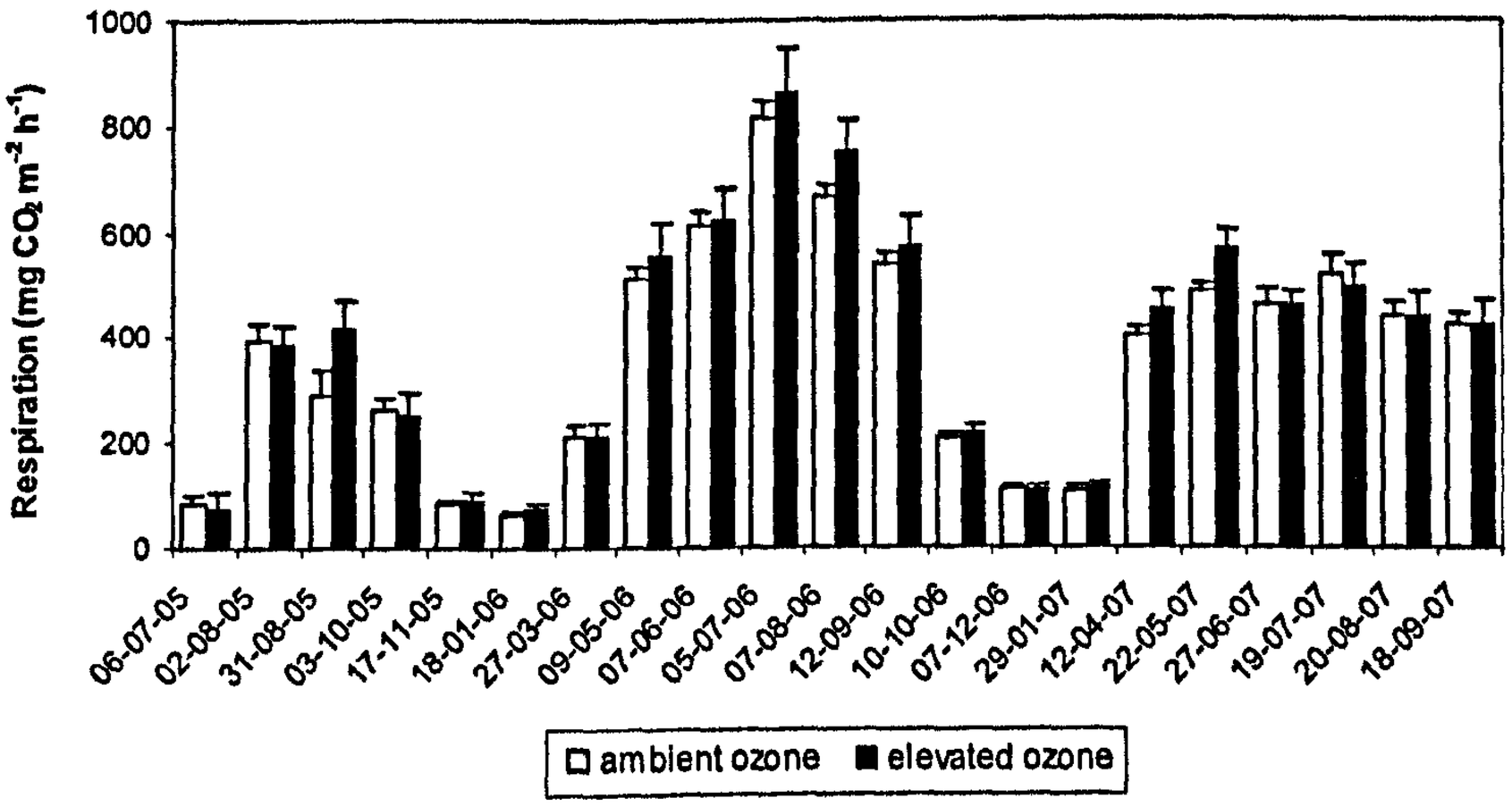


Figure 80: Changes over time in dark respiration values from mesocosms treated with ambient and elevated ozone. The error bars represent the standard error of 8 mesocosms.

4.4.2 Plant assessment

4.4.2.1 Growth and Biomass of *S. papillosum* and *E. vaginatum*

Sphagnum papillosum showed an expected increase in length increment over the summer months, but ozone did not significantly affect the extension growth (Table 36, Figure 81). Neither did the green leaf density of *Eriophorum vaginatum* indicate any significant effects of O_3 during the summer of 2007 (Table 37, Figure 82).

Table 36: Results of the RM- ANOVA of effects of ozone and time on length increment. The effects of ozone, time and the interaction of ozone and time are shown. Post- hoc differences are significant at $P< 0.05$.

Length increment	*Effect of time*	*Effects of treatment*	*Time*treatment*
Sphagnum papillosum	**0.000**	0.889	0.694

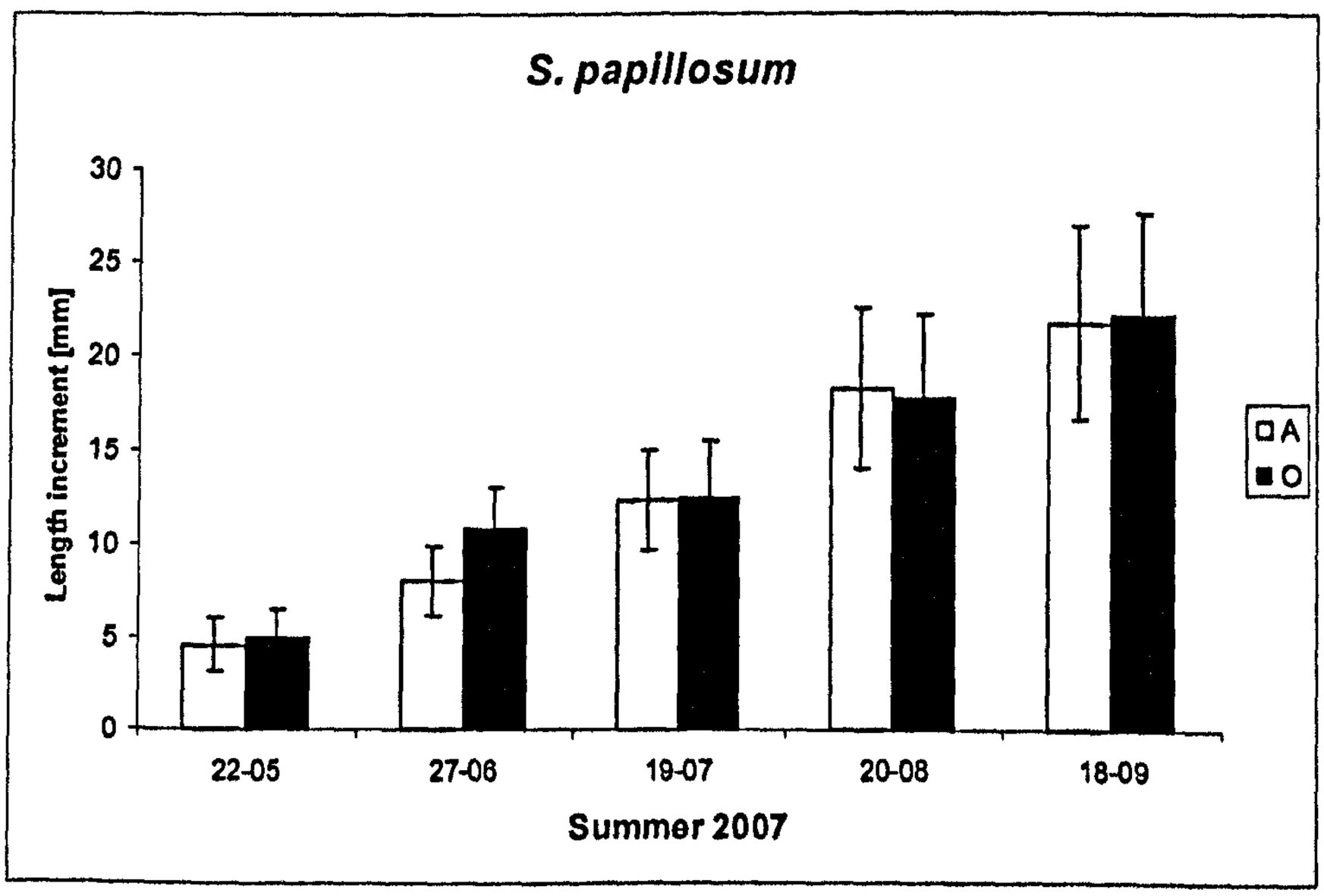


Figure 81: Variation over time in extension growth of *S. papillosum* treated with ambient (A) or elevated (O) ozone. The error bars represent the standard error of 8 mesocosms.

Table 37: Results of the RM- ANOVA of effects of ozone and time on green leaf density. The effects of ozone, time and the interaction of ozone and time are shown. Post-hoc differences are significant at $P< 0.05$.

Green leaf density	*Effect of time*	*Effects of treatment*	*Time*treatment*
Eriophorum vaginatum	0.347	0.932	0.176

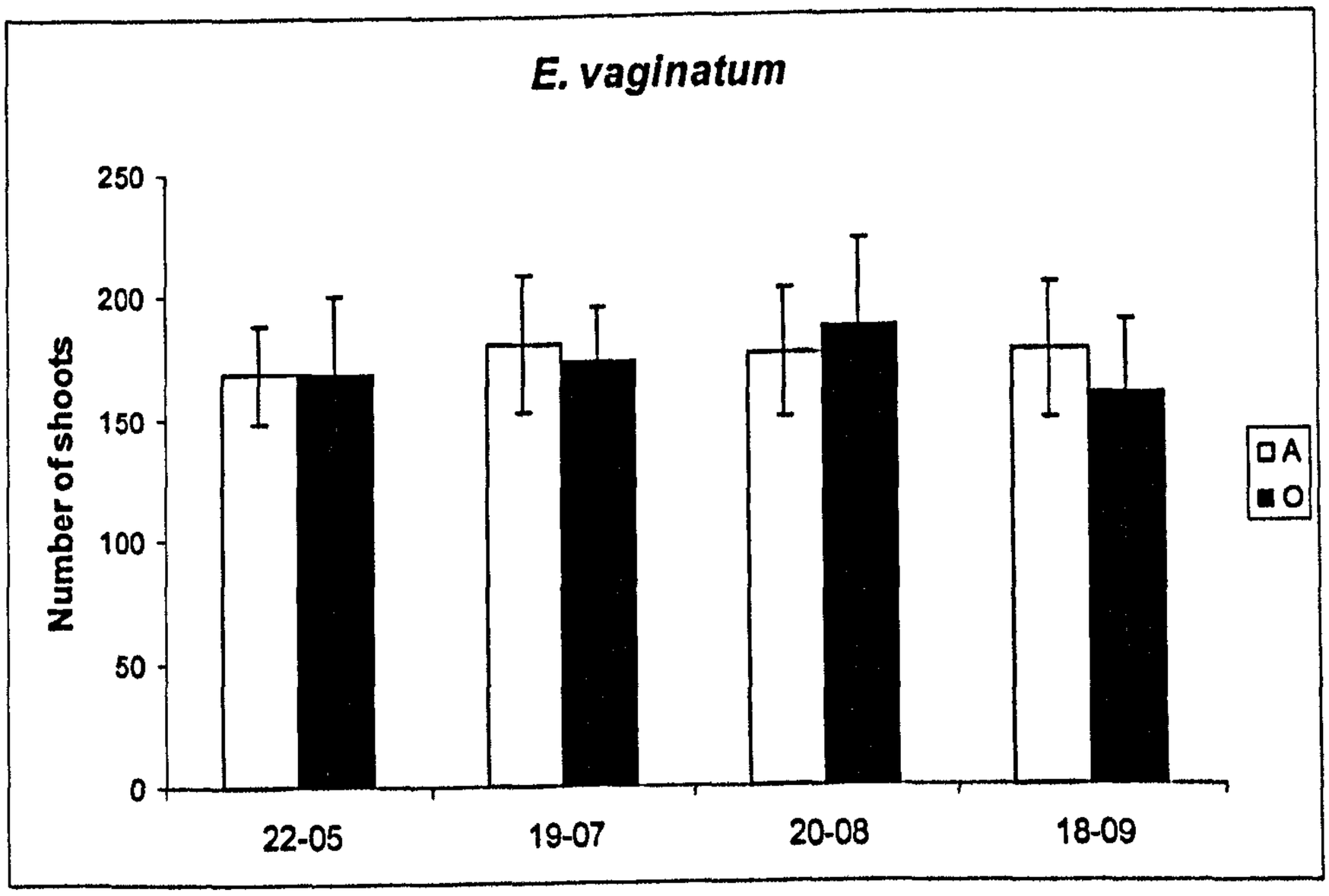


Figure 82: Variation over summer 2007 in the shoot density of *E. vaginatum* treated with ambient (A) or elevated (O) ozone. The error bars represent the standard error of 8 mesocosms.

Figure 83 (a-d) summarize the development of the capitula density, the individual capitula dry weight, the grown shoot biomass and the biomass production of *S. papillosum* over the period 2005-2007. All indices show an increase in the second year. In comparison to the results from 2006, in the year 2007 hardly any increase of the capitula density was found, and the shoot biomass increase even decreased. However, the biomass production increased,

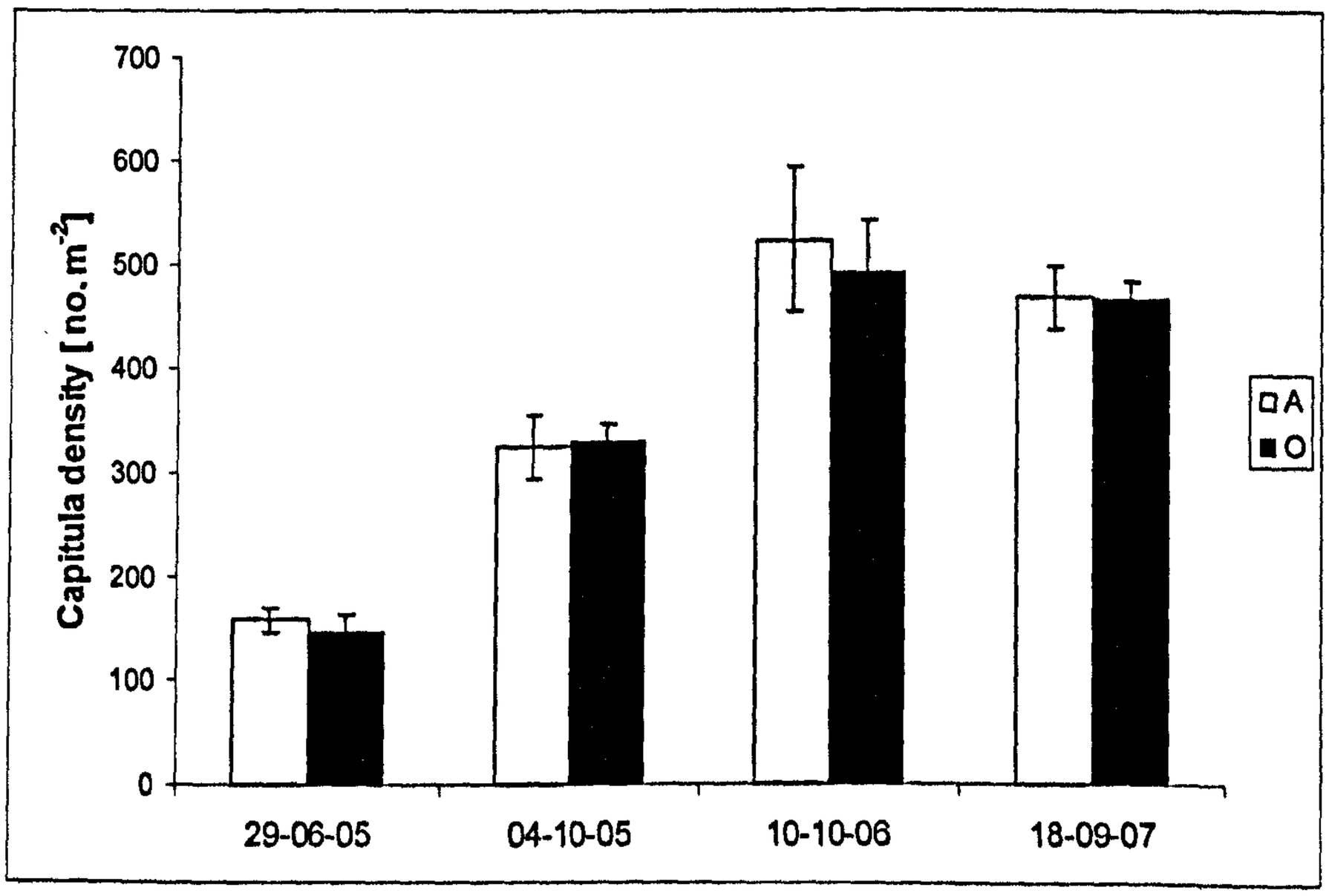
which was probably due to an increase in the capitulum dry weight. No significant effects of ozone were found (Table 38).

Table 38: Results of independent non- parametric samples test of effects of ozone treatment in the summer of 2007 on capitula density, the individual capitula dry weight, the grown shoot biomass and the biomass production of *S. papillosum*. P-value is significant at $P< 0.05$.

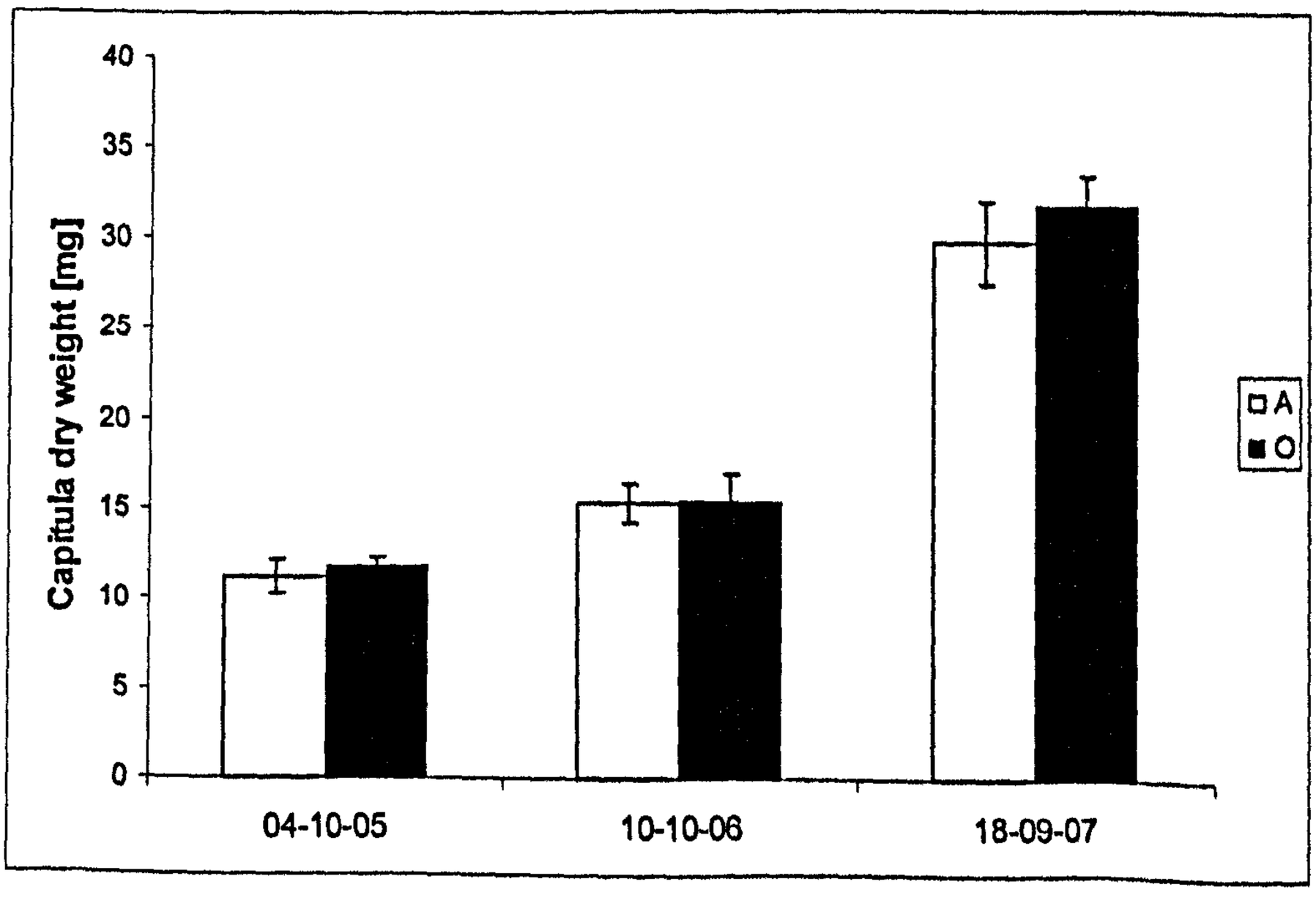
S. papillosum	***P***
Capitula density	0.43
Capitula dry weight	0.248
Shoot biomass increase	0.834
Biomass production	0.674

Figure 83 (a-d): Growht of *S. papillosum* treated with ambient (A) or elevated (O) ozone over the period 2005-2007. Error bars represent standard errors.

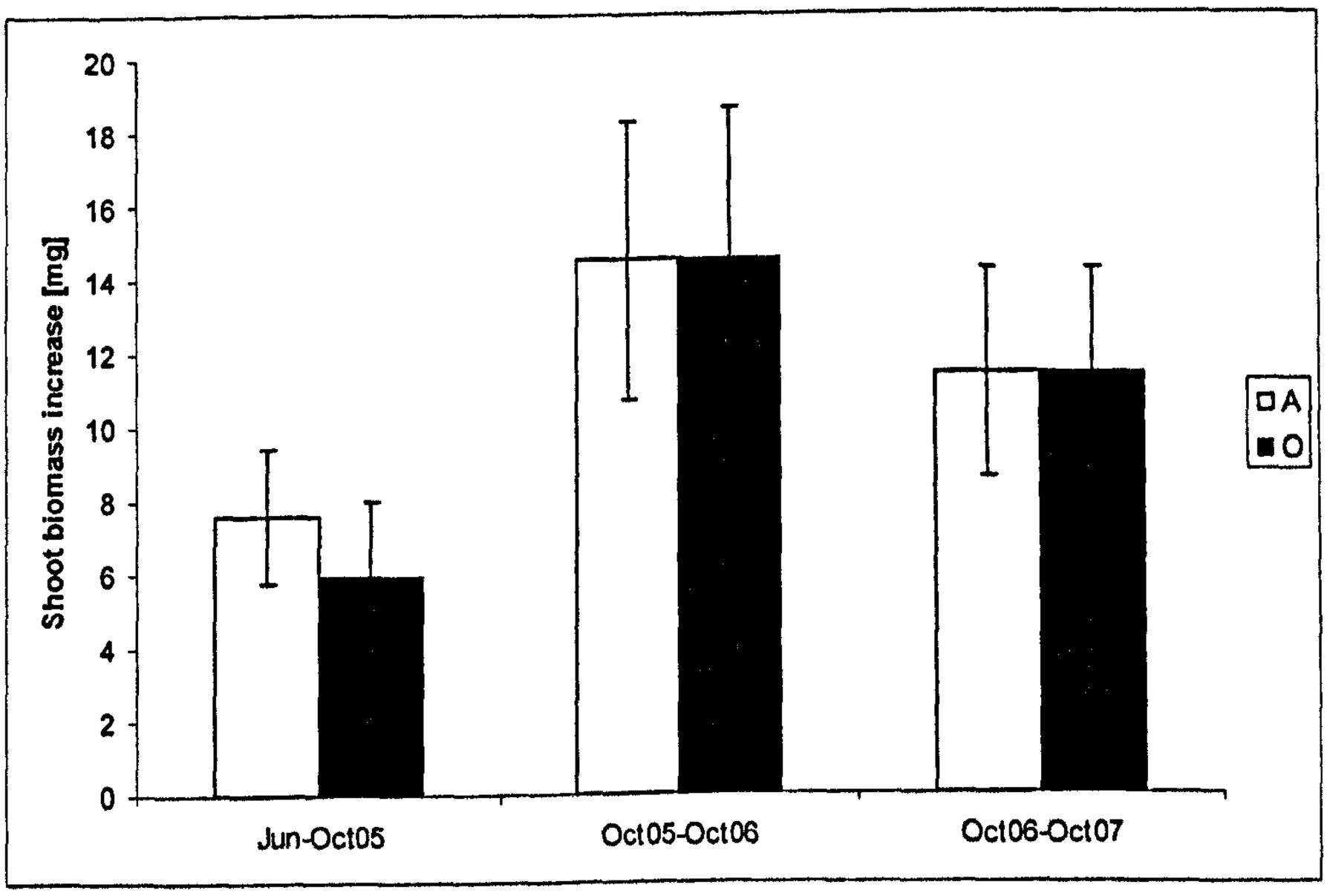
a) Capitula density



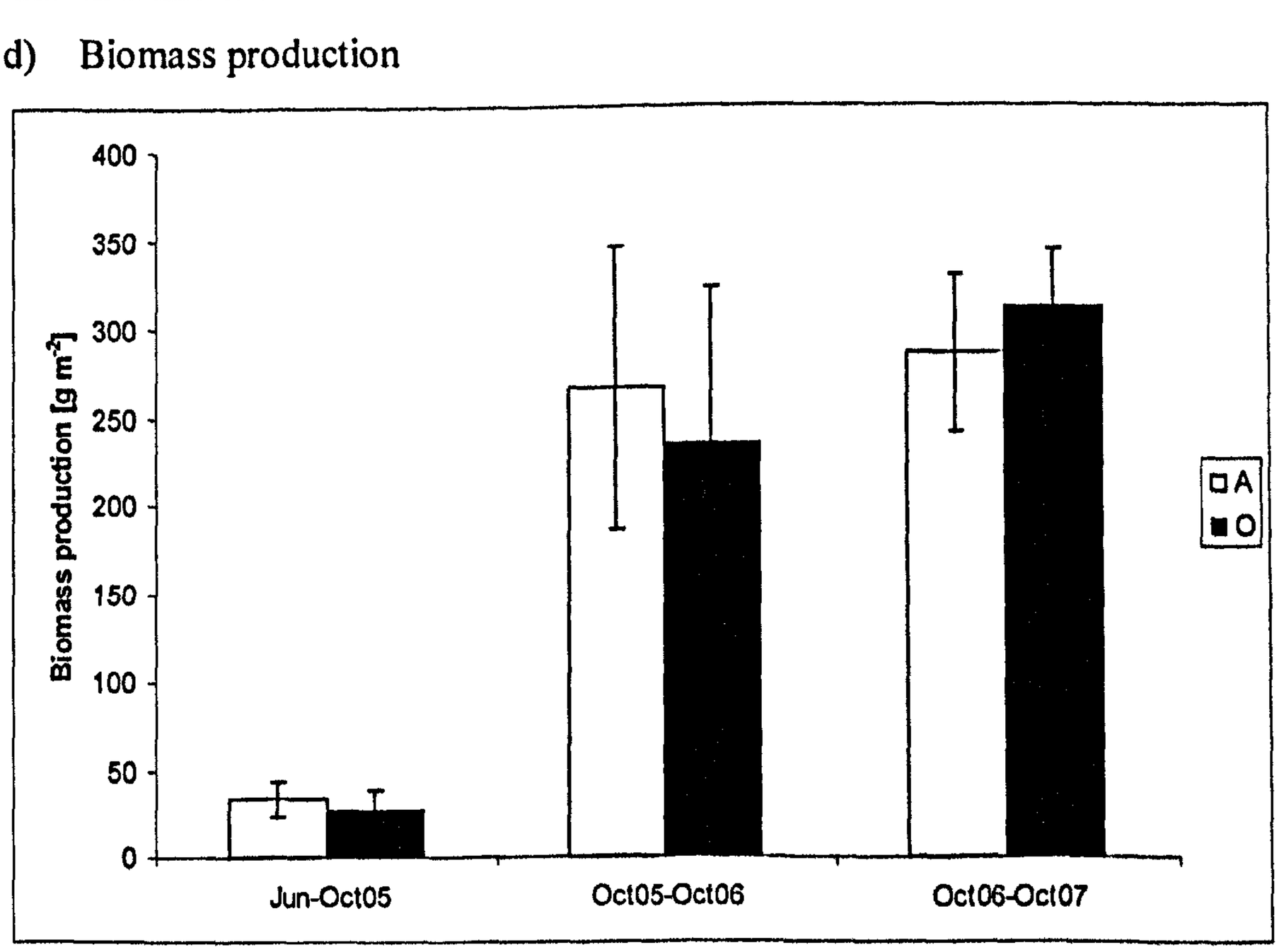
b) Capitula dry weight



c) Shoot biomass increase



d) Biomass production



4.4.2.2 Membrane permeability of *S. papillosum*

The membrane permeability showed a different pattern in 2007 than in 2006 (Figure 84, Figure 85). The membrane permeability of the water leachates showed no significant effects in 2006, and did not do so in 2007 (Table 39). Nonetheless, the permeability increased in 2007 in both treatments. The membrane permeability of the total leachates did not show an overall increase compared to the results above. The K^+ concentrations for total leachates were significantly lower in the elevated treatment than in the ambient treatment (P= 0.027) but not on the conductivity. In addition to that these results must be interpreted carefully as the effect is small and according to a Bonferoni correction, this seems to be more a trend. Nonetheless, the size effect was about 38% lower in the O_3 treatment, and there was an effect in water leachtes, even if data were very variable.

Table 39: Independent non-parametric samples test of effects of ozone treatment on membrane permeability and conductivity in 2007. P- value is significant at P< 0.05.

Parameter	*Leachate water*	*Leachate total*
K^+ concentrations	0.563	**0.027**
Conductivity	0.674	0.248

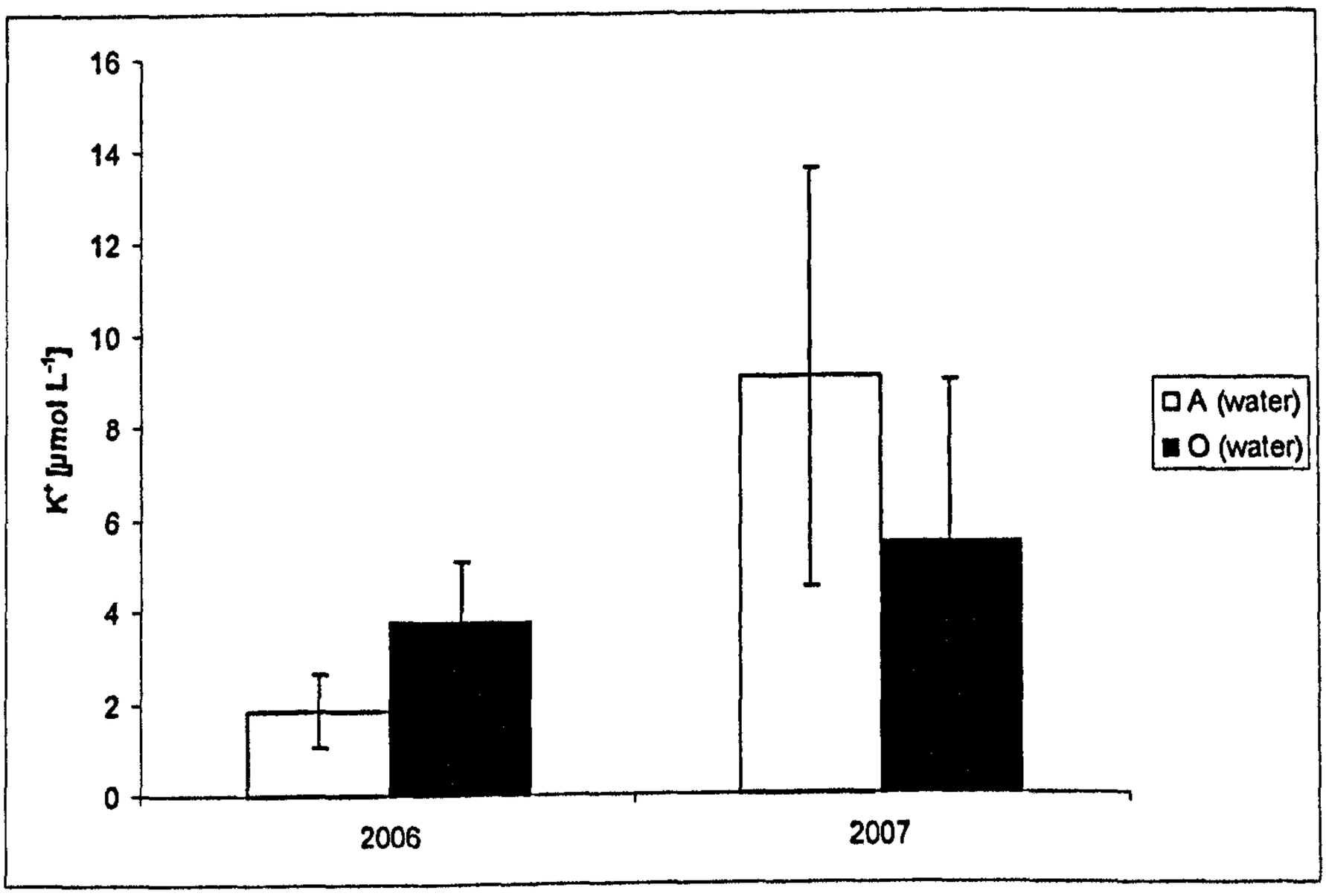


Figure 84: The potassium concentration [µmol l^{-1}] of the membrane permeability water in ambient air and O_3 enriched air of 2006 and 2007.

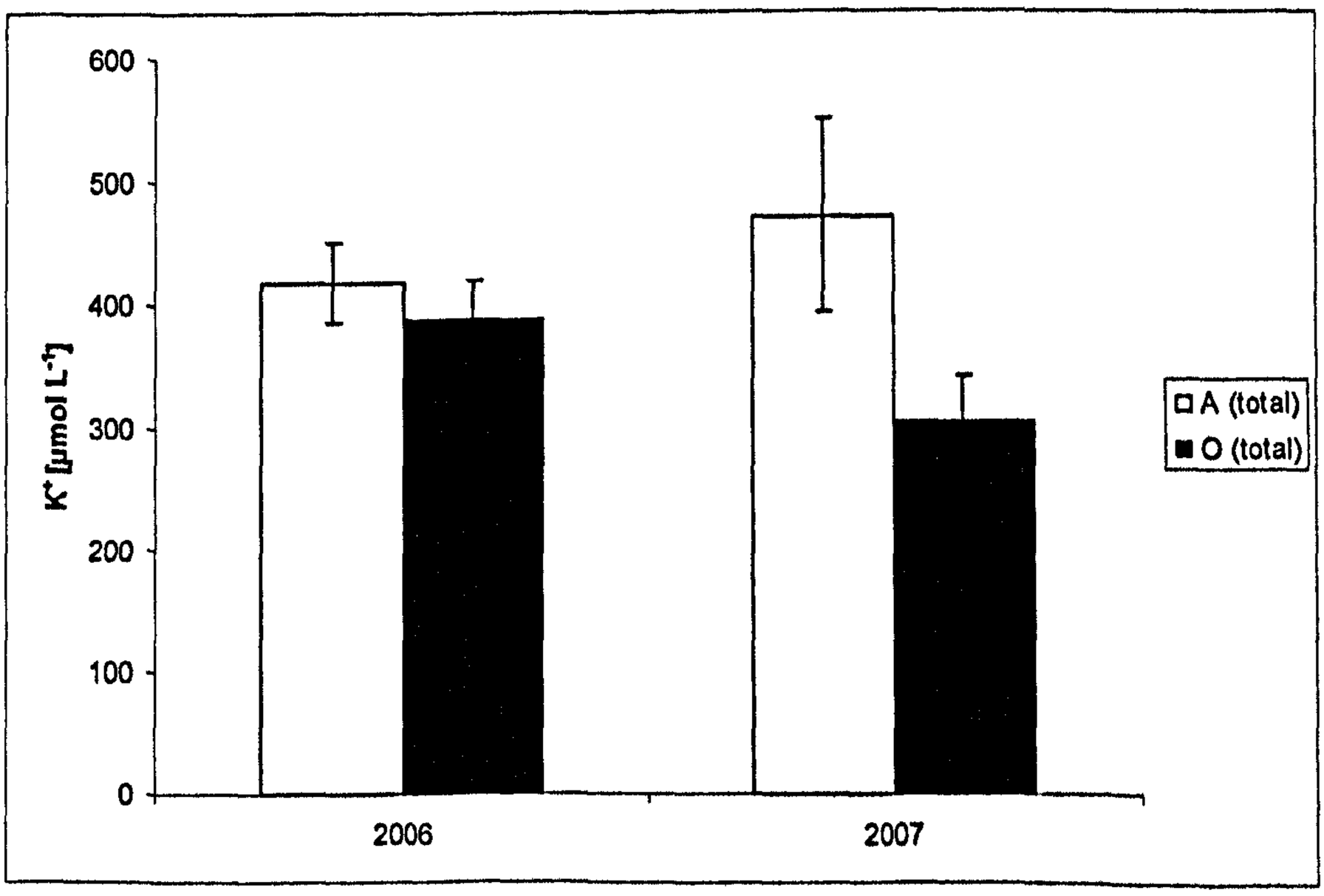


Figure 85: The potassium concentration [µmol l^{-1}] of the total membrane permeability water in ambient air and O_3 enriched air of 2006 and 2007.

4.4.2.3 Vascular plant growth

The above-ground live and senescent biomass of vascular plants at the end of the experiment is shown in Figure 86. *E. vaginatum* showed a significant decline in its live biomass when exposed to elevated ozone, but no effect on senescent biomass.

The biomass of *E. vaginatum* was reduced significantly by 28% (P= 0.036) in the elevated ozone treatment compared to the ambient treatment (Table 40). *Ericacea tetralix* and *Andromeda palifolia* showed greater biomass in the elevated ozone treatment. The biomass of *E. tetralix* was significantly greater in the elevated treatment than in the ambient treatment (P= 0.037), but the biomass of *A. palifolia* was not significantly affected by the ozone treatment. The effect on senescent biomass was negligible.

Table 40: Results of the UNIANOVA of effects of ozone on vascular plant biomass. The effects of ozone on the live and the senescent biomass of *E. vaginatum*, *E. tetralix* and *A. palifolia* are shown. P-levels are significant at P< 0.05.

Biomass	***Effects of treatment live biomass***	***Effects of treatment on senescent biomass***
E. vaginatum	**0.036**	0.147
E. tetralix	**0.037**	n.a.
A. palifolia	0.438	0.198

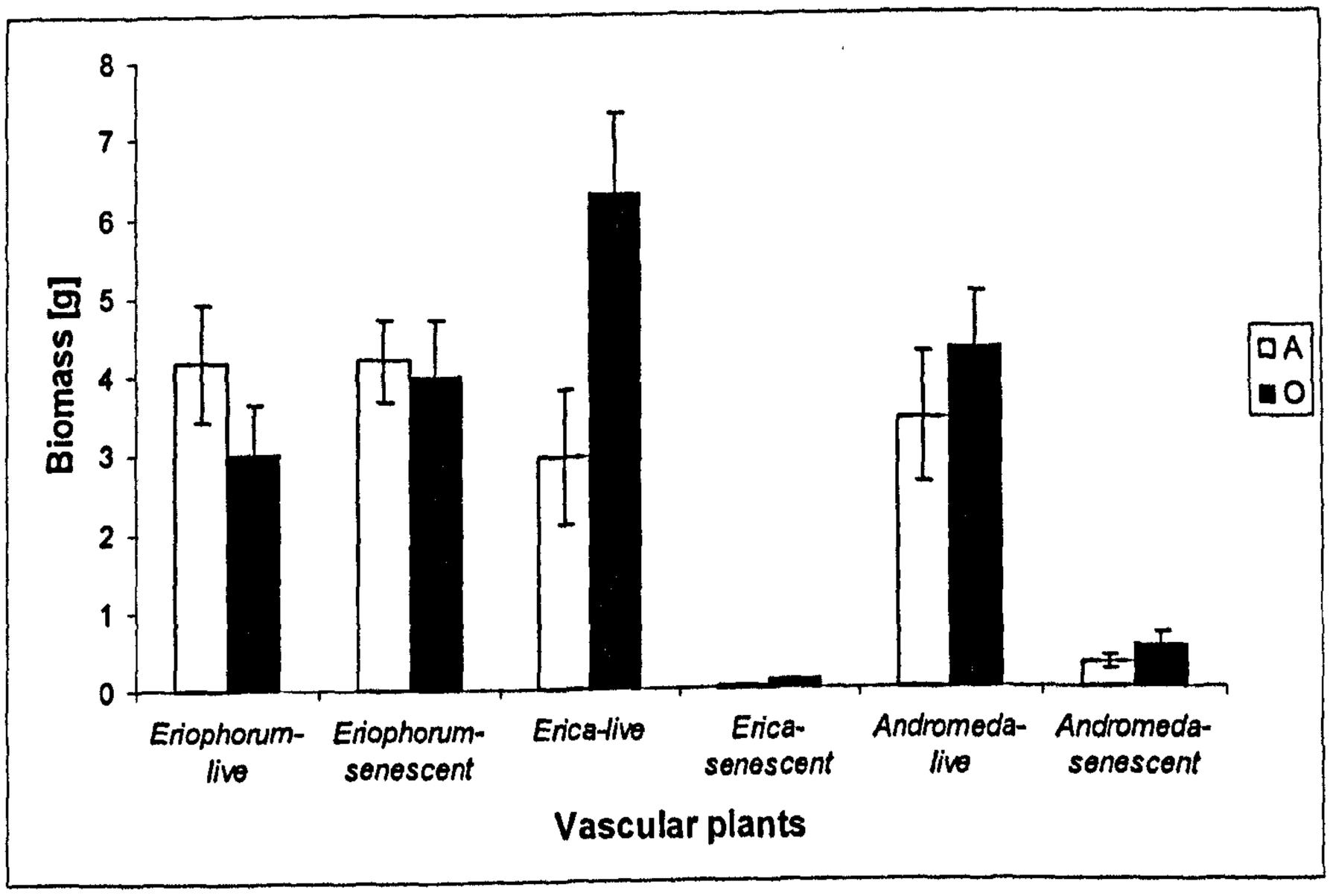


Figure 86: Mean above-ground live and senescent biomass of each species at final harvest in ambient (A) or elevated (O) ozone. Error bars represent standard errors.

4.4.3 Soil assessment

4.4.3.1 Soil temperature

The soil temperature and the pH at both depths followed a similar pattern over the growing season (Figure 87, Figure 88).

At 9 cm depth significant effects were found for the measurements on 18/09, with the temperature in the elevated treatment being significantly lower (about 1.5%) than the ambient treatment ($P = 0.023$). At 17.5 cm depth, significant effects were observed for the measurements of 18/09 and 27/06. On 18/09 ($P = 0.019$) the elevated treatment showed significantly lower soil temperatures (about 1.2%) than the ambient treatments, whereas on 27/06 ($P = 0.02$) about 3% significantly higher soil temperatures were found in the elevated treatments (Table 41, Figure 87). A RM- ANOVA was not carried out due to the not normally distributed soil temperature data.

Table 41: P- values from the independent non-parametric samples test of effects of ozone on soil temperature at 9 cm and at 17.5cmin 2007. Values in bold are significant at $P < 0.05$.

Soil temperature	***9 cm***	***17.5 cm***
22/05	0.11	0.204
27/06	0.069	**0.020**
19/07	0.915	0.913
20/08	0.435	0.373
18/09	**0.023**	**0.019**

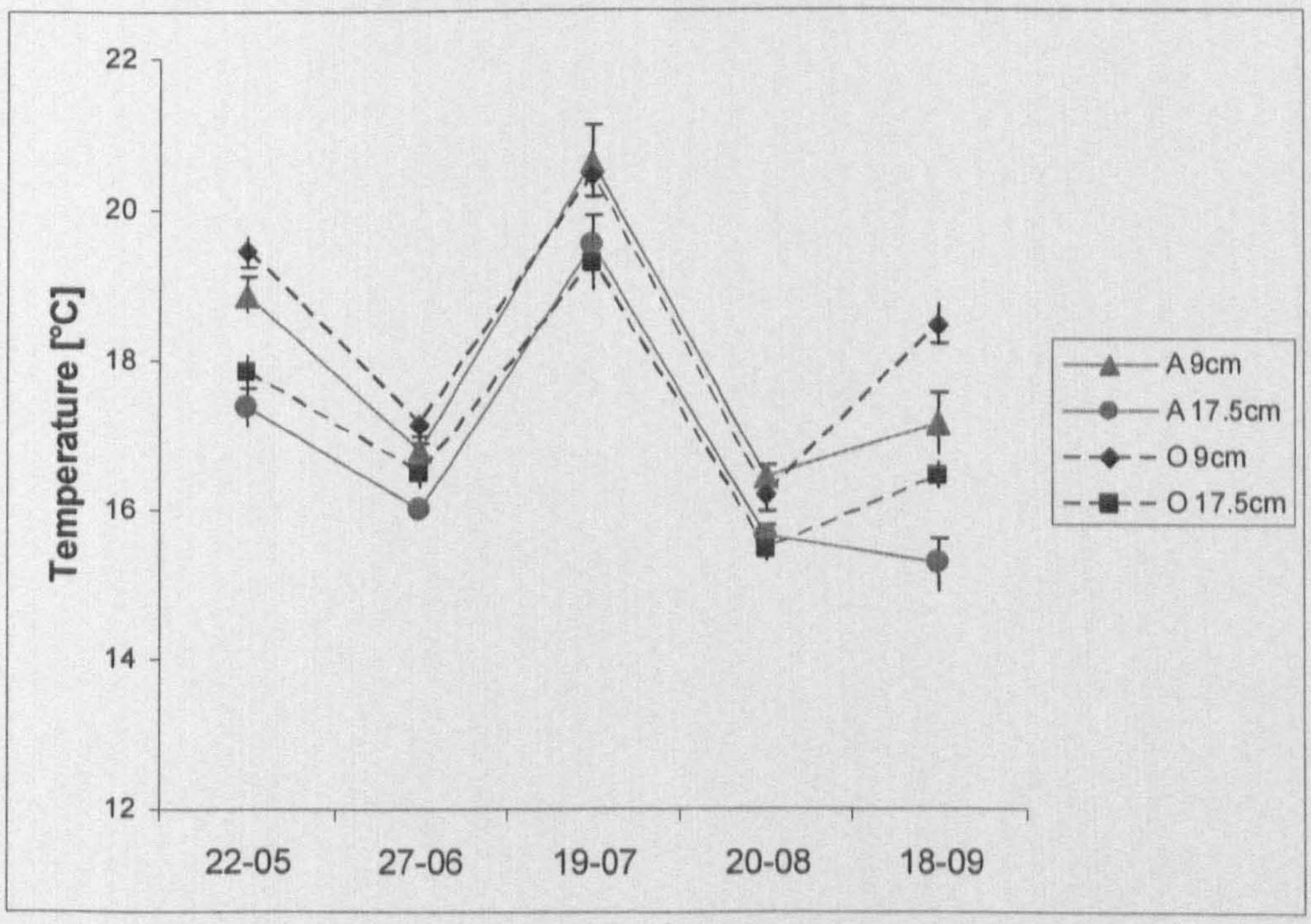


Figure 87: Soil temperature at 2 different depths of ambient (A) or elevated (O) ozone treatment. Error bars represent standard errors.

4.4.3.2 Soil solution chemistry

Significant effects were detected for pH at 10 cm for time (P = 0), time*treatment (P = 0) and treatments (P = 0.027) (Table 42, Figure 88). On 27/06 (P = 0.04) and also on 18/09 (P = 0.001) a significant difference between the two treatments was observed. The soil water of elevated treatment showed a significantly higher pH than the ambient treatment on both occasions. On 27/06 the pH was about 4.9% higher and on 18/09 about 1.5% higher (Table 43, Figure 88).

For the pH at 20 cm, significant effects were found for time (P = 0) and time*treatment (P = 0), but not for treatments (Table 42) pH measurements on 18/09 showed significant effects of ozone with the elevated treatment being about 0.12% significantly higher than the ambient treatment (P = 0.008) (Table 43, Figure 88).

Table 42: P- values from the RM- ANOVA of effects of ozone and time on soil water pH measured at 0- 10 cm and at 10- 20 cm in 2007. The effects of ozone, time and the interaction of ozone and time are shown. Values in bold are significant at P< 0.05.

Depth	*Effect of time*	*Effects of treatment*	*Time*treatment*
0-10 cm	**0**	**0.027**	**0**
10-20 cm	**0**	0.274	**0**

Table 43: P-values from the independent samples test of effects of ozone on soil water pH at 0-10cm and 10-20 cm in 2007. Values in bold are significant at $P < 0.05$.

	pH (0-10 cm)	***pH (10-20 cm)***
22/05	0.344	1.0
27/06	**0.040**	0.157
19/07	0.697	0.909
20/08	0.491	0.954
18/09	**0.001**	**0.008**

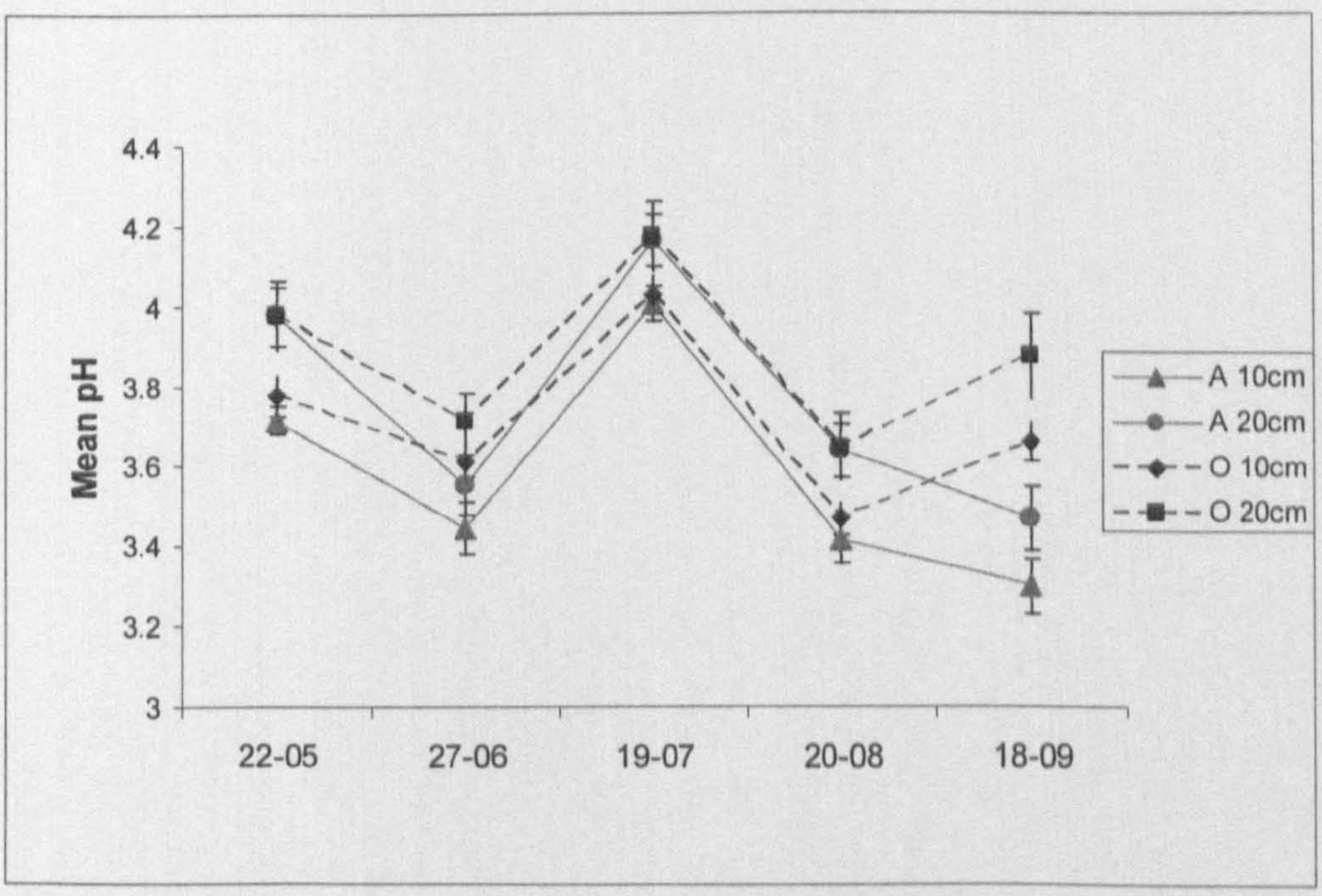


Figure 88: pH of soil water from ambient (A) or elevated (O) ozone treatment at 2 different depths in 2007. Error bars represent standard errors.

The dissolved organic carbon concentrations were overall higher in the lower part of the soil, although DOC concentrations were not significantly

affected by the ozone treatment (Table 44, Figure 89 and Figure 90). Both the DOC of the lower and the upper part of the soil only showed significant differences for the effect of time.

Table 44: P-values from the RM-ANOVA of effects of ozone and time on dissolved organic carbon measured at 0-10 cm and at 10-20 cm. The effects of ozone, time and the interaction of ozone and time are shown. Values in bold are significant at $P < 0.05$.

Depth	*Effect of time*	*Effects of treatment*	*Time*treatment*
0-10 cm	**0**	0.548	0.57
10-20 cm	**0**	0.227	0.111

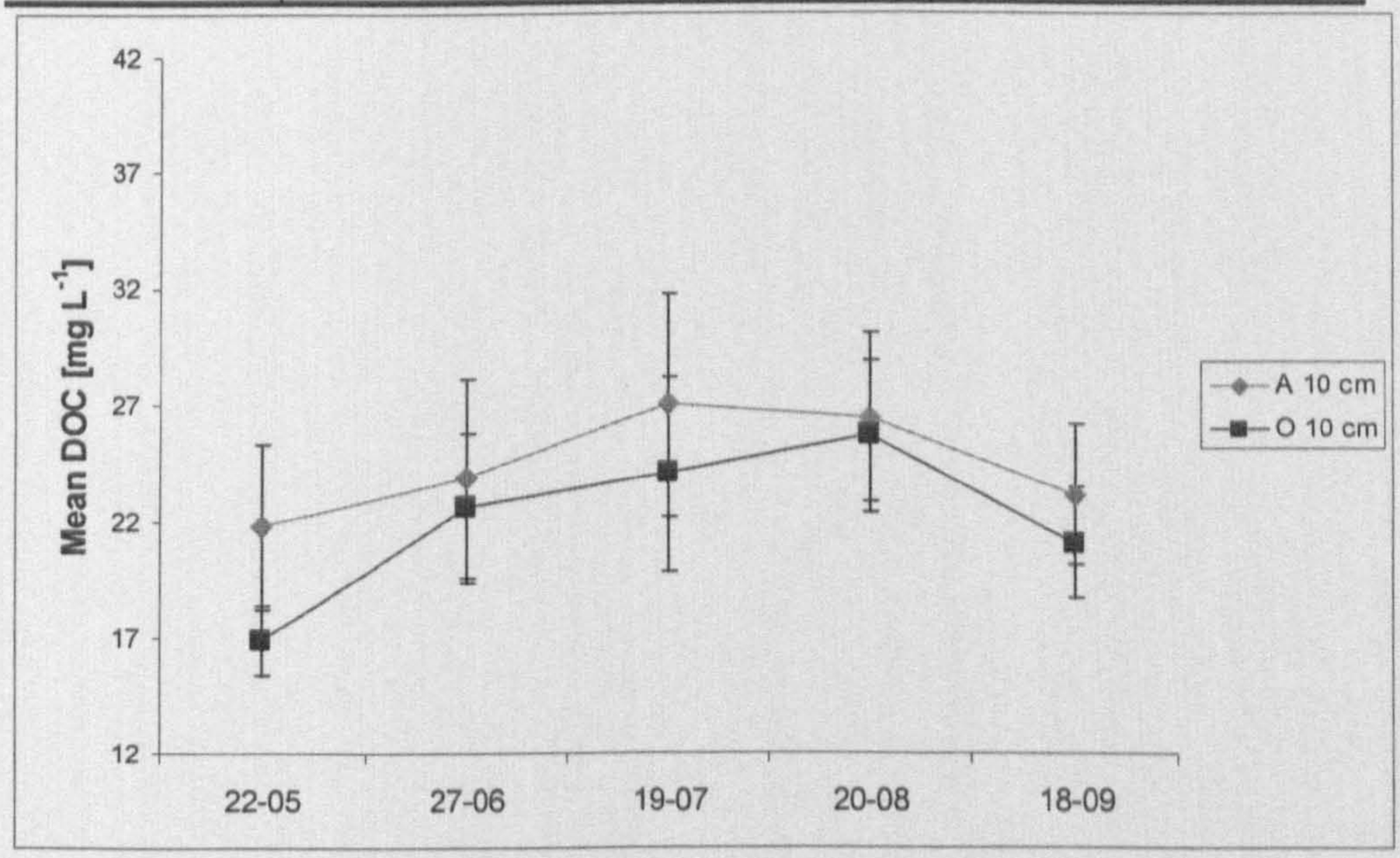


Figure 89: DOC concentrations (0-10 cm) of soil water from ambient (A) or elevated (O) ozone treatment in 2007. Error bars represent standard errors.

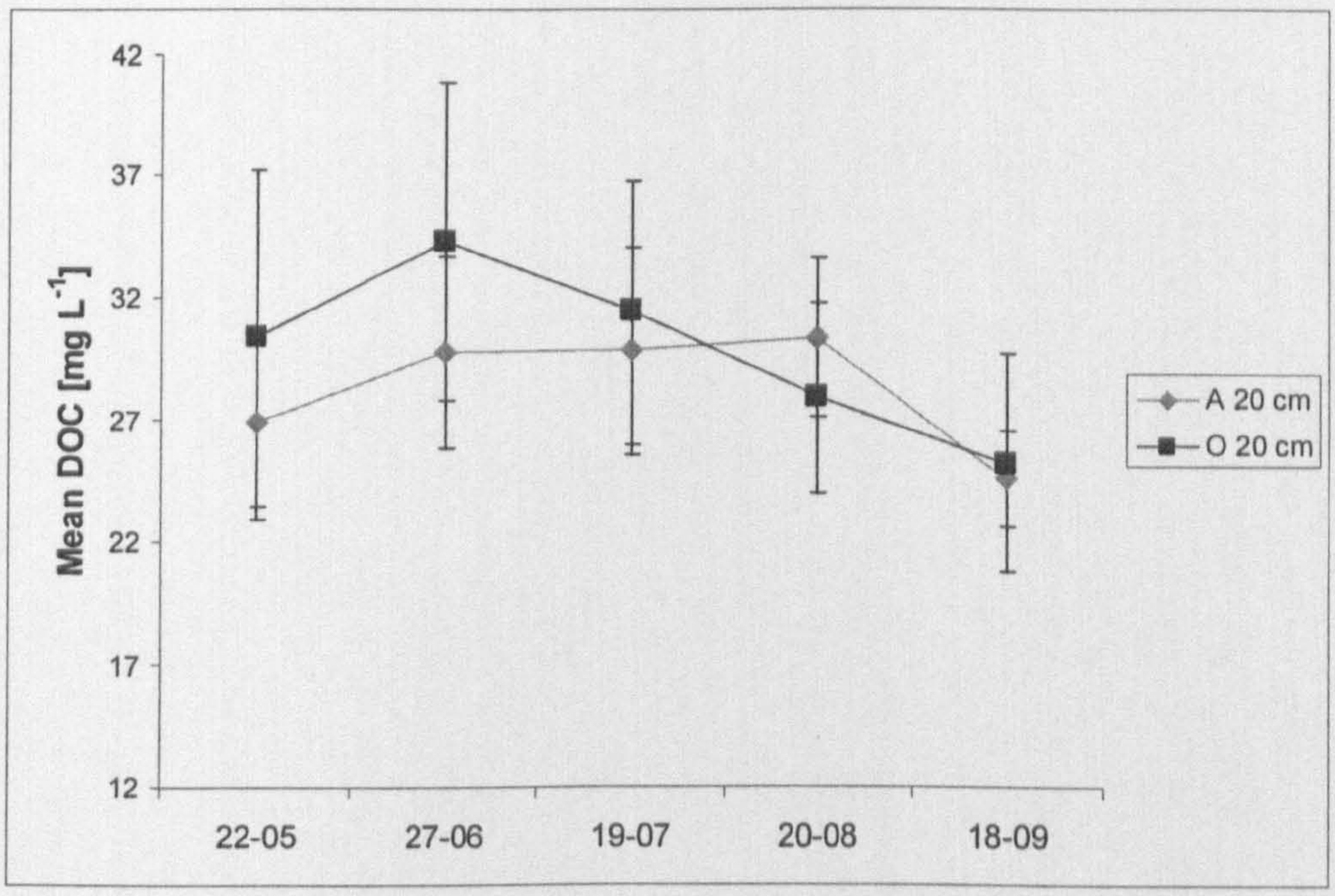


Figure 90: DOC concentrations (0-20 cm) of soil water from ambient (A) or elevated (O) ozone treatment in 2007. Error bars represent standard errors.

The ammonium concentrations of the soil water showed different temporal trends in the two treatments, at both depths. This resulted in higher levels of ammonium at the beginning, and lower levels at the end, of the growing season in the elevated ozone treatment as compared to the ambient ozone treatment (Figure 91 and Figure 92).

At 10 cm depth, significant effects were found for time (P = 0) and time*treatment (P = 0), but not for treatments (Table 45). Significant effects were detected at three points in time. The $NH4^+$ concentration were higher on 22/05 in the elevated treatment than in the ambient treatment (P = 0.002). In contrast, on 20/08 (P = 0.048) and on 18/09 (P = 0), the NH_4^+ concentration was significantly higher in the ambient treatment (Table 46). At 20 cm, significant effects were found for time (P = 0) and time*treatment (P = 0), but not for treatments (Table 45). As at 10 cm, the $NH4^+$ concentration was higher on 22/05 in the elevated treatment than in the ambient treatment (P = 0.006), but on 20/08 (P = 0.026) and on 18/09 (P = 0) the NH_4^+ concentration was significantly higher in the ambient treatment (Table 46). Whereas ammonium concentrations at both depths in the elevated treatments were on 22/05 about 15% higher and on 19/07 5% higher than in the ambient treatment, on 18/09 ammonium concentrations in the elevated treatments were about 25% lower than in the ambient treatment. The nitrate concentrations of both treatments and depths were negligible on all dates and are therefore not shown.

Table 45: P-values from the RM-ANOVA of effects of ozone and time on soil water ammonium concentrations measured at 0- 10 cm and at 10- 20 cm in 2007. The effects of ozone, time and the interaction of ozone and time are presented. Values in bold are significant at P< 0.05.

Ammonium	*Effect of time*	*Effects of treatment*	*Time*treatment*
0-10 cm	**0**	0.286	**0**
10-20 cm	**0**	0.714	**0**

Table 46: P- values from the independent samples test of effects of ozone on the ammonium concentrations at 0-10cm and 10- 20 cm in 2007.Values in bold are significant at P<0.05.

	NH4+ (0-10 cm)	***NH4+ (10-20 cm)***
22/05	**0.002**	**0.006**
27/06	0.975	0.349
19/07	**0.048**	**0.026**
20/08	0.436	0.735
18/09	**0**	**0**

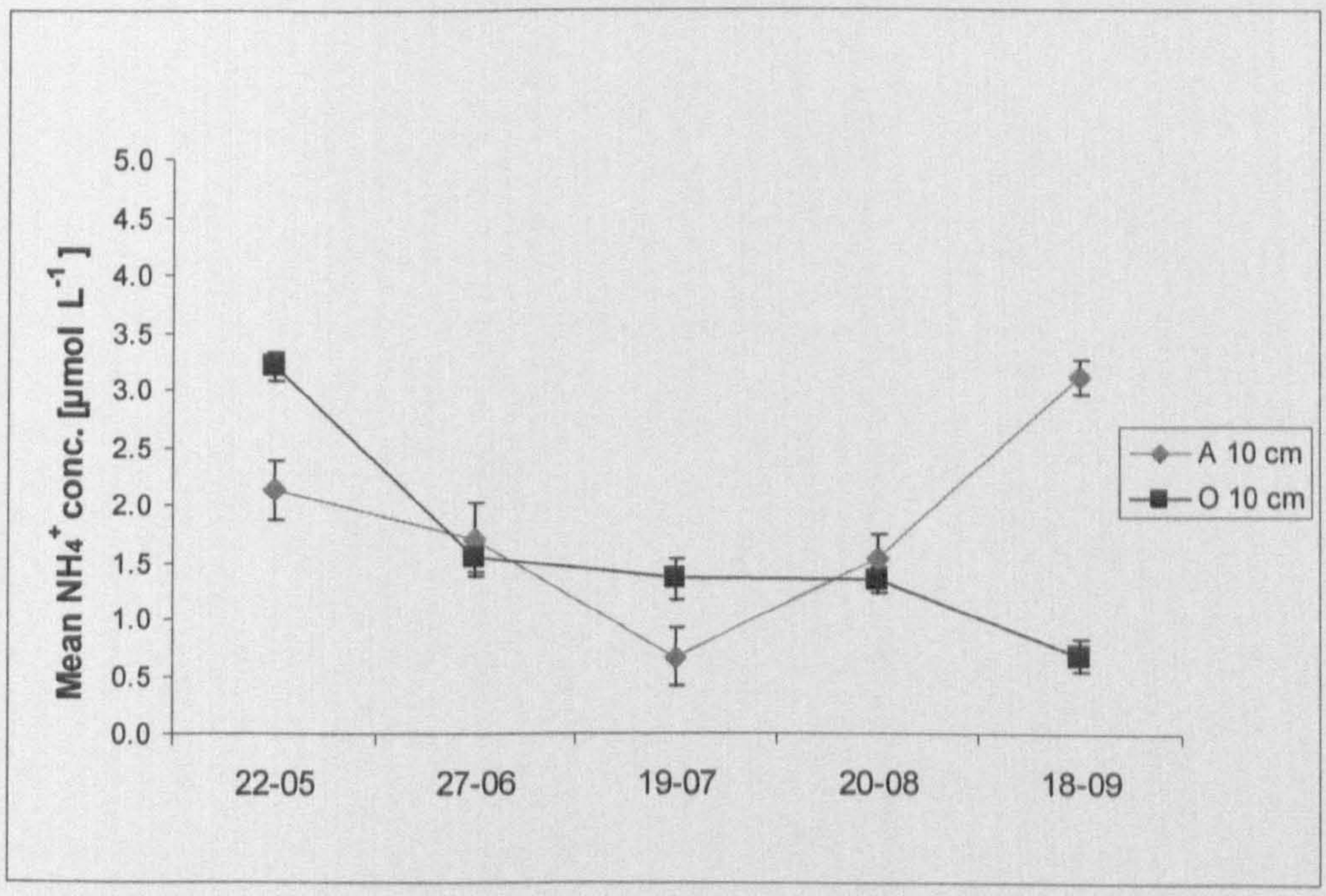


Figure 91: NH_4^+ concentrations in soil water at (0- 10 cm) for ambient (A) or elevated (O) ozone treatment in 2007. Error bars represent standard errors.

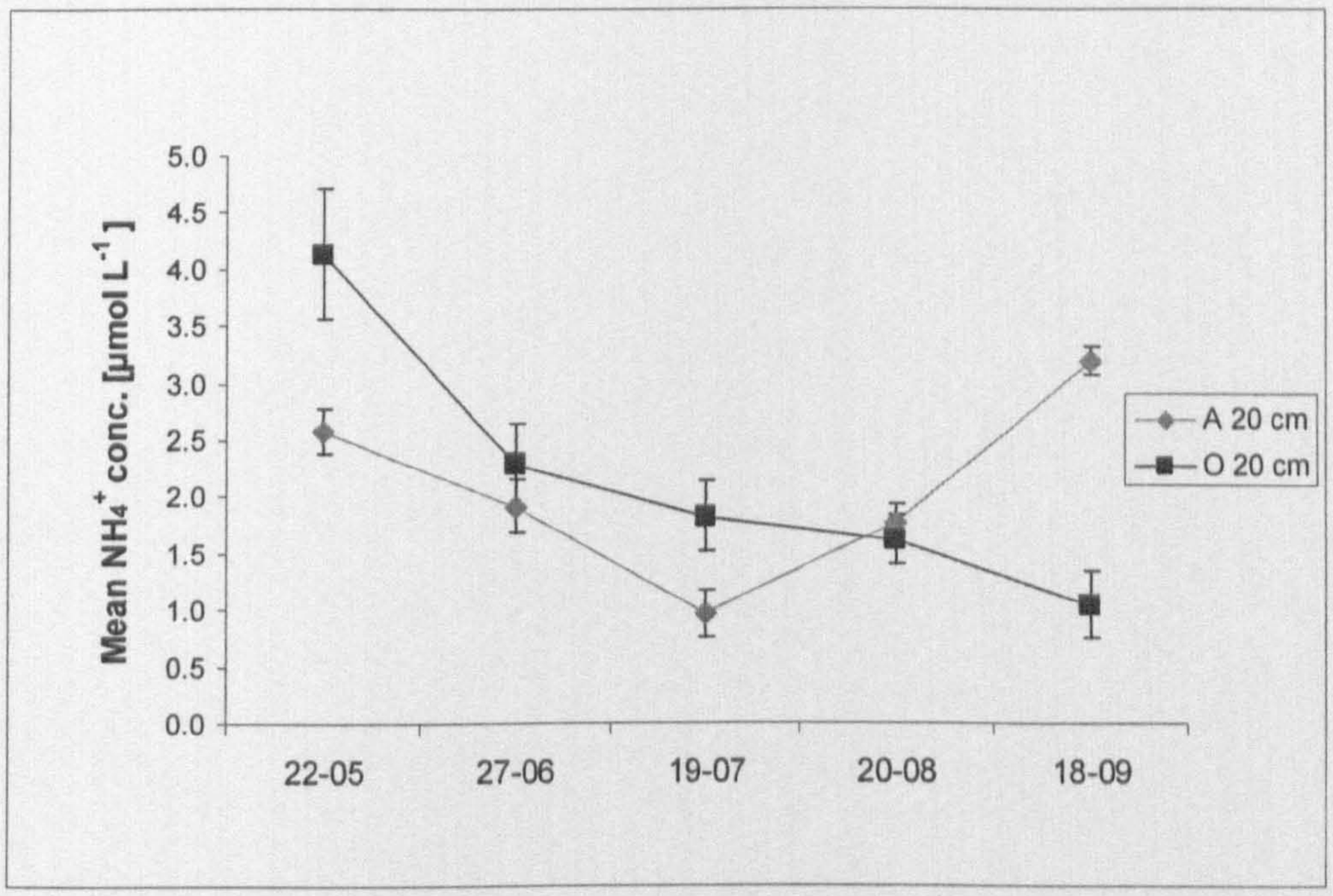


Figure 92: NH_4^+ concentrations in soil water at (0-20 cm) for ambient (A) or elevated (O) ozone treatment in 2007. Error bars represent standard errors.

At the final harvest, the data for soil extracts sampled form 0-10 cm revealed only small and non-significant differences for pH, DOC and extractable nitrate concentrations (Table 47 and Table 48).

Table 47: P-values from the independent non-parametric samples test of effects of treatment on chemistry of soil extracts at 0-10 cm in 2007.

Soil extractions (0-10cm)	***P***
K^+ concentrations	0.21
NH_4^+ concentrations	0.67
NO_3^- concentrations	0.35
pH	0.88

Table 48: Soil analysis results from 0-10 cm soil sampled in 2007 treated with ambient air or elevated ozone. Values are means and standard errors.

	pH	*DOC* [mg L^{-1}]	*K+* [mg kg^{-1}]	NO_3^- [mg kg^{-1}]	NH_4^+ [mg kg^{-1}]
Ambient	4.04 ± 0.132	13.23 ± 1.44	282.53 ± 51.76	0.931 ± 0.621	18.6 ± 4.031
Elevated	3.94 ± 0.053	12.37 ± 1.73	178.23 ± 38.86	1.38 ± 1.493	31.09 ± 13.3

4.4.4 Litter experiment

Microbial respiration

Litter respiration rates were measured using litter derived from different ozone exposure and two different soil water treatments (ozone-treated). Ambient air and elevated ozone treatments showed a similar pattern of microbial respiration over time, when ambient-treated soil water was used (Figure 93) which suggests that there is no effect of ozone treatment via the litter. However, there was a very strong and clear effect of soil water treatment in the first week of the experiment. The rates of microbial respiration were significantly reduced in the ozone-treated soil water as to about 10-15% to the values of the ambient ones (Figure 93).

Significant effects were detected for the ambient treatment (A, A*) within the soil water treatments for time (P = 0) but not for time*treatment (P = 0.113), and not between the soil water treatments (Table 49), although there was trend affecting the ambient litter with P=0.07. So, there was no consistent ozone effect, suppressing the microbial respiration, which micht have to do with the fact, that the litter was only soaked one time with the soil water and only remoistened with deionised water.

For the elevated treatment (O, O*) significant effects were found within the soil water treatments for time (P = 0) and time*treatment (P = 0.007), but not between the treatments (Table 49). As mentioned above, there was no

consistent effect of ozone on the microorganism, as the litter was not supplied by anymore soil water.

In addition to that the data of 1st and the 3rd day revealed a significant difference between the soil water treatments (P = 0.001 and P = 0.011), which were significantly lower in the elevated soil water treatment than in the ambient soil water treatment (Table 50). On the 1st day microbial respiration of ambient and elevated litter treatment were reduced by 15%, on the 3rd day microbial respiration of the ambient litter was reduced by 15% whereas microbial respiration of the elevated litter treatment was reduced to 10%. These results show that the soil water treated with elevated ozone was the main effect on the reduced microbial respiration during the first three days. At day 6 the microbial population metabolism was probably inhibited by the loss of essentiel nutrients such as nitrogen for example.

Additional results of a C/N analysis of *Eriophorum* biomass and litter, which did not show any significant effects of ozone are not shown here but can be found in Appendix 7.4.

Table 49: P- values from the RM- ANOVA of effects of ozone and time on litter respiration. The effects of ozone, time and the interaction of ozone and time are shown. Values in bold are significant at P< 0.05.

Respiration	*Effect of time*	*Effects of treatment*	*Time*treatment*
Ambient litter	**0**	**0.078**	0.113
Elevated litter	**0**	0.188	**0.007**

Table 50: P- values from the 2-Way- ANOVA of effects of litter and soil water treatment on litter respiration. The effects of litter, soil water and the interaction of litter and soil water are shown. Values in bold are significant at $P < 0.05$

	Effects of litter	***Effects of soil water***	***Litter*soil water***
Day 1	0.848	**0.001**	0.727
Day 3	0.888	**0.011**	0.527
Day 6	0.547	0.981	0.523
Day 9	0.668	0.806	0.23
Day 16	0.999	0.387	0.53
Day 22	0.692	0.19	0.594

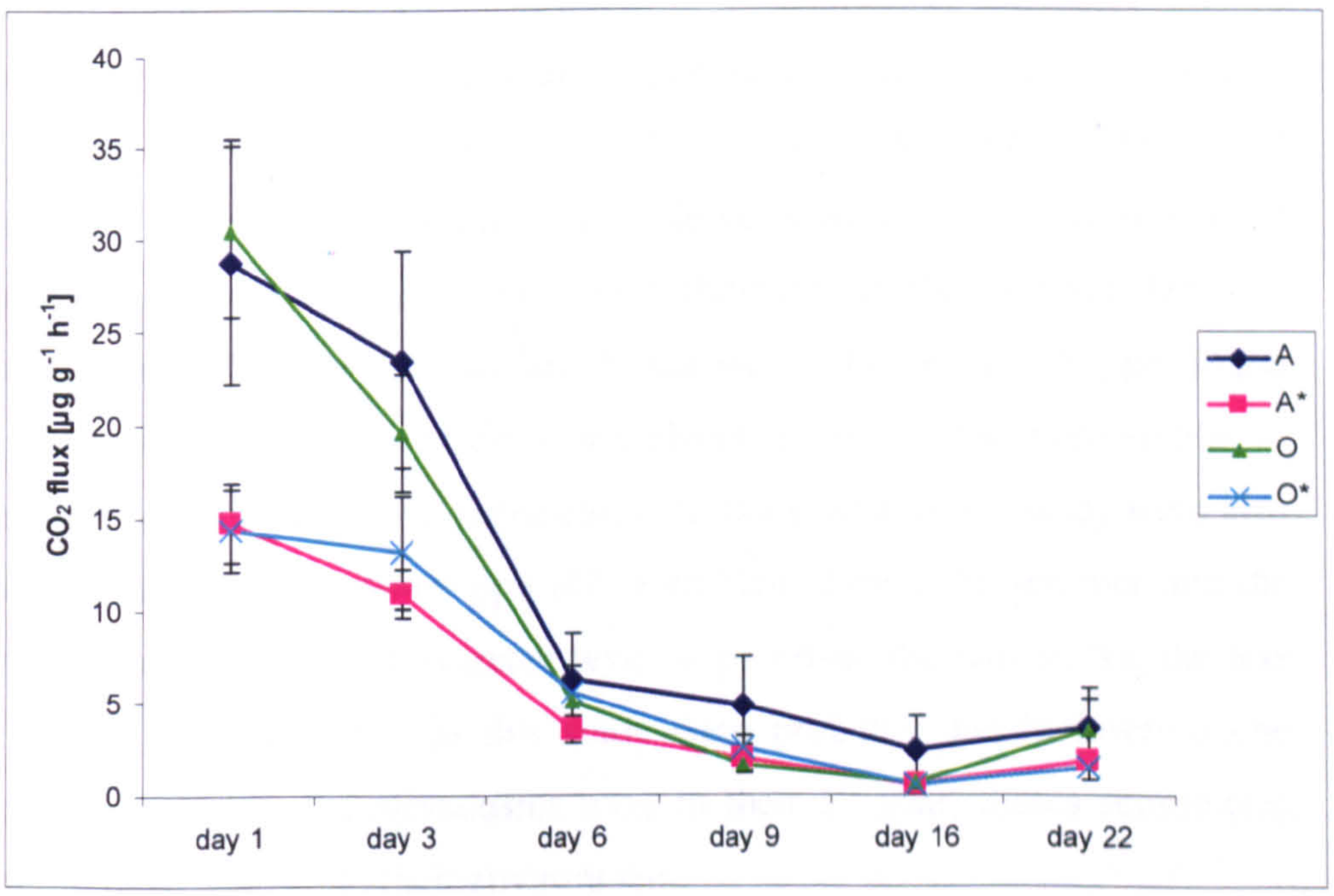


Figure 93: Litter respiration rates for ambient (A) or elevated (O) ozone treatment of litter in 2007. Error bars represent standard errors. A* and/or O* indicate that the litter from either ambient or elevated treatment was infused with soil water that derived from the elevated ozone treatment.

4.5 Discussion

Ozone effects on trace gas fluxes

In this experiment, methane fluxes decreased under elevated O_3 during the summer periods of 2005 and 2006. However, the measurements in 2007 showed less pronounced effects of ozone in the final year. This was probably due to a cooler climate and non-significant reducing fluxes and lower ozone concentrations. This suggests that the effects of ozone are not due to a cumulative change in the system, but rather due to shorter- term responses.

These findings are quite different to the other studies in the literature. Niemi *et al.* (2002) and Rinan *et al.* (2003), for instance, measured methane fluxes over a short period and observed a significant increase in methane fluxes under elevated ozone ($P< 0.05$). Niemi *et al.* (2002) explained the O_3 response by either higher primary production or stimulation via released substrates, such as root exudation, decay and litter input, or facilitated transport through *E. vaginatum* leaves. In contrast to this experiment, their study only ran for 6 weeks in growth chambers in the summer. Obvious significant differences on methane fluxes were observed at 100 ppb ozone. In contrast this study used open- top chambers, which are more similar to natural conditions and the ozone concentrations used in this study were also lower, ranging from 40-60 ppb above ambient during the summer and the mesocosms were also fumigated with 10 ppb over the winter. So, the less reduced methane fluxes in this study were probably due to lower ozone exposure, and as the mesocosms were in their 3rd year, theses ecosystems might have adapted to their environment.

Rinnan *et al.* (2003) conducted a similar short-term experiment with peatland microcosms in growth chambers for seven weeks. Interestingly, in one part of the experiment they removed all the sedges, showing that the significantly higher methane emissions in 100 ppb and 200 ppb O_3 only occurred in microcosms with *E. vaginatum* present. This suggested that the sedge was mainly responsible for higher methane emissions and that this was due to transport via its aerenchyma.

An additional incubation experiment of the soil alone was conducted at the end of the experiment. It showed a significant stimulation of the CH_4 production potential in the presence of *E. vaginatum*, the value was about 42% higher than in the *Sphagnum* microcosms. However, there was no effect of ozone on the microbial capacities to produce or consume CH_4 in peat. Consequently, the increased CH_4 efflux under high ozone treatments was explained by Rinnan *et al.* (2003) with alterations in the physiology or morphology of *E. vaginatum.* More recently, Mörksy *et al.* (2008) looked at the structure of *E. vaginatum* exposed to elevated ozone for four years in an open-air exposure field. They did not find any effects of ozone on the aerechyma, suggesting that the shoots of *E. vaginatum* are quite tolerant to ozone.

Overall there were no significant ozone effects on dark CO_2 fluxes during the 3 years. Niemi *et al.* (2002) and Rinnan *et al.* (2003) also assessed net CO_2 exchange and dark respiration. During their short- term experiments, both found an increase in the dark respiration rate in the elevated ozone treatment. Niemi *et al.* (2002) explained their findings through changes in plant physiology (stimulated dark respiration ,with reduced photosynthesis, often occurs in response to ozone probably due to the increased respiration associated with repair mechanisms) or as a result of stimulated microbial activity in surface water. Ozone biodegrades organic compounds into easily assimilable molecules and this furthermore could increase microbial growth, which might contribute to O-induced increases in methane emissions (Van der Kooij *et al.*, 1989). Rinnan *et al.* (2003) not only found an increase in the dark respiration but also observed higher respiration rates in the presence of *E. vaginatum* than without, suggesting that the dominance of the sedge leads to an increased release of CO_2 or to a stimulation of microbial respiration.

However, since the conditions in these experiments (e.g. Niemi *et al.*,2002; Rinnan *et al.*, 2003) are quite different to those in this long- term study, it is not surprising that the ozone responses differ. Meaningful differences were observed in the short-term experiments at 100 ppb ozone under controlled chamber conditions. Niemi *et al.* (2002) not only measured the dark

respiration rates during summer but also during autumn, showing low dark respiration rates, with no significant changes in response to O_3 treatment, probably due to lower temperature and less sunlight.

Ozone effects on plant growth, membrane permeability and biomass

There was no effect of O_3 on the capitula biomass of *S. papillosum*. As for the biomass of the other vascular plants, *E. vaginatum* indicated a significant response to elevated ozone and so did *Erica tetralix*. The latter effect has to be interpreted cautiously as *E. tetralix* was not equally dispersed. At the start of the experiment the mesocosms were carefully assigned to treatments to ensure an even distribution of *E. vaginatum* between treatments. Because of this, the reduced above-ground green biomass of *E. vaginatum* may be a real significant effect. Studies by Rinnan & Holopainen (2004) showed negative responses in sensitive structural paramters of *E. vaginatum* such as size of chloroplasts and amount of starch, and Mörsky *et al.* (2008) did not find an effect of ozone on the shoot length or the structure of the leaves of *E. vaginatum*, however they also observed an increase in the total number of leaves towards the end of the experiment. Growth measurements of *S. papillosum* showed no significant effects of ozone and neither did the green leaf density of *E. vaginatum*. These results may also be due to the relatively low ozone exposures and low temperatures during the summer of 2007, another reason is that. *S. papillosum* might be less sensitive to ozone than other *Sphagnum* species (Potter *et al.*, 1996a). However, ozone reduced the total K^+ leakage in *S. papillosum*. Potter *et al.* (1996a) looked at the membrane leakage of *S. capillifolium, S. cuspidatum, S. papillosum* and *S. recurvum*. Only *S. recurvum* showed an increase in potassium leakage when exposed to elevated ozone, which also appeared to be strongly correlated with changes in photosynthesis and fluorescence characteristics. However, in their study the species were exposed to 150 ppb for just 6 hours, whereas in this study the moss species had been exposed to 40-60 ppb for three years. This could imply that *S. papillosum* does not react sensitive to ozone, as potassium leakage has been used in the past to indicate cell injury, or a lower potassium content of the plant.

Ozone effects on soil and soil solution chemistry

The soil water pH showed a partially significant effect of the elevated ozone treatment. Interestingly, these were significant treatment differences at the two different depths, which indicate an alteration in the soil water chemistry, which might be associated with ecosystem related processes such as the storing of nutrients by the vegetation for instance. Elevated ozone may have increased pH, which probably led to more cation exchange. This is not consistent with the lower K^+ concentrations found in the membrane leakage. However, *Sphagnum* mosses can actively control the acidity of their environment by exchanging H^+ with cations (Na^+, K^+, Ca^{2+} and/or Mg^{2+}) in the mire water (Clymo, 1984). When rain dilutes cation concentrations, the binding of cations to the exchange sites of *Sphagnum* cell walls increases and raises the pH of the mire water (Andrus, 1986). Elevated ozone concentrations did not have an effect on the dissolved organic carbon concentration. In a study of impacts of elevated ozone (low background 20 ppb, high background 45 ppb; plus low and high peaks) on peatland below-ground DOC characteristics by Jones *et al.* (2009), changes in DOC were only reported for fens, while bogs were tolerant. They suggested that the vegetation type played the critical role as bogs, with their large amount of *Sphagnum,* which do not possess roots and do not release exudates. However, they reported a significant decline in DOC in fen cores exposed to elevated ozone, and rising values of pore water SUVA (Specific UV Absorbance), which suggested that the light molecular fraction became depleted. As CH_4 production is correlated to acetate rather than to total DOC (Ström *et al.*, 2003), this is unlikely to be a mechanism for effects of ozone on trace gas fluxes. Although there were no effect of elevate ozone on the DOC concentrations in this study, the composition of DOC may still be affected by elevated ozone, which needs clarification in further studies.

An interesting development was the change in soil water ammonium concentrations during the summer of 2007. The ammonium concentration in the elevated O_3 treatment was significantly different from the ambient treatment on three measuring dates. The ammonium concentrations for the ambient treatment were lower at the start of the vegetative growth than those for the elevated O_3 treatment, and rose again at the end of the growing

season, when the plants reduced their nitrogen uptake. The ammonium concentrations for the elevated O_3 treatment fell even more which indicates a change in the soil chemistry, and this is partly supported by the findings for pH. The higher pH in the elevated treatments means also that the soil water is less acidic. The significantly reduced ammonium concentrations were probably due to oxidation and nitrification, although these processes are slow when the pH is below 5.5. Additionally, NH_4^+ in a less acidic environment, the equilibrium changes towards ammonia production which leaves the soil in gaseous form. However, ammonia volatilization is minimal in soils with high buffering capacity or high exchange capacity (Camberato, 2001). Holmes *et al.* (2006) studied nitrogen cycling in a Northern temperate forest ecosystem under elevated carbon dioxide and O_3 concentrations (in the Aspen FACE experiment) for about four years. They detected decreased gross N-mineralization, but not ammonium immobilization, under elevated ozone, which indicated reduced N-availability. This was attributed to altered rate of litter production and not to changes in the litter chemistry.

In this study the differences in ammonium concentrations between the two treatments might be due to modified mineralization caused by altered litter production Also, the soil water revealed very low nitrate concentrations and considering that the ammonium concentrations were falling at the end of the season, the already in nutrients limited mire ecosystem might become even more N-limited in the elevated treatments. Ammonium is attracted to the soil particles so only a portion of the ammonium is in the soil water at any time. In soils of higher exchange capacity, such as peatlands, the amount of ammonium in solution is insufficient to support optimum nitrogen uptake by the plants (Camberato, 2001). This might increase the competition of plant roots and microbes for mineralized N, and might have an impact on N-cycling.

However, the partially significantly different soil temperature has to be taken into consideration. The soil temperature might confound the effects of ozone. Higher soil temperature can lead to higher methane and carbon dioxide fluxes and higher growth rates; and the lower soil temperature, as

found at the end of the vegetation period, might slow down nutrient turnover for instance.

Ozone effects on litter respiration

Microbial respiration was significantly reduced in litter treated with ozone-treated soil water as compared to the ambient air treated water on the first day, but no difference was detected between the ambient air treated litter and the elevated air treated litter used in the experiment. As shown by the significant differences between treatments in the NH_4^+ concentrations of the soil water in September, this leads to the interpretation that elevated O_3 had an indirect impact on the microbial community itself through changes in N-availability in the soil.

In a long-term study on the effects of ozone on vegetation, microbial community and methane dynamics of boreal peatland microcosms, Mörsky *et al.* (2008) found that microbial biomass was higher in peat mesocosm receiving elevated ozone and they observed the same trend in organic acids. They suggested that microbial biomass increased because of the higher substrate availability, although methane production did not reflect that. They observed only slight increases in methane flux in the elevated treatment towards the end of the experiment.

Islam *et al.* (2000) studied the interactions of tropospheric CO_2 and O_3 enrichment and moisture variations on microbial biomass and respiration in soil. They showed that elevated O_3 significantly decreased CO_2 flux by 20-36%, under restricted moisture conditions as well as under well-watered conditions. However, the effect was more pronounced under dry conditions.

Chung *et al.* (2005) investigated fungal metabolism and composition at the Aspen FACE project. They found a shift in the fungal community composition caused by litter produced under elevated ozone. Overall there seemed to be a strong connection between plant biomass, litter production and soil microbial activity.

There are numerous studies (Findlay *et al.*, 1996; Scherzer *et al.*, 1998; Kainulainen *et al.*, 2003) of O_3 effects on litter chemistry and decomposition and it has been suggested that litter produced under elevated ozone conditions might have lower N concentrations or higher phenolic

concentrations, but not necessarily reduced decomposition rates, which is consistent with the findings of this study. In this study, elevated ozone had no effect on the C/N ratio of both the biomass and the litter of *E. vaginatum* (Appendix 4). This seems to support the results from the microbial respiration, in which the litter from the elevated treatment did not show any effects. Nevertheless, a more detailed analysis of litter composition is needed to confirm this.

4.6 Conclusion

The results of this experiment very clearly demonstrate the potential for ozone

1. to affect CH_4 fluxes from wetland ecosystems,
2. to reduce *E. vaginatum* biomass and microbial activity in soil solution, and
3. to cause changes in the soil solution chemistry such as ammonium.

Because methane emission and dark respiration are closely related to the presence of *E. vaginatum*, the reduced biomass could be the cause of a reduced microbial activity and a suppression of nutrient turn-over. However, the literature also suggests that more easily assimilable molecules of organic compounds might be available as they have been biodegraded by ozone (Van der Kooij *et al.*, 1989). The reduced methane emissions and the suppressed microbial respiration in the elevated treatment also support this conclusion as well. According to the Aspen FACE project (Holmes *et al.*, 2006) findings of elevated O_3 effects on nitrogen transformation, elevated O_3 could lead to a negative feedback on N- availability, which could also be a reason for the ozone induced changes in biomass as well as in soil water chemistry and/or microbial respiration. Similarly, Mörksy *et al.* (2008) found increased organic acids, higher microbial biomass and a changed microbial community under elevated ozone

In order to support these findings and/ or to approach a better understanding of elevated ozone on the carbon cycle, further investigation is needed of factors such as the C/N ratio of the litter, the phospholipid acid composition of the soils, the root biomass of the mesocosms, with a focus on *E. vaginatum*, and the aerenchyma of *E. vaginatum*.

Nevertheless, the results from this long- term experiment were quite different from the reported findings of short-term experiments of ozone effects on peatlands. Whereas methane emissions seemed to be rather stimulated by high O_3 concentrations during short term exposure, the long-

term exposure used in this experiment lead to a decline in methane emissions, although in this final year the decline was not significant. Therefore short- term experiments might overestimate the effect of elevated O_3 on the ecosystem and underestimate the ability of the ecosystem to adjust to the higher O_3 concentrations, which also depends on the magnitude of ozone elevation. As elevated ozone and methane are both greenhouse gases, which rank only behind CO_2 in their contributions to anthropogenic climate forcing (Ramaswamy *et al.*, 2001), and rising temperatures due to the global climate change also make the soil drier, the methane oxidation might be enhanced (Del Grosso *et al.*, 2000) and the balance of the CO_2 sink/CH_4 source of the mire ecosystem negatively affected. Therefore, more studies of climate effects on peatland C fluxes that induce ozone are needed.

5. General Conclusions

5.1 Summary of key findings: ozone impacts in a community context

In the current study the effects of elevated ozone on two semi-natural ecosystems were investigated. The first semi- natural ecosystem was a grassland in the uplands of the Northern Pennines, which was fumigated with elevated ozone concentrations in a free air fumigation gradient system for two years. It was shown that long- term exposure to ozone affects the balance of functional groups and has an effect on the biomass of individual species (Chapter 2). Another component of the study examined exposure to ozone within different parts of the canopy, and effects on stomatal conductance, for different functional groups. It was shown that the ozone concentration within the canopy was significantly lower than the ozone concentration above the canopy, and the elevated ozone concentrations led to an increased stomatal closure in *Trifolium repens* and *Briza media* (Chapter 3).

The second semi-natural ecosystem was a lowland mire community from lowland Cumbria, exposed to elevated ozone concentrations in open-top chambers in Newcastle for three years. This part of the study aimed to assess the effects of ozone on C- cycling (Chapter 4). It was shown that long-term exposure to ozone reduces methane emissions, reduces the biomass of sedge species, which are believed to provide key pathways for methane fluxes from the peat to the atmosphere. Ozone has also shown to reduce soil water ammonium concentrations at the end of the growing season, and resulting in a reduction in microbial respiration.

Evidence of effects of ozone on the balance of functional groups, and the biomass of individual species, in the grassland study was found in the 2nd year. The functional group that was most affected by ozone was the forbs, which showed a significant reduction in biomass in the elevated ozone plots. Overall,

only a small percentage of the variation in the between- plot variation of forb species was explained by ozone. However, a stepwise canonical analysis revealed that ozone contributed as much to the explaination of the variation in forb species composition between experimental plots as other identified key drivers of between-plot variation, such as soil water content, nitrate concentrations and calcium concentrations. The biomass of other functional groups (grasses and legumes) was not affected significantly by ozone, nor was total above-ground biomass. However, the grass *D. glomerata* showed an increased biomass with increasing ozone concentrations. The forb species which showed a significant negative response to increasing ozone were *Rhinanthus minor* and the members of *Ranunculacae*.

The percentage reduction in ozone concentrations at 20 cm and at 5 cm above the soil surface was constant over the range of conditions in which measurements were made. Taking account of uncertainties in the measurements, plant species at 20 cm were exposed to 60- 67% of the ozone concentrations above the canopy, while at 5 cm the ozone exposure was reduced to 27- 34% of that above the canopy. Measurements of the fractional leaf area index showed that 70% of the biomass of forbs was found between 5 cm and 20 cm whereas only 30% was found above 20 cm. In contrast, legume and grass biomass were equally distributed between the two heights. This suggests that, despite their greater response to ozone treatments, forbs were likely to be exposed to lower concentrations, since more biomass was found deeper in the canopy. However, this might be different for the individual species, such as the larger forb species (*Rumex acetosa, Rhinanthus minor* or *Ranunculacae*).

Ozone reduced the stomatal conductance of the legume species *Trifolium repens* and the grass species *Briza media*. Whereas *B. media* received a constant elevated concentration of ozone in the open- top chambers, this was not the case for *T. repens* in the free air fumigation gradient system. The

stomatal conductance of *T. repens* was significantly reduced in the elevated ozone treatments on one day when the measured ozone concentrations were quite high. The stomatal conductance of *B. media* showed no negative response to the elevated ozone treatment before the harvest in July but did so after the cut in September. Hence effects of ozone on stomatal conductance were not constant but may reflect both short-term high exposures and cumulative season exposures to the pollutant.

Evidence of negative impacts of ozone on carbon cycling was found in the mire mesocosms. Ozone exposure significantly reduced the biomass of the key species *Eriophorum vaginatum.* Although significant reductions of the methane fluxes by ozone were found in previous years, no significant differences were found for methane emissions in the third year of the exposure. The reduced above-ground biomass of *E. vaginatum,* if reflected in reduced below-ground production could reduce methane fluxes because (a) production and exudation of organic C from roots stimulates methanogenesis (Rovira, 1969) and (b) although >90% of the produced methane can be oxidised by methanotrophic bacteria (Sundh *et al.*, 1995), the aerenchyma of *Eriophorum* provides a pathway through which methane can escape oxidation and reach the atmosphere.

Ozone also reduced the ammonium concentrations in soil water, and increased soil water pH, at the end of the growing season. The ammonium concentrations increased after July in the ambient treatment probably because the plants reduced their nitrogen uptake, whereas the ammonium concentrations in the elevated treatments decreased which was probably due to a change in the soil chemistry. Differences in soil water chemical or microbial composition also affected the microbial respiration associated with litter decomposition. Microbial respiration was significantly reduced on the first day of the experiment in the soil water from the elevated ozone treatment. The reduced availability of ammonium within the soil water of the elevated treatment could

have inhibited microbial activity leading to reduced rates of litter decomposition. Depending on the plant residue (N-rich or N-poor), microbial growth can be limited by N-availability, which can also be enhanced by the competitiveness of plants roots for mineralized N at N-poor sites for instance, and with it changes the microbial N turnover rate (Wang & Bakken, 1996).

5.2 Wider significance of the findings

This study has demonstrated a range of impacts of ozone on two semi- natural ecosystems. The wider significance of these findings is discussed below. As ozone had an effect on the biomass of different species, and the balance of grasses and forbs, this suggests that it could affect the diversity of species, the species composition and the species richness of semi-natural grasslands more widely. If the grass/ forb balance changes and grass species dominate, then species richness tends to decline and so might the nutritive quality of the grassland. The field site at High Keenley Fell is managed under the Higher Level Stewardship Scheme (HLS), for creation, maintenance and restoration of species-rich, semi-natural grassland. The North Pennines present one of the target areas of the HLS. The objectives of these are: preservation of Biodiversity, Landscape, Historic Environment, Resource Protection and Access. Within this area nationally and internationally important habitats including blanket bog, juniper woodland, upland hay meadows, calaminarian (metaliferous) grasslands, dry heath, limestone and base rich flushes and mires occur. A vast array of nationally scarce species occupies these habitats and the area contains nationally important breeding bird communities including breeding waders and black grouse[13]. The two ecosystems discussed here, the upland grassland at High Keenley Fell and the mire mesocosms represent important habitats for scarce and rare species. If under elevated ozone the diversity and species richness changes within one of these habitats, these habitats might become unsuitable to such rare species, which might be

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dependent on the species richness and diversity, and key characteristics of these ecosystems.

Volk *et al.* (2006) studied effects of ozone on a semi- natural grassland over a five year period in a free- air fumigation system. They found that the total above-ground biomass in the elevated O_3 plots decreased by 23%, and this was also accompanied by a strong negative response of the legume biomass. Samuelsson *et al.* (2006) found, in a temperate natural grassland study, that after two years of ozone fumigation the more tolerant grasses were favoured over the less tolerant forbs, but at the species level only the grass species *Briza media* and *Phleum bertolonii* were strongly reduced by ozone. During the last two years, biomass of the legume *Lotus corniculatus* also declined (Peacock, pers. comm.), but there were no significant effects on any of the forb species (the stomatal conductance measurements were also made within these mesocosms). Rämö *et al.* (2006) studied seven species in a lowland hay meadow mesocosm experiment, which were exposed to both, to O_3 and CO_2 in open- top chambers. Two forb species (*Campanula rotunidifolia* and *Fragraria vesca*) and two legume species (*Trifolium repens* and *Vicia cracca*) showed significant reductions and/or injuries at elevated O_3 concentrations. The grasses *Agrostis capillaris* and *Anthoxanthum odoratum* and the forb *R. acris* were not affected by O_3 concentrations of 40- 50 ppb. Hence, while the effects on forb species observed in this study were consistent with some, but not all other studies, the lack of effects on legume species is inconsistent with all other community studies, which report a negative effect of ozone on one or more legume species.

Turning to effects on individual species, Mortensen *et al.* (1993) found significant growth reductions for *Ranunculus acris* at an O_3 concentration of 90ppb in a controlled environmental chamber study. In contrast, at the lower concentrations (40- 50 ppb) of the study of Ramö *et al.* (2006) no effect was found on this species. However, there was a decline of the family

Ranunculacae in this study, suggesting that *Ranunculus* species other than *R. acris* may have been affected by the elevated ozone treatment.

In this study *D. glomerata* increased its biomass with increasing ozone concentration, which is consistent with the literature. Hayes *et al.* (2007) observed a stimulation of *D. glomerata* by ozone and similar effects of ozone stimulating *D. glomerata* production in a grassland community were reported by Thwaites *et al.* (2006). However, it is unclear whether the stimulation of *D. glomerata* biomass by ozone is the outcome of reduced competition at the field site or a direct effect of ozone.

Thwaites (1996) showed that the biomass of hemi-parasite *R. minor* was generally reduced when grown in the ozone treatment, either alone or with host plants. The effect of ozone in reducing *R. minor* biomass in this study thus agrees with the findings of Thwaites (1996). The loss of this species from this ecosystem, or a large reduction in its cover, has more severe implications than a large reduction in its biomass. *R. minor* usually moves through the vegetation, changing hosts. Patches heavily infested with the hemi-parasite will quickly decline in grass abundance, leaving neighbouring uninfected patches with higher grass abundance more suitable for the seedlings of the next generation. The remaining vegetation will recover quickly and will be suitable again for *R. minor* (Press & Phoenix, 2005). Losing this species due to higher elevated concentrations restricts the distribution of *R. minor* in the ecosystem, which might have negative consequences for the species diversity of the grassland. Area exposed to high ozone concentrations would probably not get effected by the hemi- parasite at all, making the establishment of other species difficult due to the dominance of grasses, and depending where *R. minor* reseeds due to wind dispersal, the hemi- parasite species might get lost from the ecosystem over time, leading to less diversity.

The decline of the *Ranunculacaea* and *R. minor* in the ozone treatment could be related to a reduced competitive ability through an inability to re-allocate nutrient resources to roots; in this situation, other species, such as *D. glomerata* can take advantage and increase their biomass. Less abundance in ozone of *R. minor*, which prefers grasses as a host, might also provide an advantage of *D. glomerata.* When *R. minor* dies back, gaps open up in grasslands which facilitate the invasion of other species (Joshi *et al.*, 2000).

Many authors (e.g. Wilbourn *et al.* 1995, Ashmore & Ainsworth 1995, Fuhrer *et al.*, 1994 and Gonzalez-Fernandez *et al.* 2008) have observed a decline in the biomass of *T. repens* at higher O_3 concentrations. In this study, in contrast, these reported effects of ozone on clover were not found. The amount of legumes was overall very low and there was also great variation in the frequency and abundance of legume species between the individual plots. In the study of Bassin *et al.* (2007) only nitrogen addition, but not ozone, modified the functional group composition of an alpine grassland. They concluded that the adaptation to tolerate low nutrient availability and climatic conditions of their sub-alpine plant species protected them from ozone damage. In addition to this, the proportion of legumes in their study might also be low, due to the fact that the abundance of legumes decreases with elevation (Jacot *et al.*, 2000). Legume-poor communities could also be less sensitive to ozone because they depend less on N supplied by N fixation (Rämö *et al.*, 2006).

All these changes induced by ozone can thus affect the balance of an ecosystem. The ecological effects may be much greater than the direct effects of ozone on plant biomass. For example, the decline of forbs not only reduces the species diversity of the ecosystem, since forbs also fulfil important functions within a grassland. For example, many forbs have high mineral concentrations, and therefore have a potential beneficial health effect on grazing livestock. They have also the ability to create favourable rhizosphere conditions, because of interactions of root architecture within the soil profile,

and their root exudates interact with microbial populations to influence soil mineral dynamics (Foster, 1988). Krupa *et al.* (2004) found evidence that ozone- induced changes in foliar chemistry can result in decreased nutritive quality of herbaceous vegetation for ruminant herbivores, and Muntifering *et al.* (2006) observed that elevated ozone exposure caused reductions in the digestibility and nutritive quality of clover.

The studies on the effects of ozone on carbon cycle showed that changes in one nutrient such as ammonium may be associated with effects on other processes such as microbial respiration. This in turn could also influence other related processes of the ecosystem such as nutrient turn-over. In the case of the mire ecosystems, changes in the C- cycle, e.g. through reduced below-ground production or reduced availability of N could affect the activity of the microorganisms, which then digest organic molecules more slowly, slowing down the turn-over of nutrients and reducing availability of these nutrients. Studies from the Aspen FACE study, studying the effects of O_3 and CO_2 on trembling aspen, sugar maple and paper birch, showed that ozone significantly reduced litter production, and, although elevated ozone had only little impact on litter N and lignin concentrations, the reduced litter production led to significantly lower N and lignin fluxes to the forest floor (Liu *et al.*, 2005). Holmes *et al.* (2006) reported altered soil nitrogen transformations in the same project, in which elevated O_3 decreased the rate of gross N mineralization. This was due to changes in litter production, which they suggest could lead to a negative feedback on N availability. In addition to this, Holmes *et al.* (2003) found that ozone not only had significant negative effects on the N transformation rates but also on the microbial biomass. In a study on the interactive effects of O_3 and CO_2 on a ponderosa pine mesocosm, Olszyk *et al.* (2001) found that the number of fungal colony forming units in the AC soil horizon increased with elevated O_3 while ozone decreased the bacterial colony forming units in the C soil horizon, suggesting that this might eventually result in altered mineralization and fixation rates for soil C and N. Also, Islam *et al.*

(2000) found that elevated O_3 decreased the CO_2 flux (field soil respiration fluxes, measured in soil respiration chambers in OTCs) by 20- 36% in their study of interactions of tropospheric CO_2 and O_3 enrichment and moisture variations on microbial biomass and respiration in soil. In contrast to these earlier studies, Mörsky *et al.* (2008) found an increase in microbial biomass under elevated ozone in their long- term peatland study. They also found a similar trend in concentrations of organic acids.

The main microbiological processes in a peatland, such as methane production and methane consumption, are driven by soil aeration, heat transport and organic matter mineralisation (Segers, 1998). Niemi *et al.* (2002) observed a significant increase in methane fluxes under elevated ozone, which they explained by either higher primary production or stimulation via released substrates, such as root exudation, decay and litter input or facilitated transport through *E. vaginatum* shoots. Rinnan *et al.* (2003) showed that the significantly higher methane emissions only occurred in peat microcosms with *E. vaginatum*. This suggested that the sedge was mainly responsible for higher methane emissions and that this was due to transport via its aerenchyma. The main role of *E. vaginatum* in methane flux was also reported by Greenup *et al.* (2000).

In this study, only a marginal decrease of methane fluxes was observed in elevated ozone, which is partial in agreement with the field- based results of Mörsky *et al.* (2008). They observed temporarily decreased methane emissions by elevated ozone. The methane emissions were decreased during the first growing season in 2003 but showed consistent higher methane emissions in the elevated treatment during the growing seasons 2004- 2006. They explained the reduced methane emissions with an observed decrease in photosynthesis in vascular plants. In contrast to their study, in this study the reduced methane fluxes were also observed in the years before (significant reductions in 2005 and 2006). Reduced methane emissions in this study could be due to a negatively affected net primary production. It has been shown that net primary

production is positively correlated with methane emissions (Whiting & Chanton, 1993; King *et al.*, 2002), and effects of ozone on photosynthesis could alter the substrate availability for methanogens, furthermore, methane emissions.

Peatland ecosystems are of special importance for the global carbon cycle, and because of the expected long-term increase in northern hemisphere ozone background concentrations in remote areas where peatlands are increasingly exposed to elevated ozone concentrations. The CO_2 sink/ CH_4 source balance of peatland ecosystem might be negatively affected by ozone. The results from this study suggest that elevated ozone decreases methane emissions which would be for global warming. This has also been reported by West *et al.* (2006) for instance. However, elevated ozone and methane are both greenhouse gases, which rank only behind CO_2 in their contributions to anthropogenic climate forcing (Ramaswamy *et al.*, 2001), and rising temperatures due to the global climate change could enhance the methane oxidation for instance (Del Grosso *et al.*, 2000). Climate change can lead to important feedback processes, which will affect the production of ozone precursors such as VOC from vegetation, changes in the production of lightning NOx, the release of methane from wetlands and the melting of arctic permafrost, the production of soil NOx, and the uptake of ozone by vegetation (Royal Society, 2008). Hence, any effect of ozone needs to be set in context to effects of temperature and increasing methane.

The extent of ozone fluxes to any kind of vegetation depends on the ozone concentration level at plant height and the stomatal conductance of the receptor plant. To estimate ozone concentration levels at plant height, measured ozone concentrations (often at 3 up to 50 meter above ground) are usually scaled down to the plant level under consideration of the atmospheric and boundary layer resistance. The stomatal conductance is site- and species-specific and depends on the phenological state of the receptor plant as well as on the

concurrent meteorological conditions, the soil water conditions and the ozone concentration (as it can directly harm the functioning of the stomata) (Bassin *et al.*, 2004). If the ozone fluxes will be calculated for the entire plant canopy, the LAI is also of high importance.

It can be expected that some of the findings of this study will help to further improve the development and performance of ozone flux models, such as the findings on the vertical ozone profile, the varying exposure to O_3 of the three plant functional groups due to their different contribution to the LAI at different canopy heights and the stomatal behaviour of various semi-natural vegetation species. Applications of flux models to semi-natural grasslands are still in development and have been tested only with a few species (Ashmore *et al.*, 2007). More data on this will make future risk assessments of ozone more realistic.

Jaeggi *et al.* (2006) found that the turbulence intensity above the canopy was the main direct environmental controlling parameter for the in-canopy O_3 profiles. During their analysis of O_3 profiles in a productive grassland, they found that grass species at 0.5 m were exposed to 92%, and legume species at 0.25 m to 64% of the ozone concentrations above the canopy, which is in accordance with this study. Likewise, most of the active leaf area was found in the lower half of the canopy. This vertical structure of the grassland canopy requires a two- or multilayer approach for ozone flux modelling, which takes account of the decreasing exposure to ozone inside the canopy (Baldocchi, 1998). Irradiance and spectral quality also affect stomatal conductance, which vary with canopy height, density and leaf area index (Davison *et al.* 2003) Ozone flux models are currently based on data on upper canopy leaves, and methods for whole canopy flux to grasslands are under development, with preliminary models excluding any effects of phenology and ozone on conductance (Ashmore *et al.*, 2007). Furthermore, the Jarvis- type multiplicative ozone flux models (Fuhrer, 2009) rely on defining the species-specific maximum stomatal conductance from primary or secondary data.

There are a few short-term studies on *T. repens* (Degl'Innocenti *et al.*, 2003; Crous *et al.*, 2006; Francini *et al.*, 2007) which have reported an enhanced stomatal closure when exposed to elevated O_3 concentrations. Jaeggi *et al.* (2005) found that elevated O_3 led to reduced gs and increased $\delta^{13}C$ in *Holcus lanatus, Plantago lanceolata* and *Trifolium pratense* (but not in *Ranunculus friesianus*) from non-irrigated plots. In this study, reductions in gs were found for *Briza media* and *Trifolium repens,* but not for the grass *Holcus lanatus.* This difference in the findings for *H. lanatus* could be due to the different field conditions; the field site at High Keenley Fell proved to be a water-saturated field site during the summer of 2007, which was not the case for the study of Jaeggi *et al.* (2005).

5.3 Limitations of the study

The cover of different plant species was only measured once, at the start of the experiment. It would have been insightful to repeat it at the start of the second year of fumigations, as this would have helped to assess whether the large effects of ozone on the forb species could be explained by differences at the start of this year. Also, the distribution of the forbs varied greatly between plots, which made it difficult to test effects on forbs for statistical significance. As the conditions of normality could not be met, these species had to be tested non-parametrically.

The study had to face technical problems. The porometers used had to working under high humidity conditions, which made it difficult to calibrate them. More detailed micro-meteorological monitoring next to the 2B monitors and the PAR probes at different canopy heights would have been useful to interpret the ozone profiles more clearly. Also, a longer time period for measuring the ozone profile and the stomatal conductance would have contributed to a better understanding of ozone flux in this grassland system.

Another limitation of the study was the use of a gradient FACE system and not a ring. The FACE ring provides controlled ozone exposure over a certain area every day, while the gradient system is dependent on the wind direction, and does not provide a consistent ozone exposure. Other limitations were problems with the software and the fumigation systems, which were responsible for very little fumigation occurring within the first year.

The litter experiment using collected litter from ambient and elevated treatment soaked with ambient and/or elevated ozone treated soil water was limited. As the mire mesocosms were in their final year, and the rhizosamplers membrane was so fine that microorganisms were retained from the soil water this way, the soil water for this experiment was only sampled once with destructive methods.

Studies on the impact of ozone on semi- natural ecosystems benefit from a longer duration of the study, and the free air fumigation studies give different and better insights into the responses of real ecosystems. In contrast, open- top chamber studies only partly simulate the environment, and also create side effects such as an altered microclimate through forced ventilation, although they still provide valuable information on ozone effects on ecosystems (Volk *et al.*, 2003, Bassin *et al.*, 2006). Furthermore, the few long- term studies in open-air fumigation systems on natural occurring ecosystems such as the Alpflix study in Switzerland or the Aspen FACE project in the USA provided new insights into how ozone affects not only one constituent of the ecosystem, but also how different but inter-related environmental processes are also affected. The Aspen FACE project showed how litter decomposition, nitrogen transformations and microorganisms respond to elevated ozone concentrations, carbon dioxide concentrations and their interaction in a forest ecosystem (Holmes *et al.*, 2003, 2006; Lindroth *et al.*, 2001; Liu *et al.*, 2005). The Alpflix demonstrated the effect of Nitrogen addition and elevated ozone and their interaction on a sub- alpine grassland ecosystem (Bassin *et al.*, 2007, 2009). These studies stress the importance of community scale studies on a long- term

basis under free air fumigation. Hence, it is important to continue such long-term studies of effects of ozone, and to carry out more in-depth studies which look at effects of ozone at different levels of the ecosystem and try to link them.

5.4 Suggestions for future work

Higher concentrations of ozone seemed to affect the specie *Rhinanthus* minor in particular, a hemi-parasite which is known to modify the composition of the grassland and therefore to increase its diversity. A strong trend of a declining biomass with ozone treatments was found for this species, but it could not be shown to be statistically significant.

An interesting aspect for future work would be to study the relationship between the hemi-parasite and the host. Thwaites (1996) found that the root biomass of *Trifolium pratense* and *Festuca rubra* increased when parasitized by *R. minor* in ambient air, whereas their root biomass decreased when parasitized in ozone. Parasites such as *R. minor* are defined as keystone species in grasslands as they represent the "ecosystem engineers". They modulate the availability of resources by causing physical state of changes in biotic and abiotic materials (Jones *et al.*, 1994). Root hemi- parasites often occur in nutrient poor communities and their effects on nutrient cycling within such ecosystems can be considerable, as they unlock nutrients from less available forms into more labile, available forms (Press, & Phoenix, 2005).

Effects of ozone on roots and stolon biomass were not examined. However, such effects have been reported for clover, for example, and they could play an important role in modifying the nutrient cycle. Additional studies on the nutrient cycle would have been valuable, especially since the mire mesocosm study showed indirect effects of ozone on the carbon cycle.

The soil water samples of the mire mesocosm study showed interesting changes in the seasonal patterns of ammonium concentration, but, as there was no data

available from previous years, it is not clear if this is a consistent effect of ozone. As methanogens produce methane and the methanotrophic bacteria consume methane, it would have been valuable to study, if the bacterial communities were affected by elevated ozone. Another missing part of this study, in addition to effects on the below-ground microbial community profile, was how the roots and the aerenchyma, which are all of great importance to carbon cycling in this ecosystem, are affected by ozone. Furthermore, additional carbohydrate analyses of the litter would give a better insight into the causes of the reduced microbial activity in ozone, as either quantity or quality of the litter might be responsible for the changes.

5.5 Overall conclusions

In this study elevated ozone has shown to:

1. Modify communities, by altering their composition, and by affecting functional groups and individual species negatively. These modifications might have been influenced by other factors, such as available nutrients. The responses to ozone of the individual species do only partly reflect the response of the plant community to ozone.

2. Have an effect on nutrients and on the nutrient cycle, by changing indirectly the soil chemistry. As nutrient availability is altered, only certain species or functional groups may benefit from the changed conditions, thereby enhancing competition with other species. More long- term studies of ozone on communities that assess above- and belowground processes are needed.

3. Affect the methane fluxes, the microbial respiration and the biomass of species from wetland ecosystems. Methane emission and dark respiration are closely related to the presence of *E. vaginatum*. A reduction in biomass of the sedge might be related to a reduced microbial activity and a suppression of nutrient turn- over. Elevated ozone and methane are both greenhouse gases. A warmer climate might enhance methane oxidation and affect the balance of the CO_2 sink/ CH_4 source of the mire ecosystem negatively. More studies of climate effects on peatland C fluxes that induce ozone are needed.

4. Have an effect on the stomatal conductance of individual species. This study suggests that short-term peak exposures and gradients of ozone within the canopy could also be important in determining the correct ozone flux to grasslands. Current grassland flux models do not sufficiently take into account the direct effect of ozone on the stomatal functioning of various semi-natural vegetation species, nor the vertical distribution of the biomass within the canopy (expressed as layer-specific LAI). These factors have to be implemented in ozone risk assessment studies based on the flux approach.

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Ref Type: Generic

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7. Appendices

7.1. Appendix 1

Additional diagrams of the soil analysis at High Keenley Fell in April 2007:

Figure 94 shows the nitrate concentrations across the plots. Both significant O_3 and transect effects were found, although the data show large variation between plots. At 10-20 cm, the nitrate concentrations were significantly greater in T3 than in T1, while at 0-10 cm, the nitrate concentrations were significantly higher in the control plots than in the10 ppb plots.

Figure 95 shows the ammonium concentrations across the plots. There was only a significant effect of transect at 0-10 cm, the ammonium concentrations were significantly greater in T3 and in T1 than in T2 (T3> T2; T1> T2). There was a large variation between values at 0-10 cm in T1.

Figure 96 shows the phosphorus concentrations across the plots. As for NH_4^+, there was only a significant effect of transect at 10-20 cm, the phosphorus concentrations were significantly greater in T3 than in T2 and than in T1 (T3> T2; T3> T1). There was a large variation between values, and they were generally higher in T3.

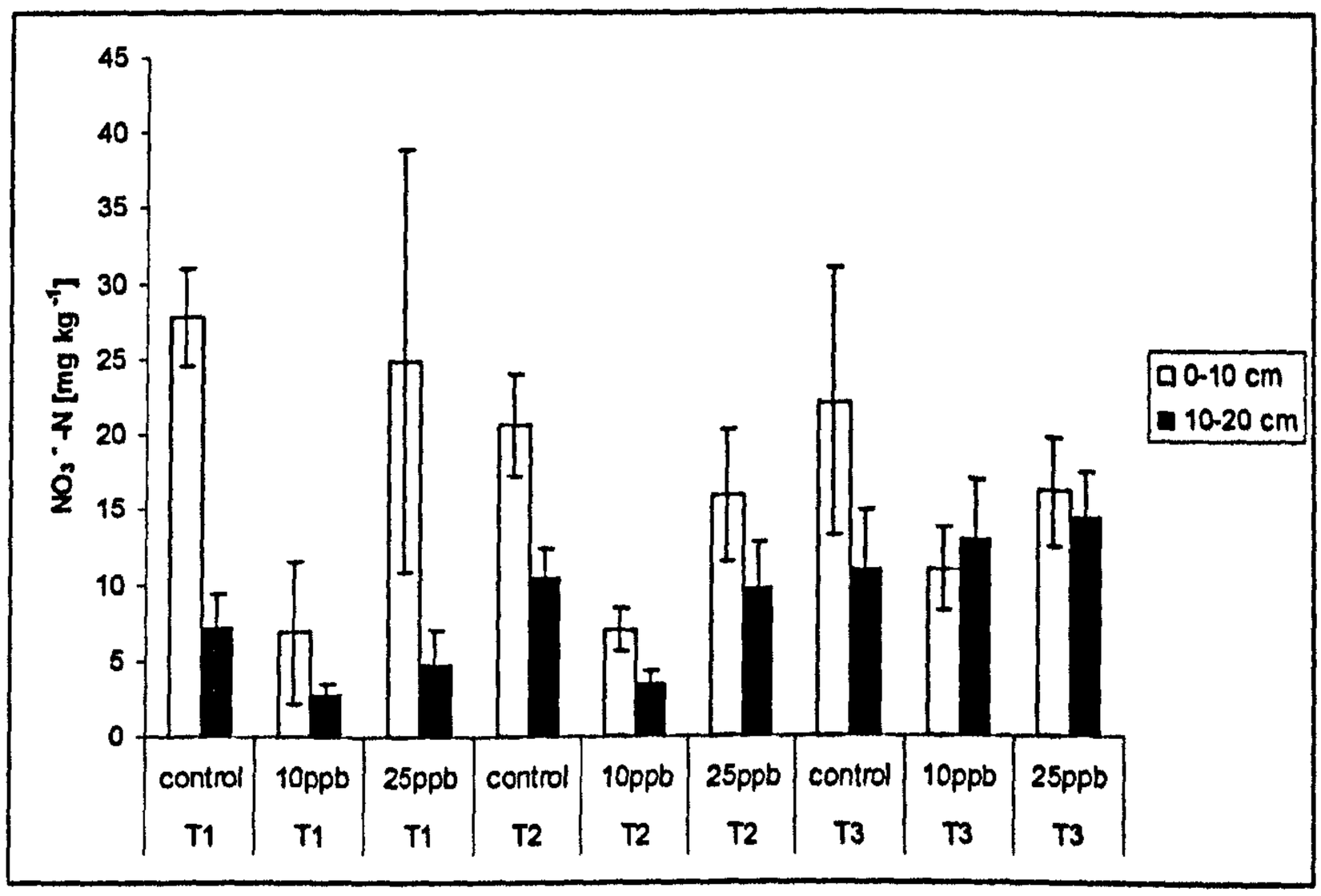

Figure 94: The mean extractable nitrate concentration (mg kg^{-1}) in April 2007 in the nine combinations of transect and treatment. Error bars represent standard errors between replicate sub-plots.

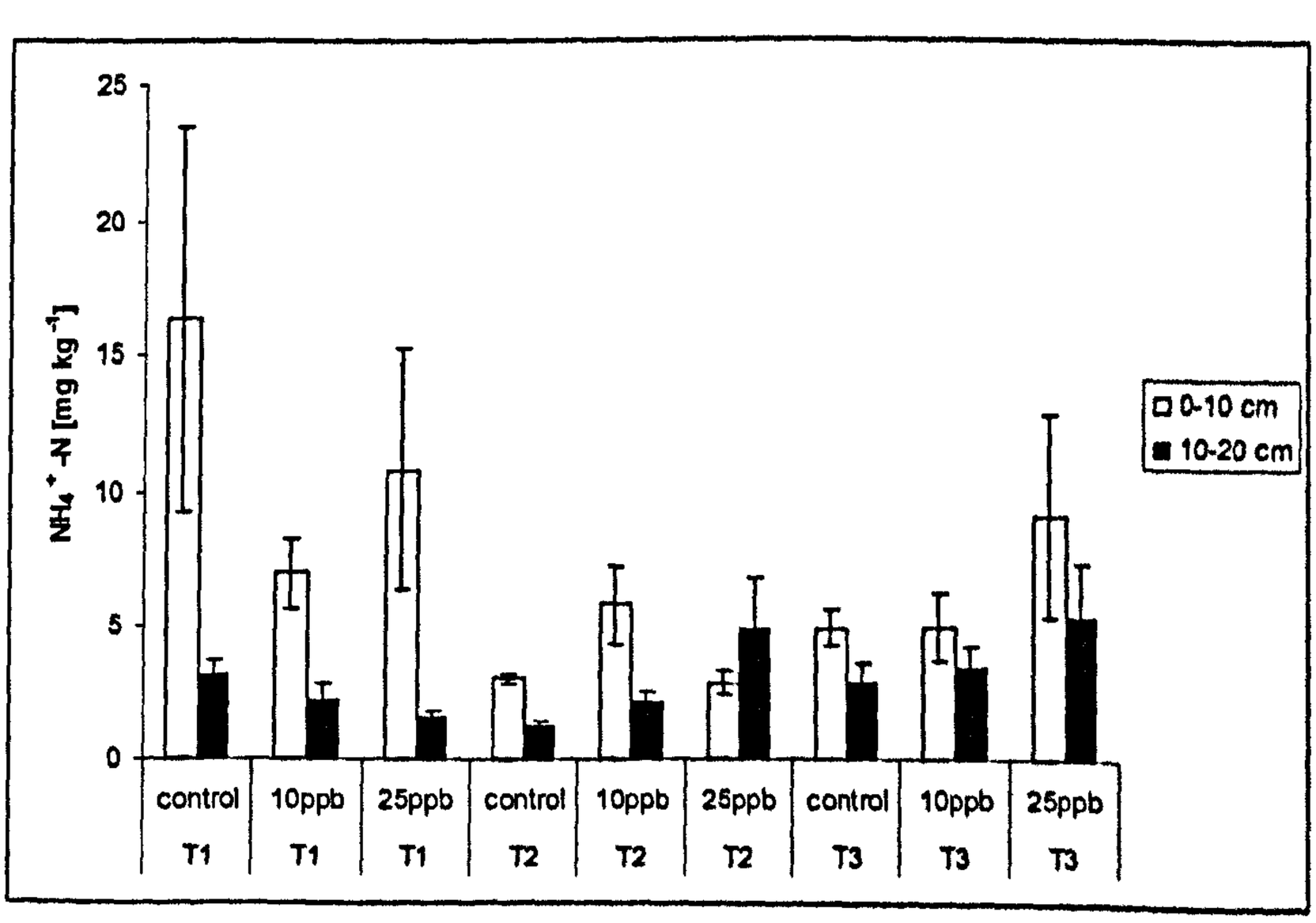

Figure 95: The mean extractable ammonium concentration (mg kg^{-1}) in April 2007 in the nine combinations of transect and treatment. Error bars represent standard errors between replicate subplots.

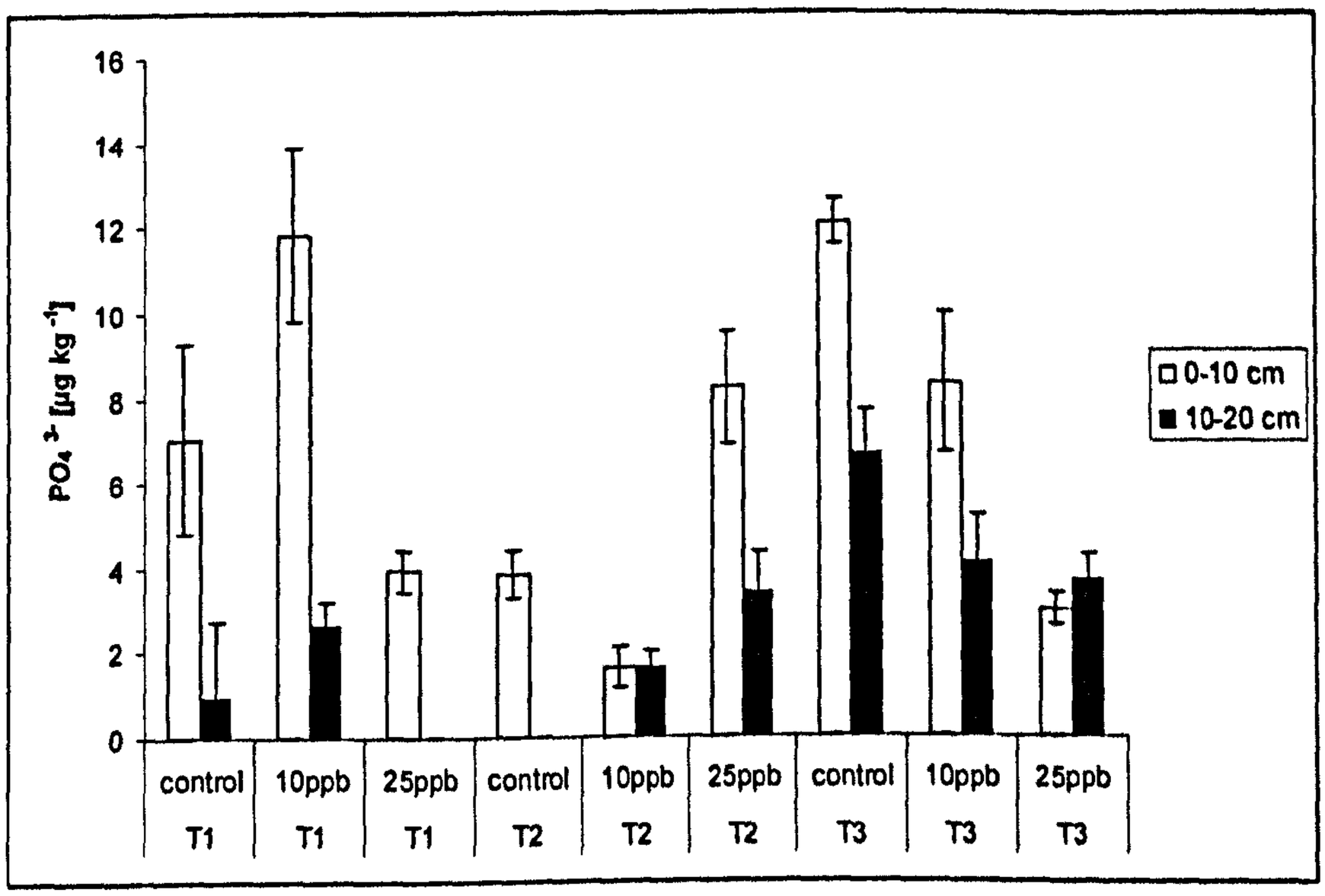

Figure 96: The mean plant available phosphorus concentration (µg kg^{-1}) in April 2007 in the nine combinations of transect and treatment. Error bars represent standard error between replicate subplots.

Figure 97 shows the potassium concentrations across the plots. Only transect effects were significant at 0-10 cm, the potassium concentrations were significantly greater in T1 than in T2 and than in T3, and T2 was also significantly lower than T3 (T1> T2 and T3; T3> T2). There was considerable variation between plots at 0-10 cm, but not much variation between the values was observed at 10-20 cm.

Figure 98 shows the calcium concentrations across the plots. Both, significant O_3 and transect effects were found. At 0-10 cm, the calcium concentrations were significantly greater in T3 than in T2 and than in T1. The calcium concentrations found at 10-20cm were also significantly greater in T3 than in T2 and than in T1 also. At 0-10cm, the calcium concentrations were significantly higher in the control plots than in the10 ppb plots (and the 25 ppb plots). The variation between plots for calcium was relatively low compared to other determinants.

Figure 99 shows the pH across the plots. There was no significant effect of transect. At 10-20 cm, pH was significantly lower in both the control and 10 ppb plots than in the 25 ppb plots.

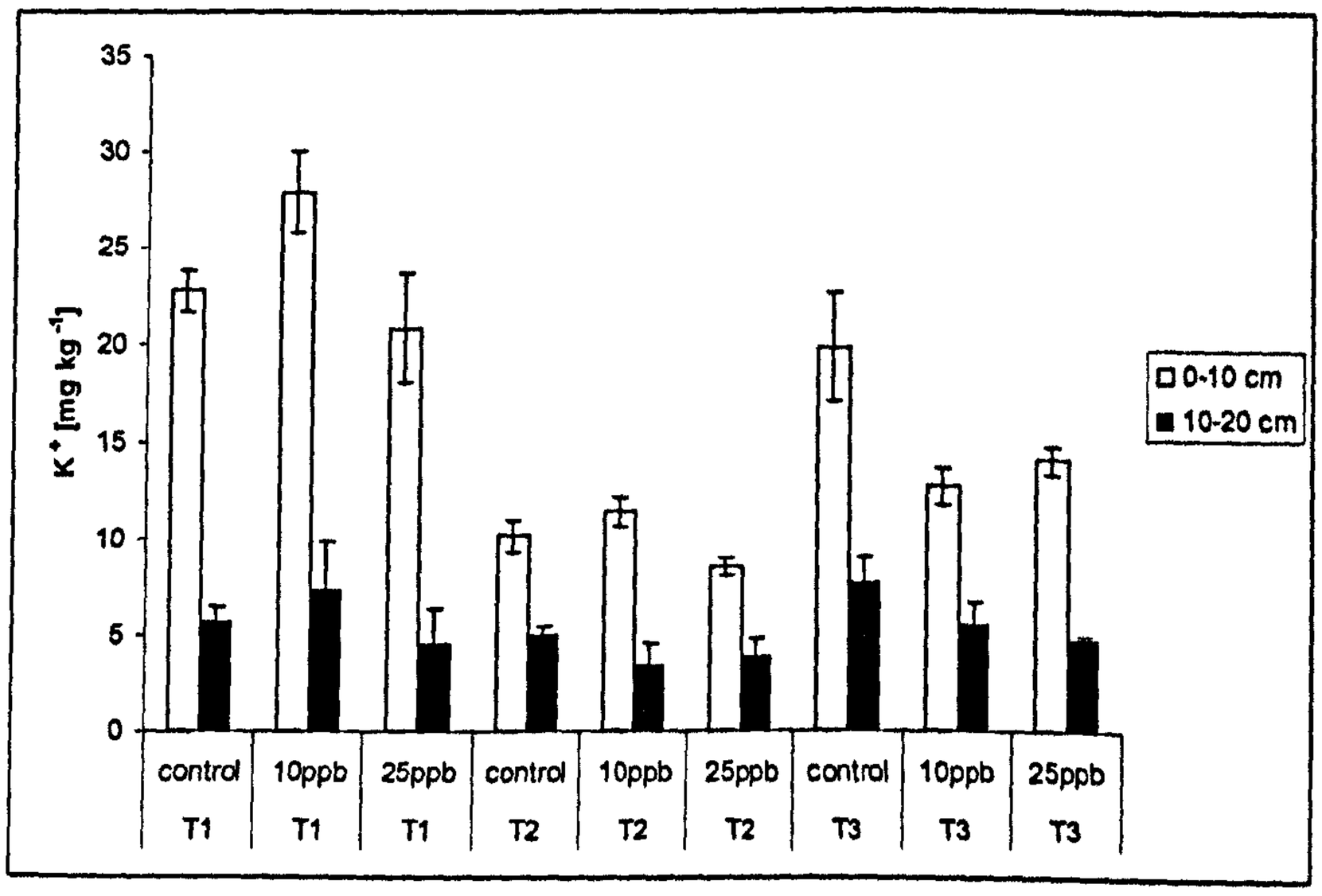

Figure 97: The mean plant available potassium concentration (mg kg^{-1}) in April 2007 in the nine combinations of transect and treatment. Error bars represent standard errors between replicate subplots.

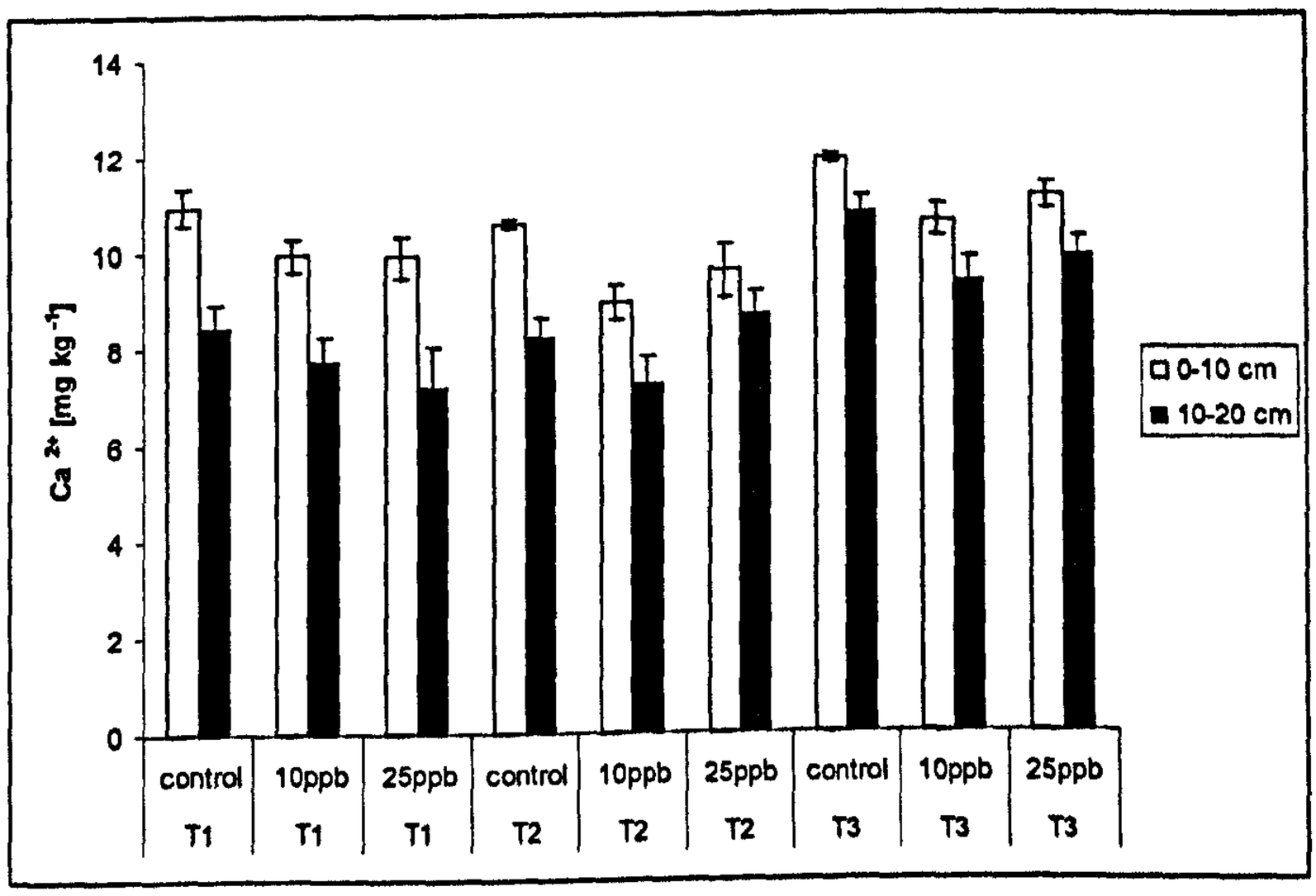

Figure 98: The mean plant available calcium concentration (mg kg^{-1}) in April 2007 in the nine combinations of transect and treatment. Error bars represent standard errors between replicate subplots.

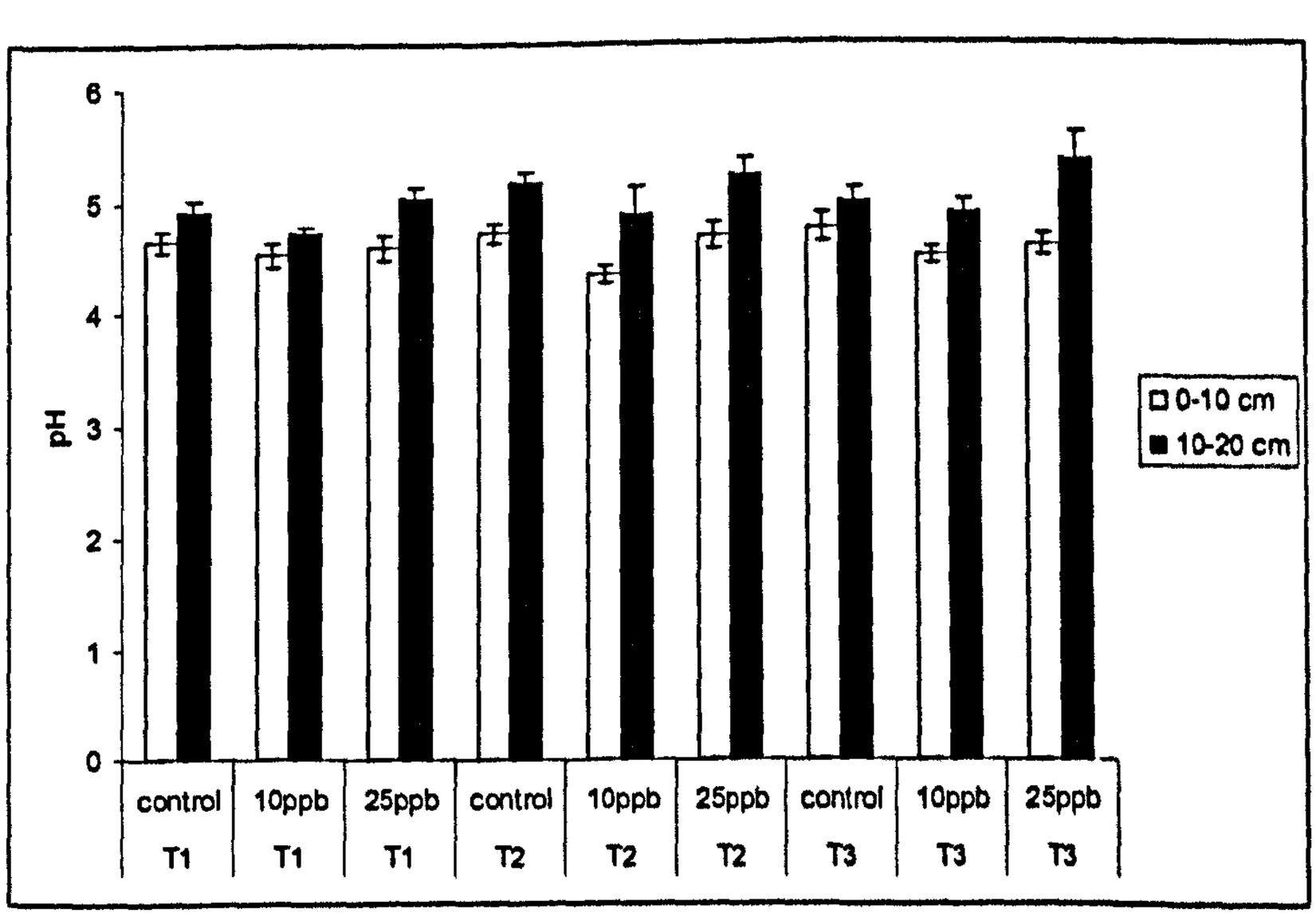

Figure 99: The mean pH in April 2007 in the nine combinations of transect and treatment. Error bars represent standard errors between replicate subplots.

Figure 100 shows the soil water content across the plots. Both, significant O_3 and transect effects were found. At 10-20 cm the soil water content was significantly greater in T3 than in T2. In terms of ozone treatments, at 0-10 cm the soil water content was significantly higher in the control plots than in the10 ppb and the 25 ppb plots. At 10-20 cm, the SWC in the control treatments was significantly higher than that in the 10 ppb treatment.

Figure 101 shows the organic matter content across the plots. Both, significant O_3 and transect effects were found but only at 10-20 cm. The organic matter content was significantly greater in T3 than in T1 and than in T2, while the organic matter content was significantly higher in the control plots than in the 10 ppb plots and the 25 ppb plots.

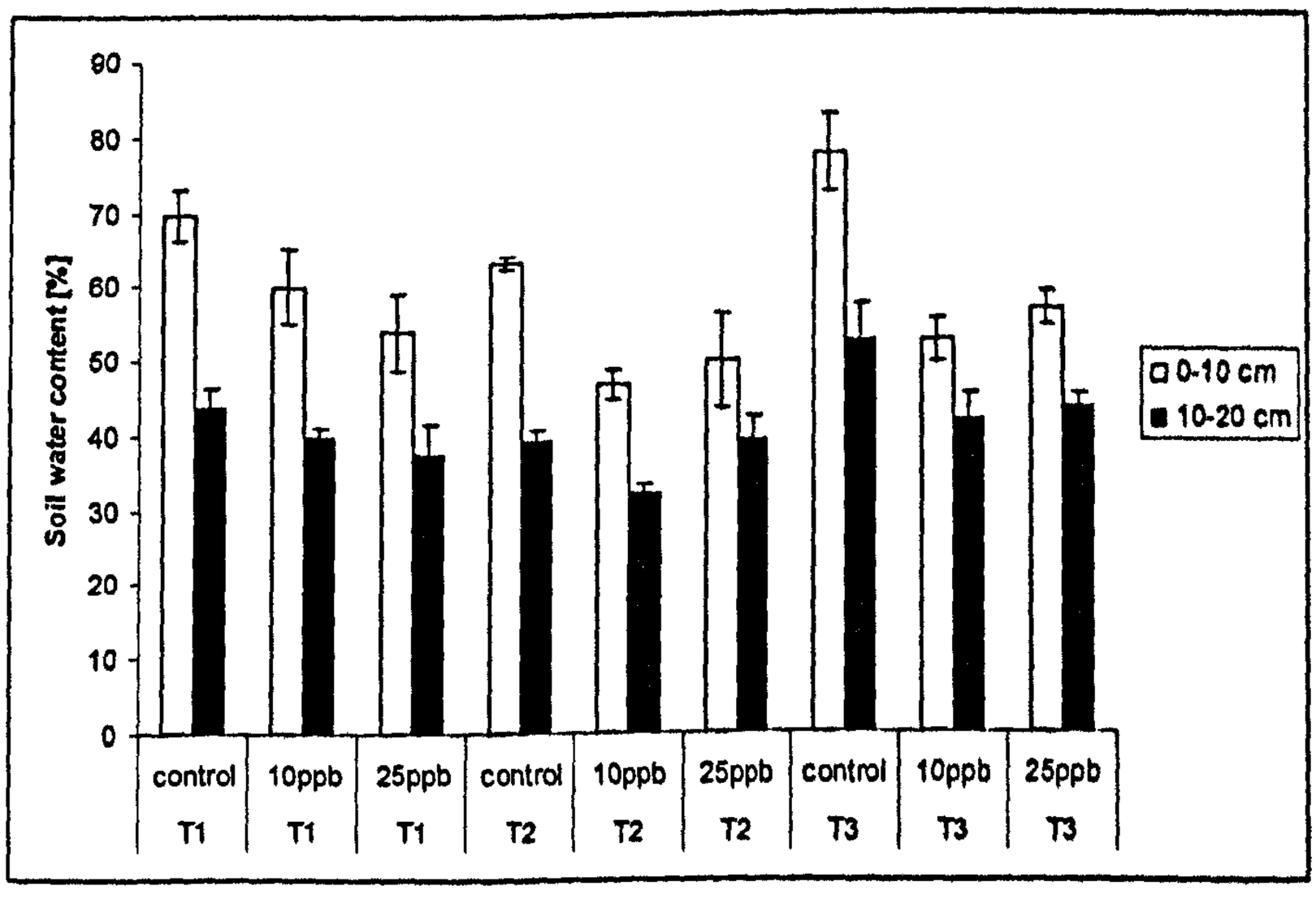

Figure 100: The mean soil water content (%) in April 2007 in the nine combinations of transect and treatment. Error bars represent standard errors between replicate subplots.

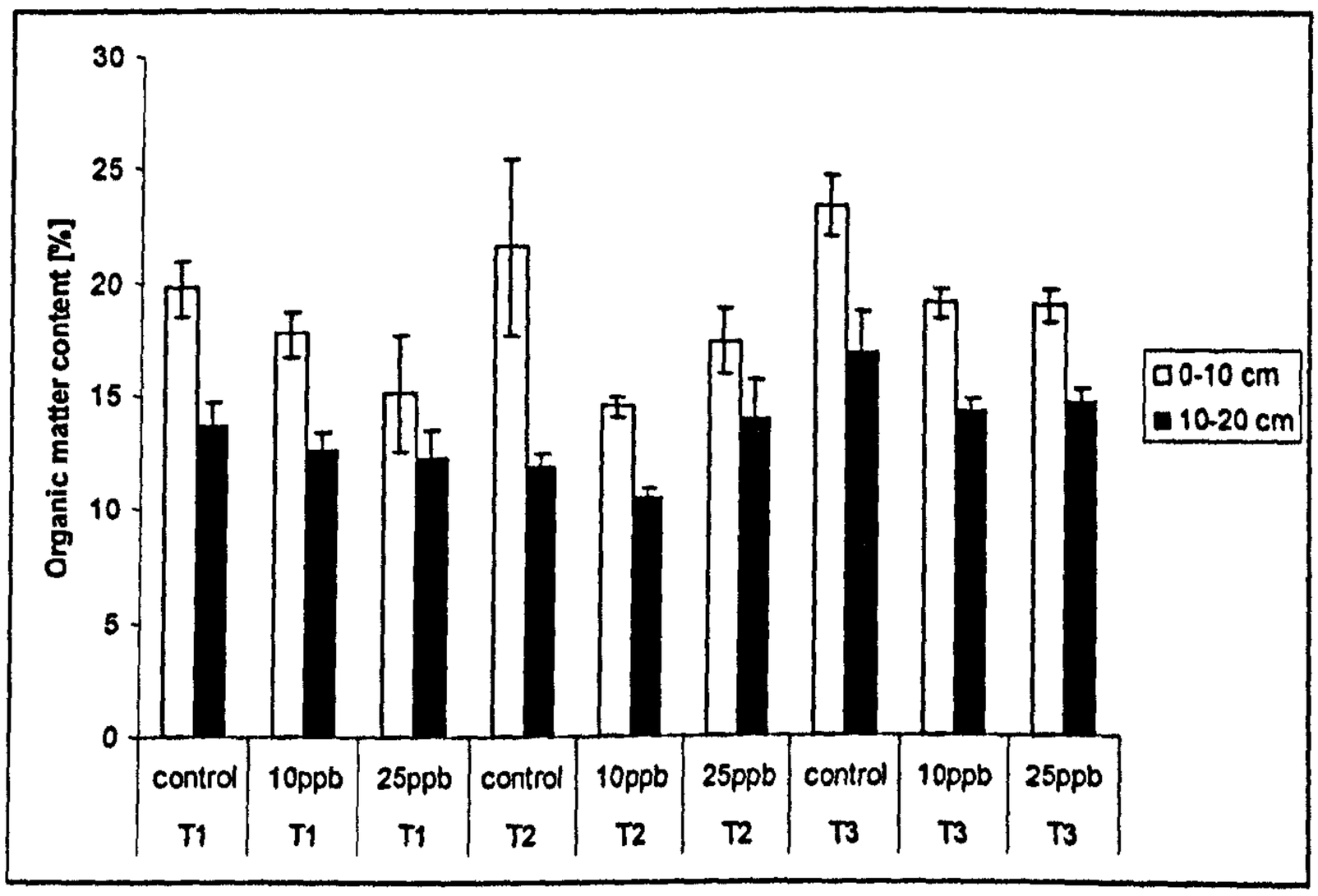

Figure 101: The mean organic matter (%) in April 2007 in the nine combinations of transect and treatment. Error bars represent standard errors between replicate subplots.

7.2. Appendix 2

Additional RDA-diagrams of the multivariate analysis, using O_3 as a cumulative environmental variable:

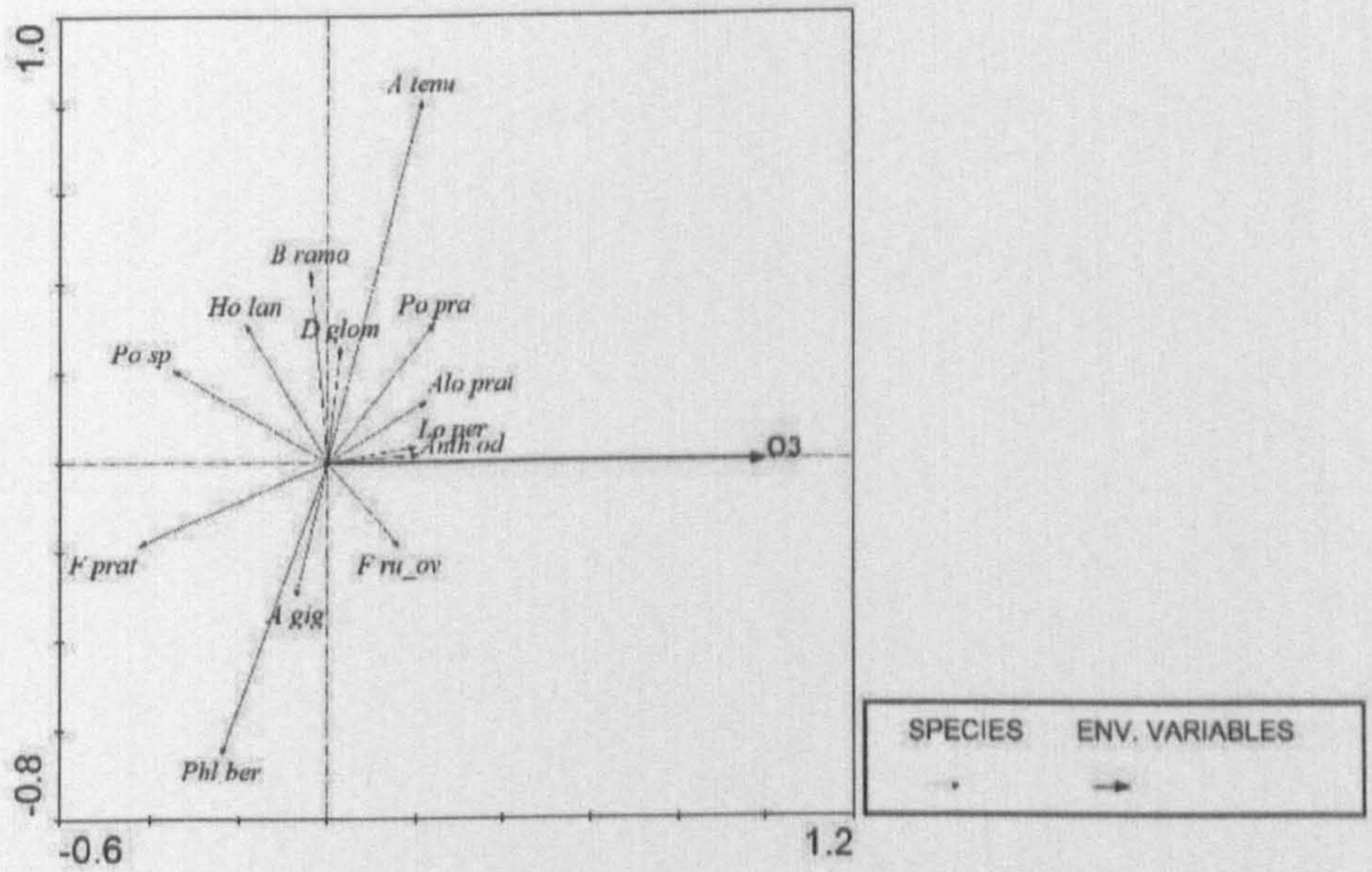

Figure 102: Redundancy analyses of the harvest in 2007. Cumulative O_3 exposure is fitted as the environmental variable. Species codes are: *A tenu= Agrostis tenuis, A gig= Agrostis gigantea, A stol= Agrostis stolonifera, Alo prat= Alopecurus pratensis, Anth od= Anthoxanthum odoratum, D glom= Dactylus glomerata, F prat= Festuca pratensis, F ru_ov =Festuca rubra/ovina, Ho lan= Holcus lanatus, Lo per= Lolium perenne, Phl ber= Phleum bertolonii, Phl pra= Phleum pratense, Po pra= Poa pratensis, Tris fla= Trisetum flavescens, Ju eff= Juncus effuses*

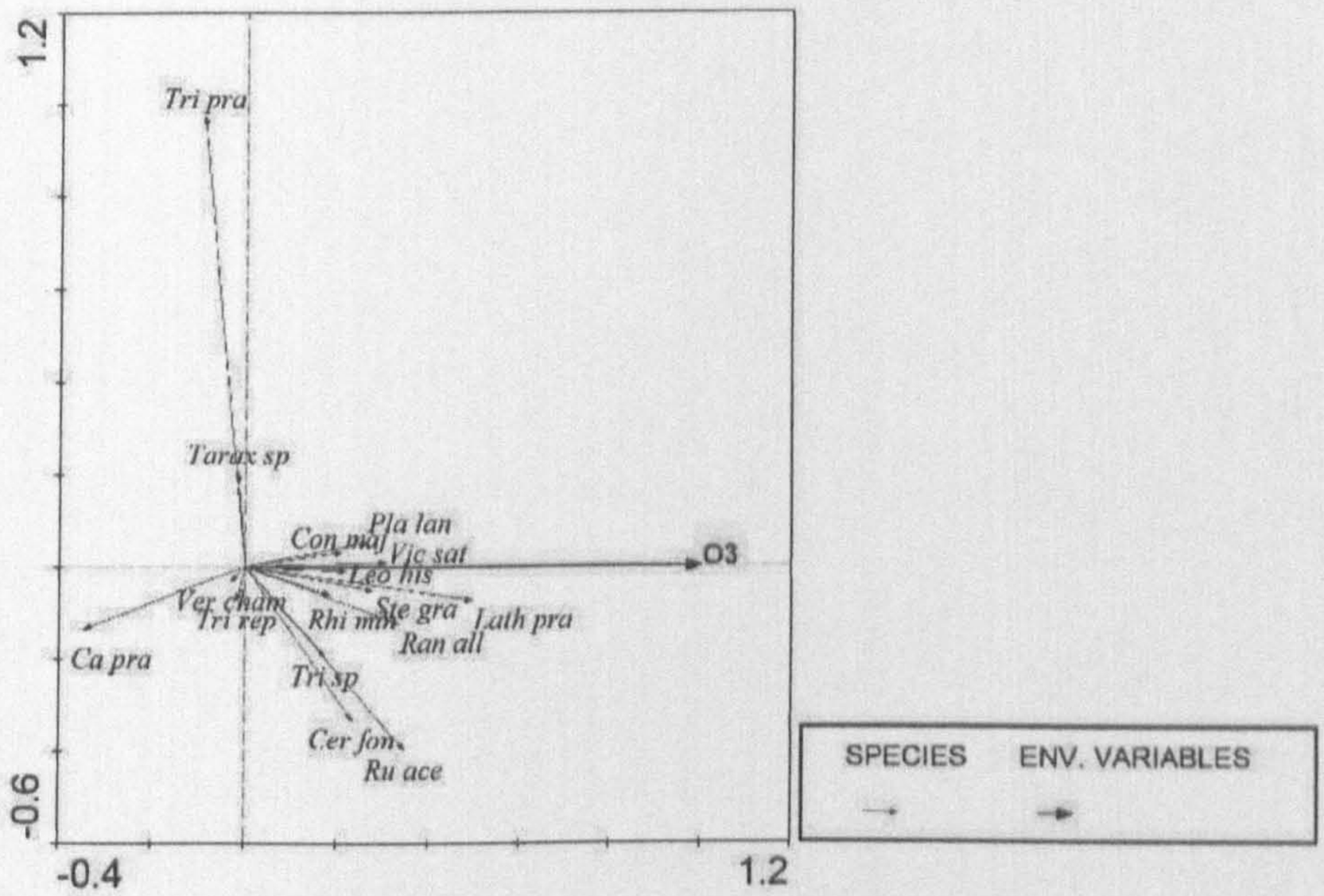

Figure 103: Redundancy analyses of the harvest in 2007. Cumulative O_3 exposure is fitted as the environmental variable. Species codes are: *Ca pra= Cardamine pratensis, Cer fon= Cerastium fontanum, Con maj= Conopodium majus, Lath pra= Lathyrus pratensis, Leo his= Leontodon hispidus, Pla lan= Plantago laneolata, Ran all= Ranunculacae, Rhi min= Rhinanthus minor, Ru ace= Rumex acetosa, Tarax sp= Taraxacum spec., Tri pra= Trifolium pratense, Tri rep= Trifolium repens, Ste gra= Stellaria graminea, Vic sat= Vicia sativa, Ver cham= Veronica chamaedrys.*

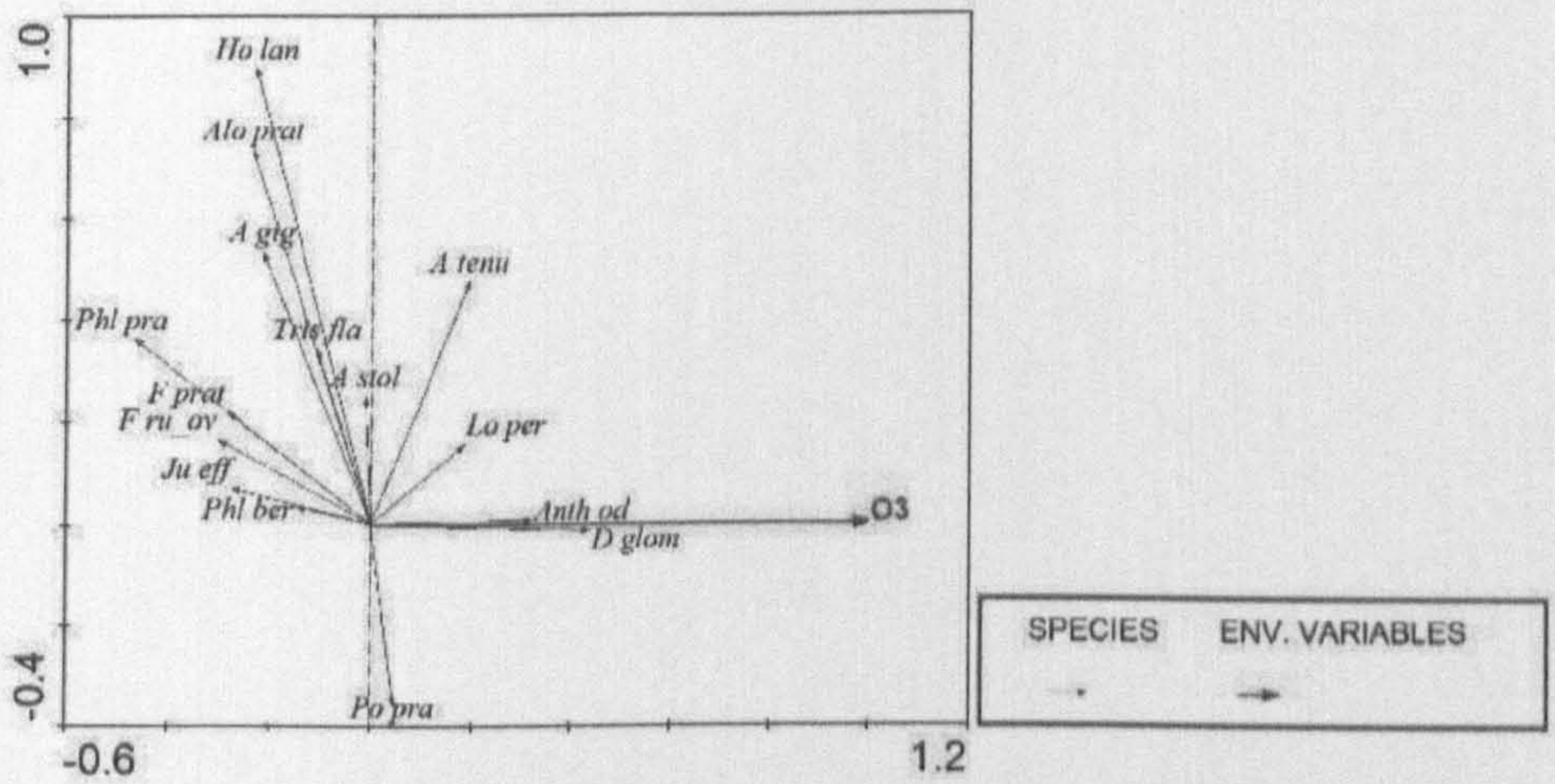

Figure 104: Redundancy analyses of the harvest in 2008. Cumulative O_3 exposure is fitted as the environmental variable. Species codes are: *A tenu= Agrostis tenuis, A gig= Agrostis gigantea, A stol= Agrostis stolonifera, Alo prat= Alopecurus pratensis, Anth od= Anthoxanthum odoratum, D glom= Dactylis glomerata, F prat= Festuca pratensis, F ru_ov= Festuca rubra/ovina, Ho lan= Holcus lanatus, Lo per= Lolium perenne, Phl ber= Phleum bertolonii, Phl pra= Phleum pratense, Po pra= Poa pratensis, Tris fla= Trisetum flavescens, Ju eff= Juncus effusus*

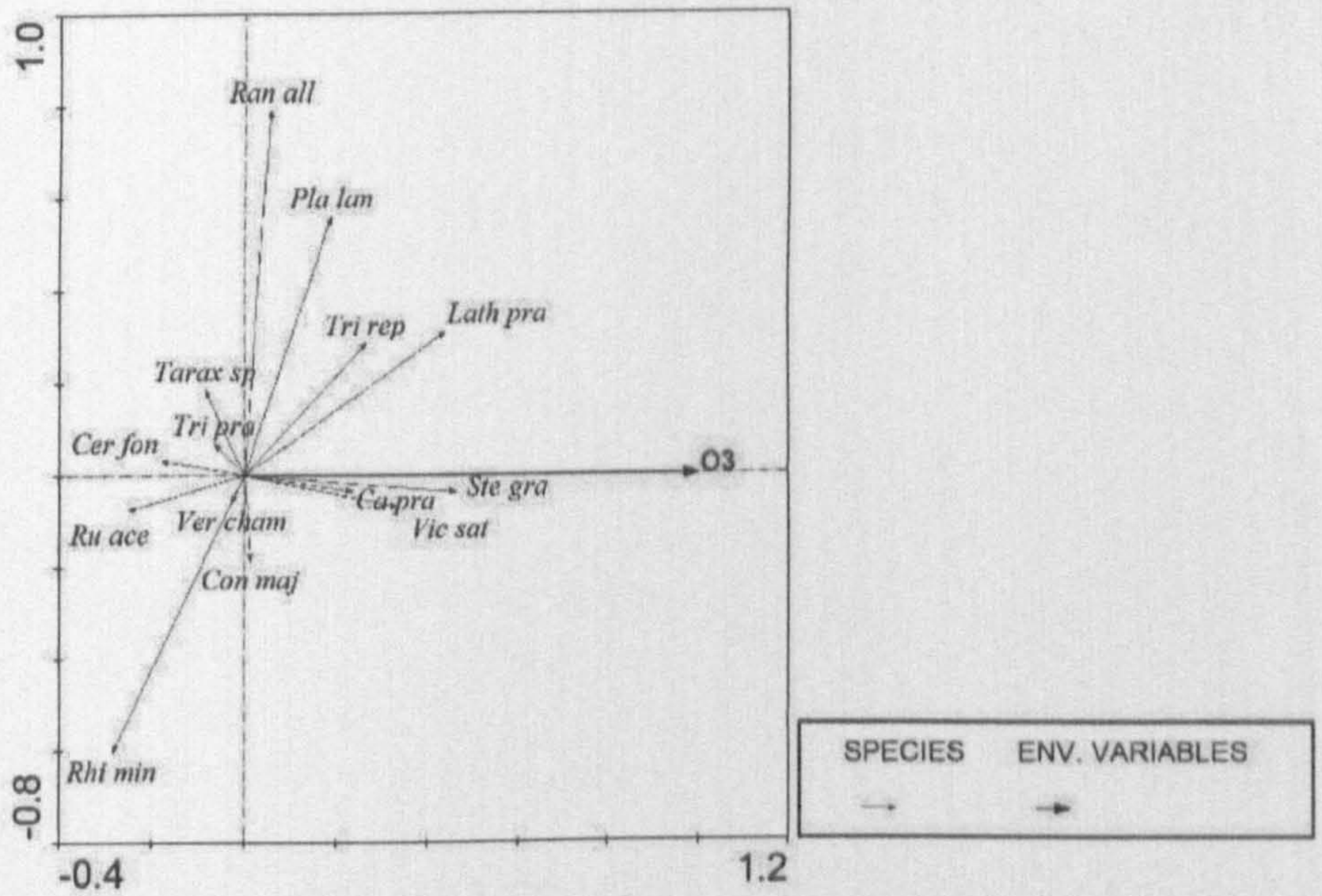

Figure 105: Redundancy analyses of the harvest in 2008. Cumulative O_3 exposure is fitted as the environmental variable. Species codes are: *Ca pra= Cardamine pratensis, Cer fon= Cerastium fontanum, Con maj= Conopodium majus, Lath pra= Lathyrus pratensis, Leo his= Leontodon hispidus, Pla lan= Plantago laneolata, Ran all= Ranunculacae, Rhi min= Rhinanthus minor, Ru ace= Rumex acetosa, Tarax sp= Taraxacum spec., Tri pra= Trifolium pratense, Tri rep= Trifolium repens, Ste gra= Stellaria graminea, Vic sat= Vicia sativa, Ver cham= Veronica chamaedrys.*

7.3. Appendix 3

Additional diagrams of the soil water analysis of the grassland mesocosms at close house in July and September 2008:

Shortly after the cut the ozone treatment and the nutrient treatment significantly increased the pH at 0-10 cm, which was about 6 to 7% significantly lower in the elevated ozone treatment and the low nutrient treatment. Two and a half months later the pH was still significantly influenced by the low nutrient treatment but not by the ozone treatment. The pH showed a decrease in the ozone treatment and in the high nutrient treatment, which was significantly lower in the elevated treatment and in the high nutrient treatment (Table 51, Table 52; Figure 106, Figure 109). Effects of ammonium concentration were only found for the nutrient treatment which was higher in the low nutrient treatment at 0- 10cm on 22nd July; on 12th September no effects on ozone and nutrients were observed (Table 52, Figure 108). The nitrate concentrations were not affected by the treatments. However, significant chamber effects were found for the pH and ammonium concentration at 10-20 cm on 22nd July and at 0-10 cm on 12th September (Table 52) As the chambers effects do not fall together with the ozone and nutrient effects just after the cut and as also the chamber effects 2 1/2 months later were too inhomogeneous to be significant, these effects will be neglected., which is illustrated in Table 53.

Table 51: Effects of ozone and nitrogen on the soil in July 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant, n.a. = not available).

22nd July	*O3-effect*	*N-effect*	*Ozone*Nitrogen*
0-10 cm			
pH	**0.003**	**0.027**	0.369.
NH_4^+-N concentrations	0.197	**0.014**	0.756
NO_3^--N concentrations	0.610	0.443	0.547
10-20 cm			
pH	0.288	0.135	0.580
NH_4^+-N concentrations	0.247	0.867	0.393
NO_3^--N concentrations	0.931	0.659	0.552
Post-hoc			
0-10 cm			
pH	50ppb>30ppb	LN>HN50ppb >30ppb	ns
NH_4^+-N concentrations	ns	LN>HN	ns
NO_3^--N concentrations	ns	ns	ns
10-20 cm			
pH	ns	ns	ns
NH_4^+-N concentrations	ns	ns	ns
NO_3^--N concentrations	ns	ns	ns

Table 52: Effects of ozone and nitrogen and on the soil in September 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant, n.a. = not available).

12th September	*O3-effect*	*N-effect*	*Ozone*Nitrogen*
0-10 cm			
pH	0.412	**0.006**	0.838
NH_4^+-N concentrations	0.283	0.489	0.396
NO_3^--N concentrations	0.814	0.376	0.702
10-20 cm			
pH	**0.001**	**0.046**	0.797
NH_4^+-N concentrations	0.180	0.627	0.895
NO_3^--N concentrations	0.136	0.220	0.091
Post-hoc			
0-10 cm			
pH	ns	LN>HN	ns
NH_4^+-N concentrations	ns	ns	ns
NO_3^--N concentrations	ns	ns	ns
10-20 cm			
pH	50ppb<30ppb	LN>HN	ns
NH_4^+-N concentrations	ns	ns	ns
NO_3^--N concentrations	ns	ns	ns

Table 53: Results of the ANOVA on chamber effects on 22nd July and 12th September (Chambers 1, 4 =elevated, Chambers 2, 3 = ambient)) on the soil in the summer 2008. Post-hoc differences are significant at P< 0.05 (ns = non significant, n.a. = not available).

P	*22nd July*	*12th September*
0-10 cm		
pH	0.552	**0.07**
NH_4^+-N concentrations	0.284	**0.043**
NO_3^--N concentrations	0.143	0.319
10-20 cm		
pH	**0.005**	0.961
NH_4^+-N concentrations	**0.026**	0.090
NO_3^--N concentrations	0.251	0.303
Post-hoc		
0-10 cm		
pH	ns	ns[14]
NH_4^+-N concentrations	ns	ns[15]
NO_3^--N concentrations	ns	ns
10-20 cm		
pH	3+1,2,4	ns
NH_4^+-N concentrations	4+1,2,3	ns
NO_3^--N concentrations	ns	ns

[14] Inhomogeneous, P set to P< 0.1
[15] Inhomogeneous, P set to P< 0.1

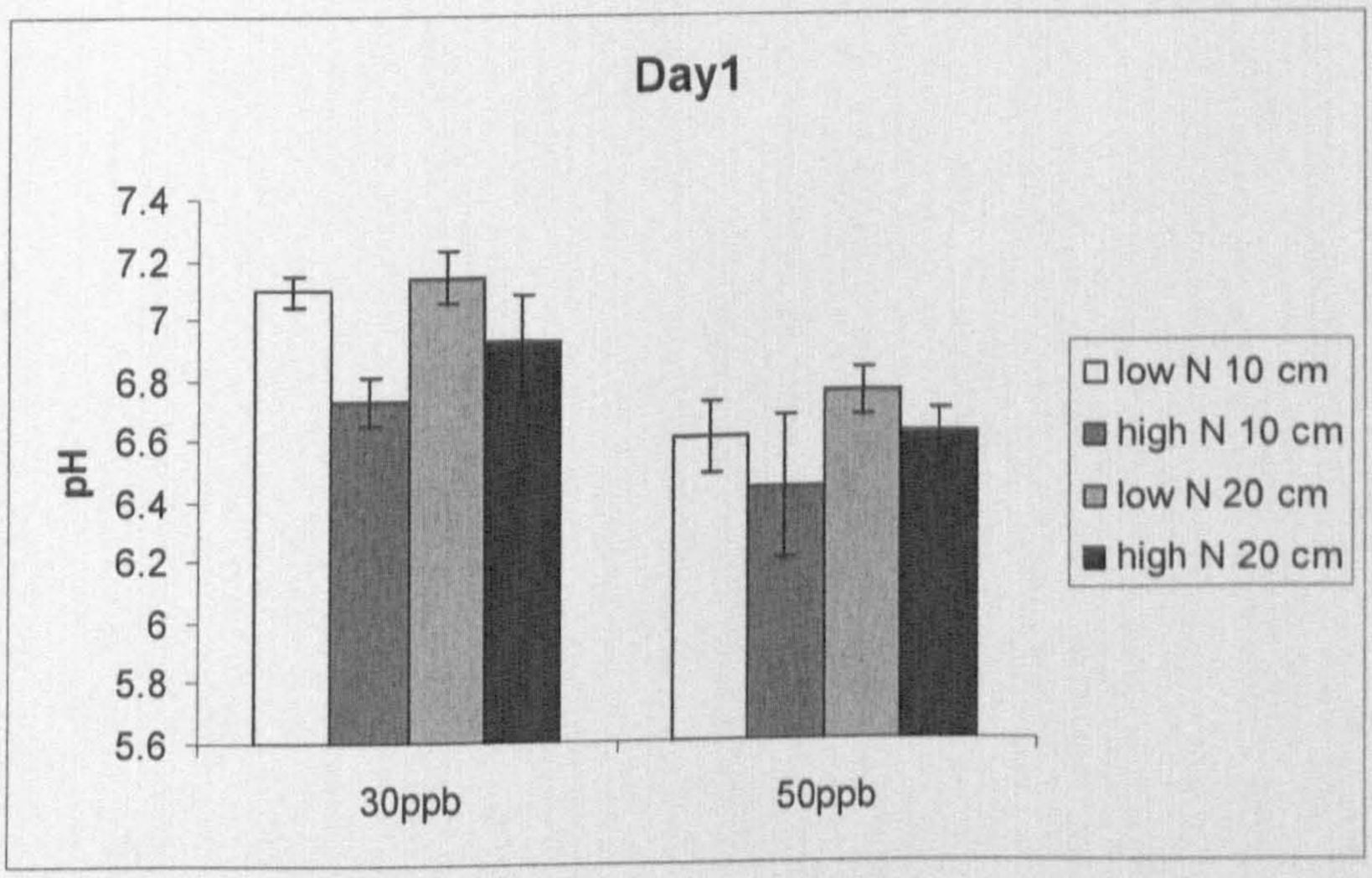

Figure 106: The mean pH at 0- 10 cm and at 10- 20 cm at two different O_3-treatments and two nutrient treatments (50 ppb, 30 ppb; low N, high N) during 2008. The error bars represent standard errors.

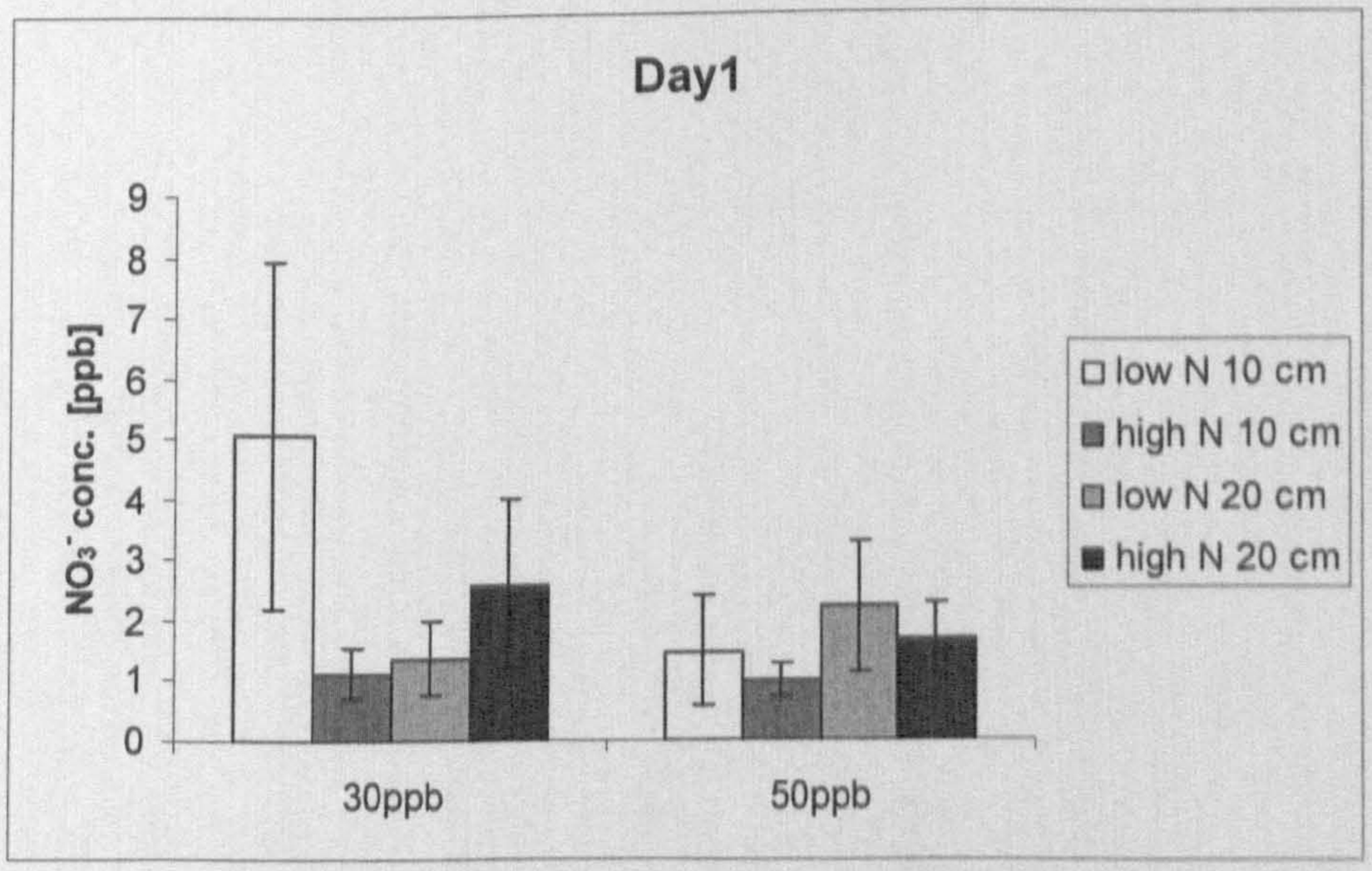

Figure 107: The mean nitrate concentrations [mg kg^{-1}] at 0- 10 cm and at 10-20 cm at two different O_3-treatments and two nutrient treatments (50 ppb, 30 ppb; low N, high N) during 2008. The error bars represent standard errors.

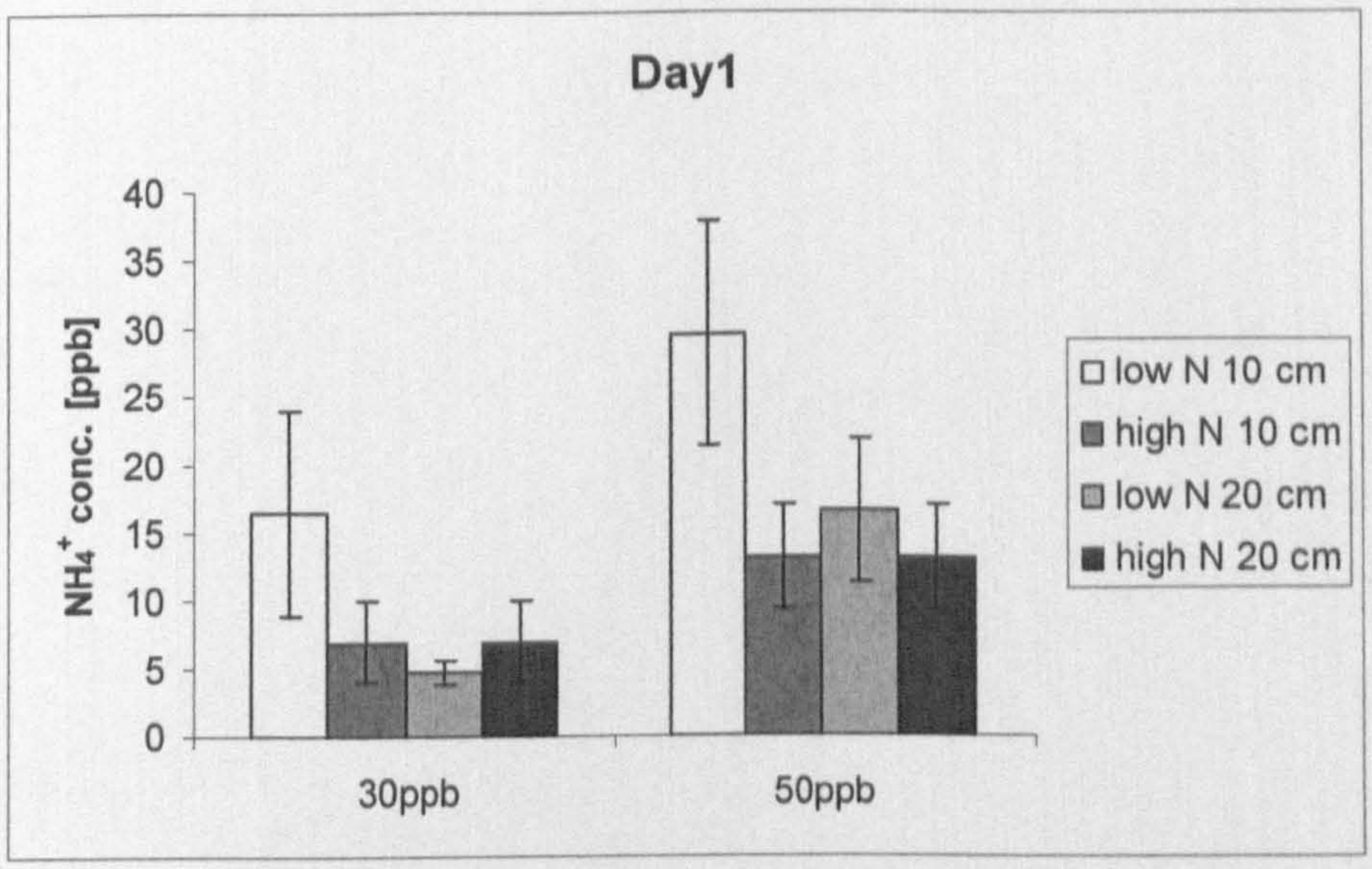

Figure 108: The mean ammonium concentrations [mg kg^{-1}] at 0- 10 cm and at 10-20 cm at two different O_3-treatments and two N-treatments (50 ppb, 30 ppb; low N, high N) during 2008. The error bars represent standard errors.

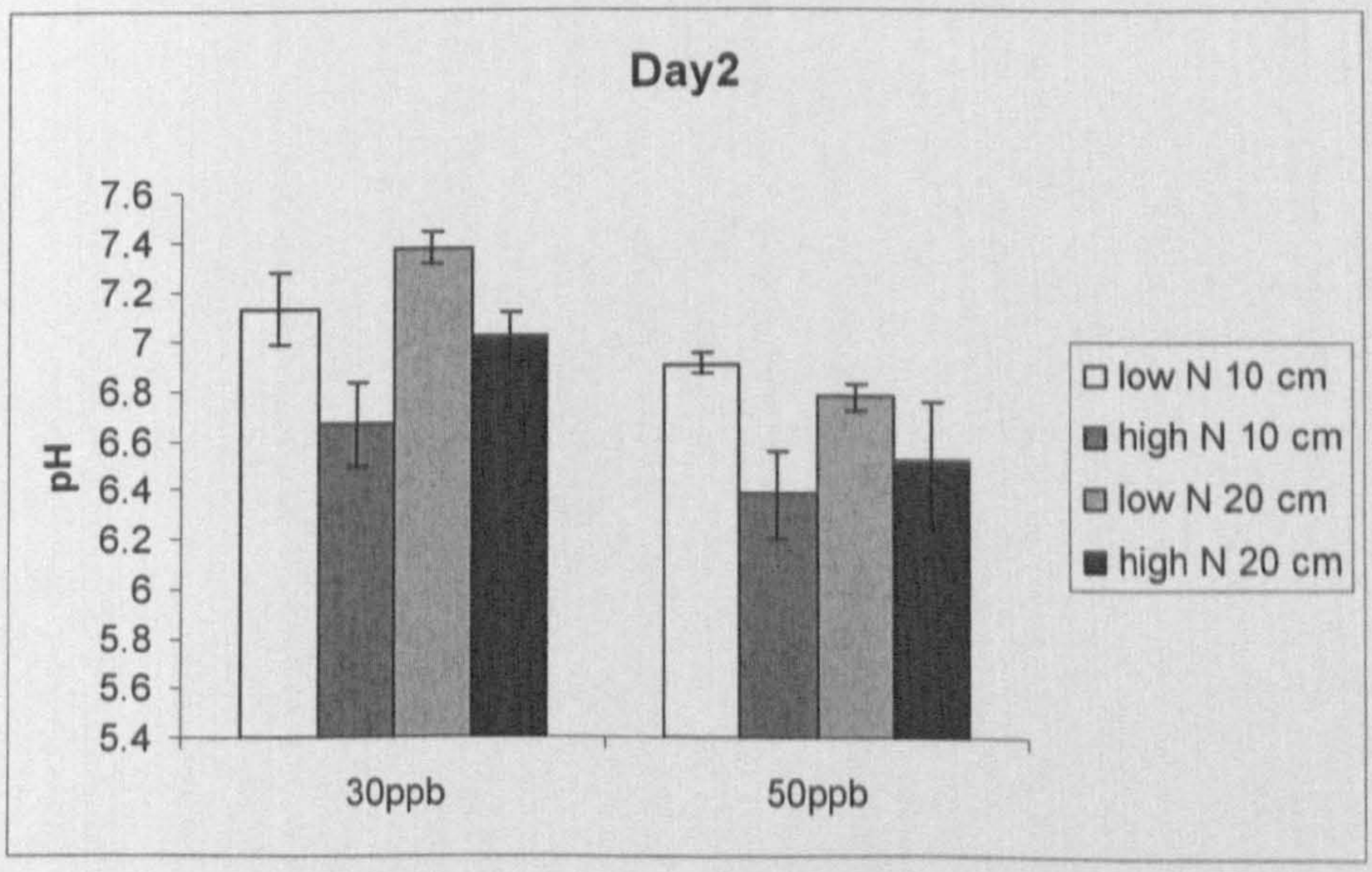

Figure 109: The mean pH at 0- 10 cm and at 10- 20 cm at two different O3-treatments and two nutrient treatments (50 ppb, 30 ppb; low N, high N) during 2008. The error bars represent standard errors.

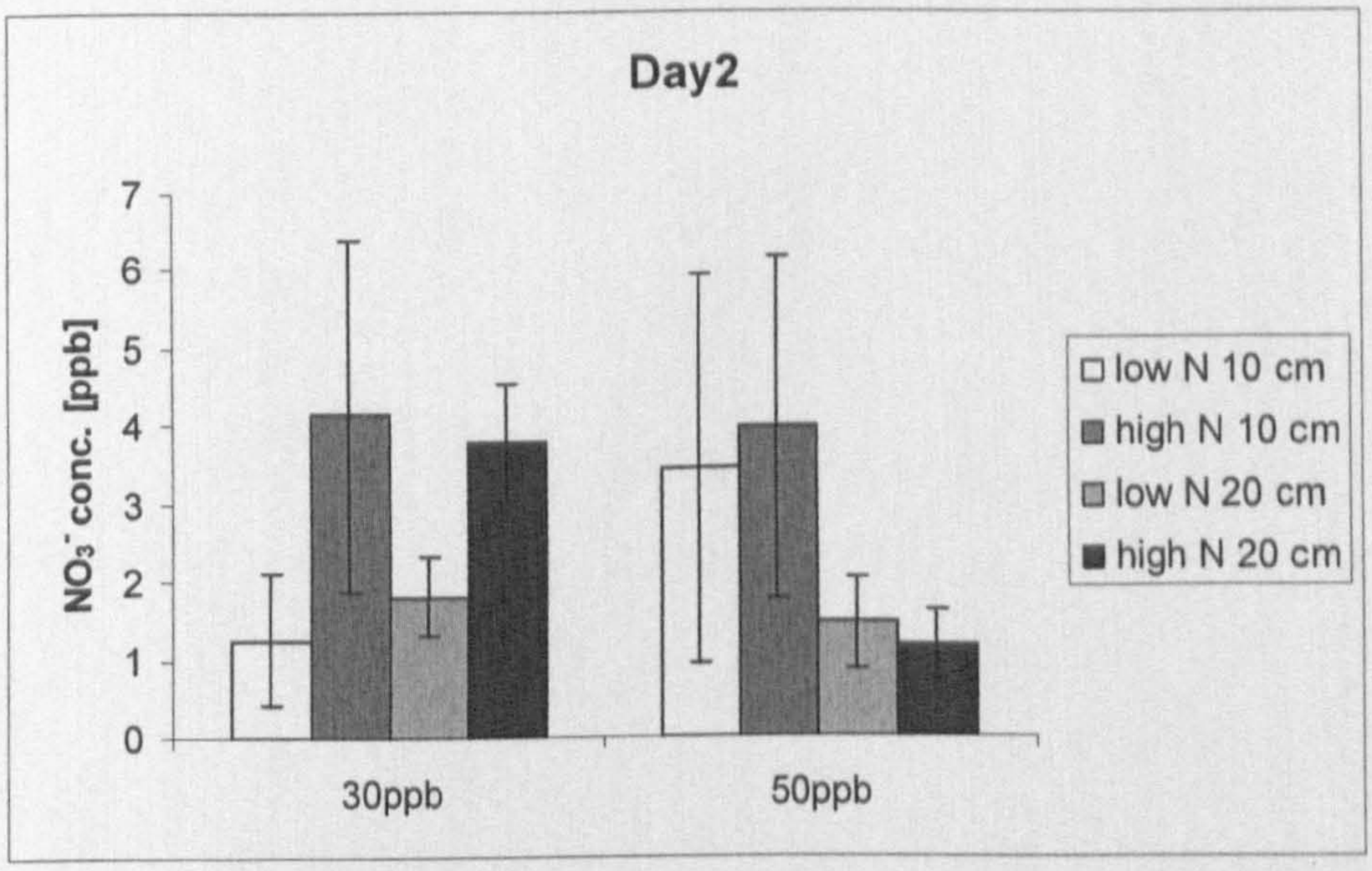

Figure 110: The mean nitrate concentrations [mg kg^{-1}] at 0-10 cm and at 10-20 cm at two different O_3-treatments and two nutrient treatments (50 ppb, 30 ppb; low N, high N) during 2008. The error bars represent standard errors.

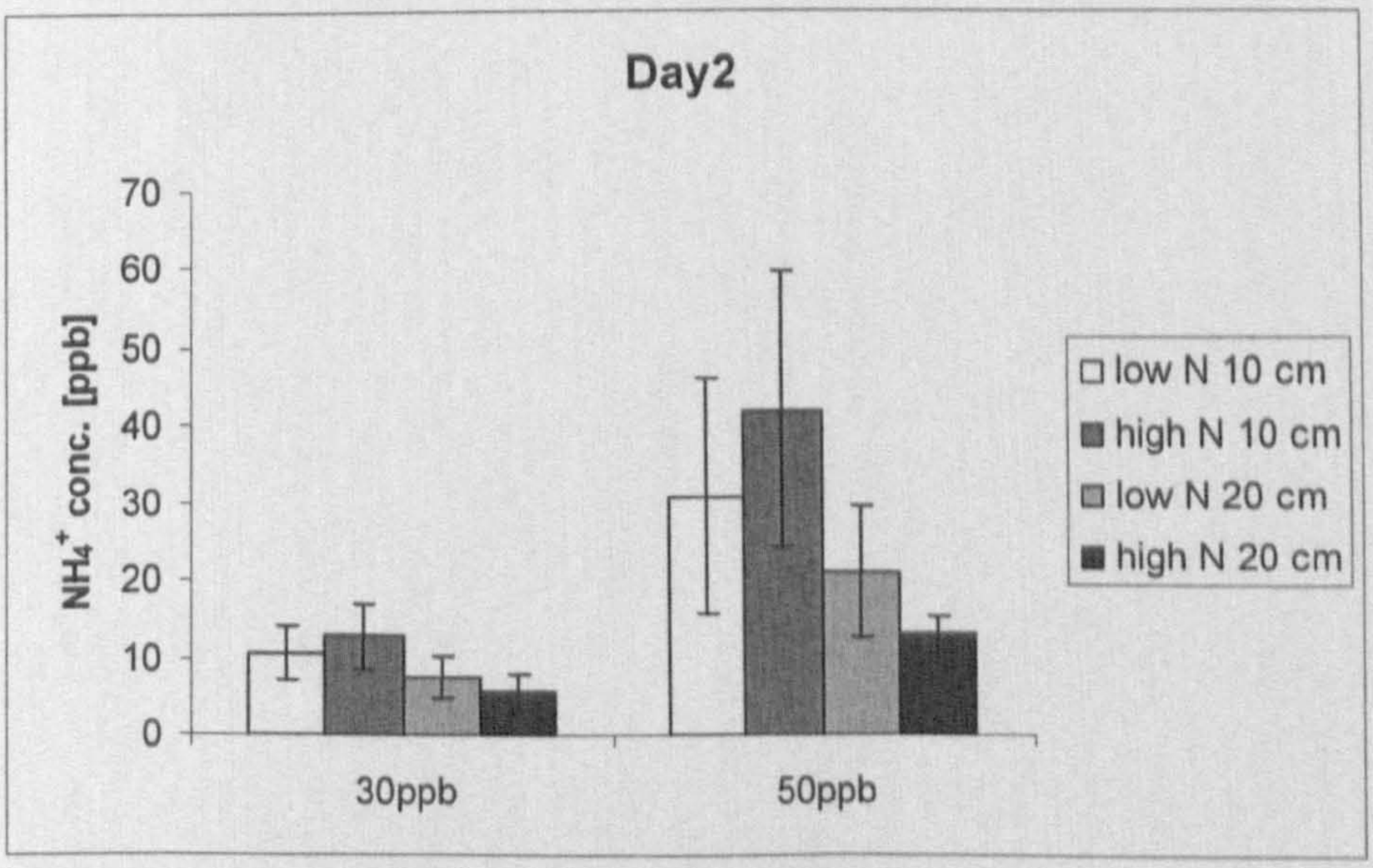

Figure 111: The mean pH at 0-10 cm and at 10-20 cm at two different O_3-treatments and two nutrient treatments (50 ppb, 30 ppb; low N, high N) during 2008. The error bars represent standard errors.

7.4. Appendix 4

The C/N ratio was higher in the *E. vaginatum* biomass than in the *E. vaginatum* litter. Significant effects of ozone were not found for the biomass and not for the litter of *E. vaginatum* (Table 54, Figure 112).

Table 54: P-values from the independent samples test of effects of ozone on C/N ratio of live *Eriophorum* biomass and *Eriophorum* litter.

C/N ratio	**P**
E. vaginatum biomass	0.356
E. vaginatum litter	0.822

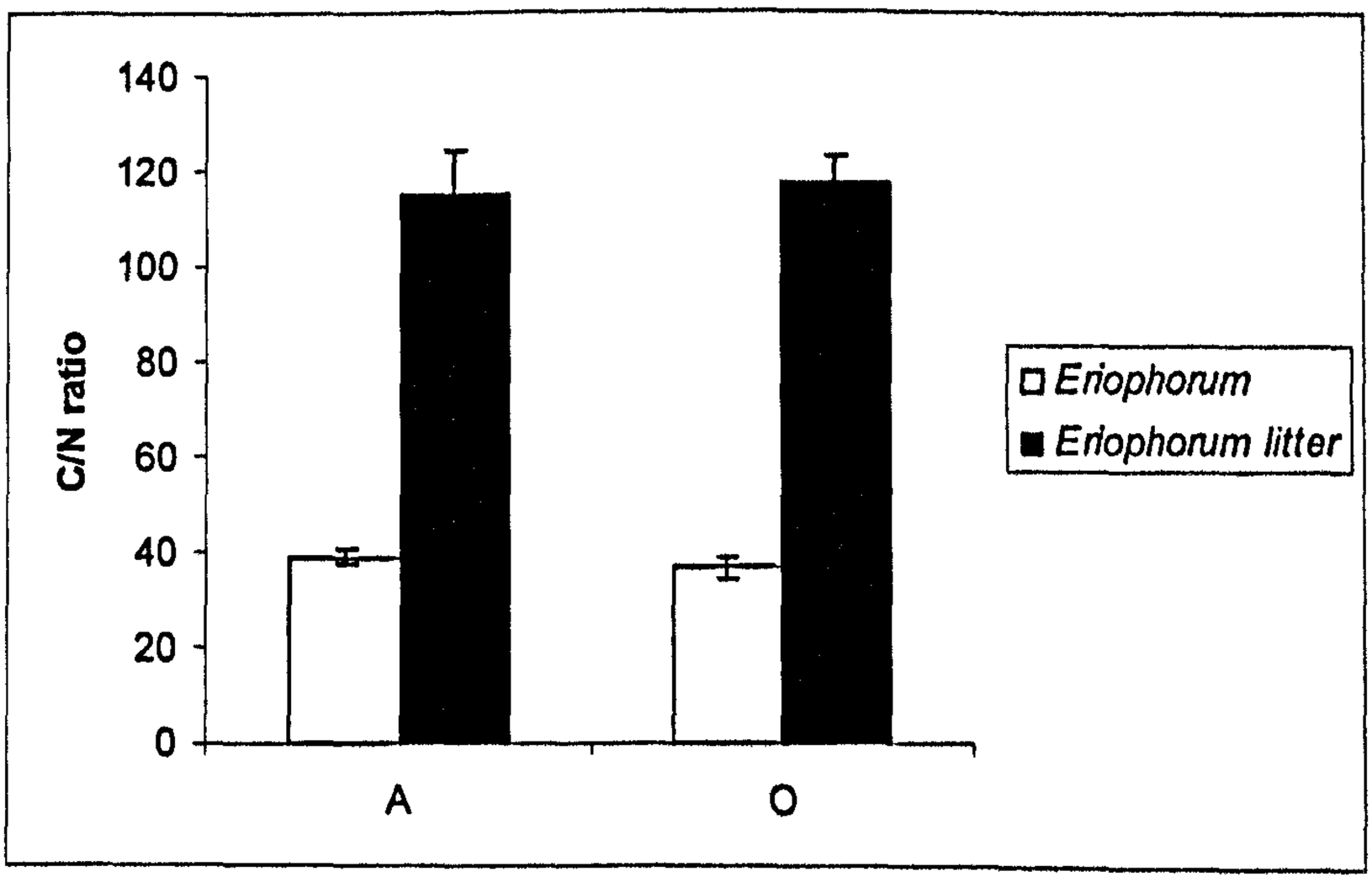

Figure 112: The C/N ratio of *E. vaginatum* biomass and *E. vaginatum* litter in ambient air (A) and O3 enriched air (O) in 2007. Error bars represent standard errors.