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Carbon and nitrogen relationships in swards of *Danthonia richardsonii* in response to carbon dioxide enrichment and nitrogen supply

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A thesis submitted for the degree of Doctor of Philosophy of The Australian National University

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Statement of originality

The work presented in this thesis is my own. Specific contributions by others are referred to in the text and acknowledgments

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Abstract

Atmospheric CO_2 concentrations are increasing steadily, although not fast enough to account for all anthropogenic emissions. Uptake of extra carbon by the terrestrial biosphere in response to the CO_2 concentration increase may, in turn, be moderating the rate of increase. However, there are uncertainties pertaining to the ability of natural ecosystems to respond to this increase in terms of productivity and carbon sequestration, when productivity is restricted by nitrogen availability. This study provides experimental data on the response of microcosms of the wild, C_3 Australian grass *Danthonia richardsonii* Cashmore to CO_2 enrichment over 4 years of growth in the Canberra Phytotron, when productivity was restricted by low nitrogen availability. Complementary experiments with isolated plants elucidated the effects of CO_2 enrichment on carbon and nitrogen acquisition and allocation. The general growth response of *D. richardsonii* to CO_2 enrichment was compared with other C_3 grasses in further isolated plant experiments.

Microcosms accumulated extra carbon under CO_2 enrichment. Microcosms were supplied continuously with 3 productivity limiting rates of N supply, 2.2, 6.7 or 19.8 g N m^{-2} yr^{-1}, at atmospheric CO_2 concentrations of 359 or 718 $\mu L\ L^{-1}$. Controlled quantities of water were supplied and periodic drought imposed. The effect of CO_2 on total plant-soil system carbon was highly significant at all N supply rates, and did not diminish over time. Increased microcosm carbon was attained without a persistent increase in leaf area index, and above ground live carbon was not increased by CO_2 enrichment after the first year. Leaf turnover was higher at high CO_2, as was the amount of total senesced leaf. Root carbon was lower at high CO_2 at low- and mid-N, but higher at high-N. Rates of water-use were lower at high CO_2, resulting in a higher soil water content. At the higher N levels both soil microbial and non-microbial carbon were increased at high CO_2, while total soil carbon and non-microbial carbon were increased at low-N. Low- and mid-N microcosms gained significant amounts of nitrogen from the environment, attributed to a combination of nitrogen deposition and free-living nitrogen fixation. Nitrogen loss from the high-N microcosms was lower at high CO_2. Green leaf nitrogen concentration and total standing leaf nitrogen were reduced by CO_2 enrichment. Increased C:N ratios of senesced leaf at high CO_2 resulted in decreased decomposition (cumulated microbial respiration) *in vitro*. This was not reflected at the microcosm level, possibly owing to increased soil water content and microbial biomass.

High CO_2 increased carbon accumulation by isolated plants when grown at several N levels (0.05, 0.2, 0.5 or 6 mg N $plant^{-1}$ day^{-1}) over 37 days. Net assimilation rate and leaf nitrogen

productivity were increased at high CO_2. Whole plant (g N g^{-1} C) and leaf nitrogen concentrations (g N g^{-1} C or g N m^{-2}) were reduced by CO_2 enrichment when nitrogen supply restricted growth. Allometric relationships showed that carbon allocation and root nitrogen concentration (g N g^{-1} C) were not affected by CO_2 enrichment. Nitrogen allocation to root, as a proportion of total plant nitrogen was increased at high CO_2, and leaf to root surface area ratio reduced, indicating a shift in investment from processes involved with carbon acquisition to those involved with nitrogen acquisition. The differences were small at the higher rates of N supply.

As an isolated plant, *D. richardsonii* exhibited a similar response in dry matter or carbon accumulation to CO_2 enrichment (~360 μL L^{-1} and ~720 μL L^{-1}) as other grasses when nitrogen supply was abundant (Hoagland solution) or growth limiting (0.4 or 1.6 mg N $plant^{-1}$ day^{-1}) over 71 days. Total transpiration (g H_2O $plant^{-1}$) was reduced, and transpiration efficiency (g C g^{-1} H_2O) increased at high CO_2 in plants experiencing nitrogen limitation, but was not determined in the plants supplied with abundant N.

D. richardsonii showed real increases in nitrogen use efficiency at high CO_2. This was expressed at the microcosm level as increases in total plant-soil system carbon gain a high CO_2 at all rates of nitrogen supply. The magnitude of this response is large enough to account for a significant proportion of global anthropogenic carbon emissions, *if* applicable to all ecosystems in the field.

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Chapter 1. Introduction

Global average atmospheric carbon dioxide (CO_2) concentration has been rising steadily since the Industrial Revolution from a concentration of about 280 $\mu L\,L^{-1}$, which was fairly constant over the preceding 18,000 years (Neftel *et al.*, 1985; Barnola *et al.*, 1987; Watson,RT *et al.*, 1990; Boden *et al.*, 1994; Schimel *et al.*, 1995), to about 356 $\mu L\,L^{-1}$ in 1993 (Boden *et al.*, 1994; Schimel *et al.*, 1995). This increase is largely due to the effects of fossil fuel burning, cement manufacture and net deforestation (Leggett *et al.*, 1992; Watson,RT *et al.*, 1992; Keeling, 1994). Modelling studies with scenarios of future anthropogenic carbon emissions (Leggett *et al.*, 1992) have shown that atmospheric CO_2 concentrations will probably reach 650 - 750 $\mu L\,L^{-1}$ by the year 2100, and may continue to rise for a significant time thereafter (Wigley, 1993; Schimel *et al.*, 1995). This increase in atmospheric CO_2 concentration, along with increasing concentrations of other trace gases in the atmosphere owing human activities may have profound impacts on the Earth's climate system (eg. Houghton *et al.*, 1990, 1992, 1995). The terrestrial biosphere may be moderating this increase in atmospheric CO_2 (Gifford, 1979; Goudriaan & Ketner, 1984; Gifford *et al.*, 1996b, c). This study is concerned with the direct effect of increased atmospheric CO_2 concentration on plant growth, plant-soil system function and carbon storage in the plant-soil system.

Current increases in atmospheric CO_2 concentration are too low to account for all of the anthropogenic carbon emissions. Estimated sources and sinks of those emissions are given in Table 1.1. The additional terrestrial sinks are possibly increases in carbon storage in the biosphere resulting from a combination of a number of processes, namely nitrogen deposition, climatic anomalies and CO_2 fertilisation (Watson,RT *et al.*, 1992; Schimel *et al.*, 1995). While the contribution of each of these processes to the additional terrestrial sink is largely uncertain (Watson,RT *et al.*, 1992; Schimel *et al.*, 1995), some models of varying degrees of complexity predict that CO_2 fertilisation may a be major component of this sink (Bacastow & Keeling, 1973; Gifford, 1979, 1980, 1992a, 1993; Goudriaan & Ketner, 1984; Hunt,HW *et al.*, 1991; Melillo *et al.*, 1995; Gifford *et al.*, 1996b). With total soil and vegetation C pools of about 1310 to 1730 Gt (10^{15} g) (Zinke *et al.*, 1984) and 500 to 600 Gt (Olson *et al.*, 1983) respectively, an annual increment in this combined pool of less than 0.1% would account for the terrestrial sink. There has been much debate over the ability of nutrient limited ecosystems to respond to CO_2 fertilisation (eg. Strain & Bazzaz, 1983; Melillo *et al.* 1990) and, as stated by Schimel *et al.* (1995),

> *Experimental confirmation* [of the CO_2 fertilising effect] *from ecosystem-level studies, however, is lacking. As a result, the role of the terrestrial biosphere in controlling future atmospheric CO_2 concentrations is difficult to predict.*

This study aims to measure the productivity and carbon storage response to atmospheric CO_2 increase of a simple, experimental model ecosystem (microcosm) of a monotypic grass sward, which had its productivity restricted by low nitrogen supply. This study was not undertaken in the field as a high degree of homogeneity of initial parameters, such as soil carbon and nitrogen concentrations are needed to measure hypothesised changes in pool sizes resulting from CO_2 increase. Such a degree of homogeneity is not available in natural systems (Ross *et al.,* 1995). Thus this study provides an important link between those at a higher level, where measurements of ecosystem CO_2 flux over short time periods are made without the measurement of pool size, and those studies at a lower level, where the response of individual plants or plant processes to CO_2 increase are determined.

The major hypothesis of this study was;

> *A simple, monotypic C_3 grass microcosm will increase its total carbon accumulation in response to atmospheric CO_2 increase over a four year period even when its productivity is severely restricted by low nitrogen supply.*

Factors pertinent to this hypothesis are addressed in this chapter, and a further set of hypotheses developed and outlined in a thesis plan.

A simple, conceptual model of microcosm function

A comprehensive review of the literature relating to the many fâcets of plant response to atmospheric CO_2 will not be presented here. Rather, a brief review of the primary mechanisms of plant response to atmospheric CO_2 and other areas pertinent to this study will be given, in line with the simple conceptual model of microcosm function presented in Diagram 1-1. Further review of literature as it relates to this study is presented in the experimental chapters.

Acquisition of carbon

Mechanisms of CO_2 response

There are two major mechanisms whereby growth at high CO_2 can impact on the accumulation of carbon by plants. The first is a photosynthetic response, the second a stomatal response, both of which involve an increase in the efficiency of use of resources, many of which are often in short supply to the plant. Atmospheric CO_2 enrichment may also effect dark respiration. The

response of dark respiration to CO_2 enrichment and its magnitude is an active area of debate, with reports of little effect of CO_2 on dark respiration relative to photosynthesis (eg. Gifford, 1995), and others of decreased dark respiration in response to CO_2 enrichment (eg. Azcón-Bieto et al., 1994). In this study effects of CO_2 on photosynthesis, stomata and respiration are not measured directly. The net effects are inferred via changes in plant growth characteristics and in plant-soil system carbon gain.

Photosynthetic responses to CO_2 enrichment

Approximately 40-45% of plant dry mass is organic carbon, which originates from the reduction of atmospheric CO_2 in the process of photosynthesis. Rates of leaf photosynthesis are very sensitive to atmospheric CO_2 concentrations in plants with the C_3 photosynthetic pathway (eg. von Caemmerer & Farquhar, 1981), which constitute about 95% of all plant species (Bowes, 1993). The major carbon fixing enzyme, ribulose bisphosphate 1,5-carboxylase/oxygenase (RUBISCO) can either act as a carboxylase, fixing carbon into an organic form of use to the plant, or an oxygenase, which is an energy wasting process producing seemingly useless products (Morell *et al.,* 1992). The photorespiratory recovery pathway metabolises these oxygenation products and recovers a proportion of the carbon, while the rest is respired as CO_2 (Morell *et al.,* 1992). Photorespiratory carbon loss accounts for a significant proportion of gross carbon fixation of C_3 plants (Bowes, 1993). As atmospheric CO_2 concentrations rise, the oxygenation function of Rubisco is competitively inhibited, and the net rate of carbon fixation of C_3 plants increases. Less energy is wasted in the photorespiratory recovery pathway, leading to increases in the efficiency of use of radiation.

Nitrogen use in relation to photosynthesis and CO_2 increase

Rubisco accounts for about 25% of total leaf nitrogen (Evans,JR, 1989), and is thought to be the most abundant protein on Earth (Bowes, 1991). Increases of net photosynthetic rate under CO_2 enrichment may result in increases in the efficiency of use of the nitrogen associated with Rubisco and other proteins involved with carbon acquisition. This may result in less investment of nitrogen in leaf proteins at the leaf level, if not for the whole plant. Sage *et al.* (1989) reported reductions in Rubisco content (g m^{-2} leaf) under CO_2 enrichment in *Chenopodium album* and *Brassica oleracea*, but not in *Solanum tuberosum* or *S. melongena,* while Rubisco always formed a lower proportion of total leaf nitrogen. In that study, leaf nitrogen surface density (expressed as mol m^{-2} leaf) was increased in all species, which was possibly related to the high level of nitrogen supply. A similar trend (not statistically significant) of decreased Rubisco content with increased CO_2 concentration above 220 $\mu L\ L^{-1}$ was noted in soybean

leaves (Campbell *et al.,* 1988). Rowland-Bamford *et al.* (1991) reported a linear decline in Rubisco content (g m^{-2}) of rice leaf with increasing CO_2 concentrations, a decline in Rubisco content as a proportion of total soluble leaf protein and a non-significant decline in leaf nitrogen surface density. Besford *et al.* (1990) and Nie *et al.* (1995) have shown, for tomato and wheat respectively, that early in leaf expansion Rubisco content (g m^{-2}) is not altered by CO_2 enrichment, although as the leaf expands Rubisco content becomes substantially lower at high CO_2. The wheat was grown in the field in a FACE experiment (Free-Air CO_2 Enrichment; Lewin *et al.,* 1994), demonstrating that this effect is expressed in the field. Reduction in investment in chlorophyll under CO_2 enrichment has sometimes been observed (Sage *et al.,* 1989; Wullschleger *et al.,* 1992; Xu *et al.,* 1994) and sometimes not (Sage *et al.,* 1989; Xu *et al.,* 1994; Nie,GY *et al.,* 1995). Thus, photosynthesis at high atmospheric CO_2 concentrations may lead to increased photosynthetic nitrogen use efficiency. In turn, this may result in a less investment of nitrogen in leaf protein associated with carbon acquisition, which may only be expressed as reductions in leaf nitrogen surface density when plant growth is limited by low nitrogen supply. Changes in leaf nitrogen concentration and surface density at high CO_2 will be assessed over a range of nitrogen availability.

Photosynthetic acclimation

In isolated plants the stimulation of leaf level photosynthesis by CO_2 enrichment is sometimes transitory. Photosynthetic rates are commonly observed to "acclimate" or "down-regulate" such that at their growth CO_2 concentration photosynthetic rates are similar between those grown under ambient CO_2 concentrations and those grown under high CO_2 concentrations (Bowes, 1991; Stitt, 1991). This phenomena is not always observed (Sage *et al.,* 1989; Gunderson *et al.,* 1993; Johnson,HB *et al.,* 1993), and while down-regulation of some species has been shown to result in lower photosynthetic rates under CO_2 enrichment (Sage *et al.,* 1989), some species have been noted to "up-regulate" under CO_2 enrichment, such that photosynthetic capacity is increased (Campbell *et al.,* 1988; Sage *et al.,* 1989; Arp & Drake, 1991). There are many hypotheses for this phenomenon, mostly related to sink feedback from carbohydrate accumulation, or differing patterns of nitrogen allocation, both within the leaf and within the plant under CO_2 enrichment (Arp, 1991; Bowes, 1991, 1993; Stitt, 1991; Besford, 1993; Xu *et al.,* 1994; Barrett & Gifford, 1995a). Complete down-regulation of photosynthesis (leaf photosynthetic rates do not differ between CO_2 treatments at their growth CO_2 concentration) does not preclude a growth response to CO_2 increase. Complete down-regulation often occurs only after CO_2 has increased leaf area (Gifford, 1992a). Integrated carbon acquisition by the leaf may also be greater under CO_2 enrichment even when complete down-regulation is

observed, as leaf gas exchange measurements are often undertaken only on fully expanded leaves (eg. Sage *et al.,* 1989) and may not provide a fair estimate of overall plant function. A decline in photosynthetic capacity is often noted with leaf age (Besford, 1993; Pearson & Brooks, 1995), and down-regulation may be associated with a more rapid decline in Rubisco content (above). The data of Besford (1990) show almost complete down-regulation at 100% leaf expansion in tomato, while visual integration of the light-saturated photosynthetic rate curve against leaf age shows a greater carbon acquisition over the life of the leaf under CO_2 enrichment.

Photosynthetic acclimation was not directly measured in this study. Growth analysis techniques are used to calculate net assimilation rate (NAR) and nitrogen productivity. Nitrogen productivity is calculated on both a leaf nitrogen (NP_L, Garnier & Vancaeyzeele, 1994) and plant nitrogen (NP_P, Ingestad, 1979) basis. NAR is defined in this study as the rate of change in total plant carbon per unit leaf area;

$$NAR = \frac{1}{A_L} \cdot \frac{dC_P}{dt}$$

where A_L is leaf area, C_P is total plant carbon and t is time. NP_L is defined as the rate of change in total plant carbon per unit total leaf nitrogen;

$$NP_L = \frac{1}{N_L} \cdot \frac{dC_P}{dt}$$

where N_L is total leaf nitrogen content. NP_P is defined as the rate of change in total plant carbon per unit total plant nitrogen;

$$NP_P = \frac{1}{N_P} \cdot \frac{dC_P}{dt}$$

where N_P is total plant nitrogen.

Stomatal responses to CO_2 increase

All carbon dioxide must pass through pores in the leaf surface, the stomata, before its carbon can be reduced into an organic form. The majority of plant water loss occurs through the stomata, as carbon is gained in exchange for water (Salisbury & Ross, 1991). Stomatal aperture, or conductance is under physiological control, and decreases with decreases in light intensity, soil water content, atmospheric humidity and increasing CO_2 concentration (Milthorpe & Moorby, 1979). CO_2 sensitivity of stomatal conductance is such that conductance is approximately 40% lower at 680 $\mu L\ L^{-1}$ than at 340 $\mu L\ L^{-1}$, and this response is similar over a wide range of species (Morison, 1985), including those with a C_4 photosynthetic pathway

(Morison & Gifford, 1983). The decrease in stomatal conductance under CO_2 enrichment can result in large increases in water use efficiency of both C_3 and C_4 plants (Morison, 1993; Samarakoon & Gifford, 1995; Samarakoon *et al.*, 1995).

Other stomatal parameters may also be sensitive to CO_2 concentrations. In some species stomatal density has been observed to decrease with increasing CO_2 concentrations, both under experimental conditions (Woodward, 1987; Woodward & Bazzaz, 1988; Knapp *et al.*, 1994) and in historical or fossil samples (Woodward, 1987; Penuelas & Matamala, 1990; Beerling & Chaloner, 1993b; McElwain & Chaloner, 1995). This decrease is not always observed (Körner, 1988; Gaudillere & Mousseau, 1989; Ryle & Stanley, 1992; Radoglou & Jarvis, 1993; Estiarte *et al.*, 1994) and the historical changes in stomatal density may be confounded by differences in temperature during growth (Beerling & Chaloner, 1993a).

Stomatal conductance and other stomatal characteristics were not directly determined in this study. However changes of whole plant and microcosm water use resulting from CO_2 enrichment were measured.

Biomass responses of isolated plants in response to CO_2 enrichment

Numerous studies of the responses of isolated plants to CO_2 enrichment, both under optimal growth conditions and where other environmental factors limit growth have been reported in the literature. These studies generally indicate an increase in biomass or carbon accumulation under CO_2 enrichment, and increases in the efficiency of use of scarce resources for carbon gain. There are many good reviews of these responses, and none was undertaken here (eg. Wittwer & Robb, 1964; Kimball, 1983; Cure & Acock, 1986; Enoch, 1990; Rawson, 1992; Morison, 1993; Poorter, 1993; Idso & Idso, 1994; Rogers *et al.*, 1994). There are some exceptions to the general pattern of increased biomass under CO_2 enrichment, with some species under some growth conditions not exhibiting increases in biomass accumulation, even under favourable growth conditions (eg. Kimball, 1983; Oberbauer *et al.*, 1986; Hunt et al., 1991). Although some of these responses may be explained by contamination of the CO_2 supply with ethylene, which suppress growth (Morison & Gifford, 1984), and other such experimental problems, this cannot account for all of these reports of non-response. Thus, in case the biomass response to CO_2 is species dependant, verification of the "normality" of the CO_2 response of the model grass species chosen for use in this study was required.

Responses of plants grown as swards

Although many studies have been completed on the response of isolated plants to CO_2 enrichment under varying environmental conditions, far fewer studies have been made of plant communities. Most of the published sward experiments have been undertaken on domesticated species, which exhibit qualitatively similar responses to those of isolated plants of the same species (Lawlor & Mitchell, 1991). Responses of wild species to CO_2 enrichment has been more variable, with some experiments showing no increase in biomass production (Billings *et al.*, 1984; Körner & Arnone, 1992; Fredeen *et al.*, 1995; Schäppi & Körner, 1996). However, when net canopy carbon exchange is measured, CO_2 enriched canopies often appear to acquire more carbon, without a measurable increase in plant biomass carbon pools (Billings *et al.*, 1984; Diemer, 1994; Fredeen *et al.*, 1995; Schäppi & Körner, 1996). This extra carbon might be accumulating in the soil. In field situations the natural variability of soil carbon concentrations are large, making the detection of such increases very difficult or impossible (Ross *et al.*, 1995). However, since the instigation of this study increases in soil carbon under CO_2 enriched canopies have been reported (Rice *et al.*, 1994; Wood *et al.*, 1994). This forms the major element of this study, to test for increases in carbon accumulation at high CO_2 by C_3 grass swards.

Acquisition of nitrogen

It has often been hypothesised that natural ecosystems will not respond to atmospheric CO_2 increase because their productivity is limited by other factors, such as water availability and nitrogen supply (eg. Strain & Bazzaz, 1983; Melillo *et al.*, 1990). However, productivity is almost always co-limited by several factors (Gifford, 1974, 1992b; Bloom *et al.*, 1985; Rastetter & Shaver, 1992), and it has been well proven, at least at the isolated plant level that increased levels of atmospheric CO_2 can increase plant growth when it is limited by a variety of other factors (Gifford, 1979; Idso & Idso, 1994). If plants are not able to increase nitrogen acquisition under CO_2 enrichment, any increase in biomass or carbon storage will be via increases in the carbon to nitrogen (C:N) ratio of the plant, or possibly due to changes in nitrogen distribution to pools of a higher carbon to nitrogen ratio (Gifford, 1992b; Shaver *et al.*, 1992). Increases in carbon to nitrogen ratios may imply reductions in decomposition rates, which may feed back onto ecosystem productivity, reducing the CO_2 stimulation (Strain & Bazzaz, 1983; Melillo *et al.*, 1990). This effect has not been rigorously assessed experimentally.

Nitrogen gain from the environment

If plant-soil system nitrogen content can be increased under CO_2 enrichment, the potential exists for increased productivity even with a substantial reduction in decomposition rate. An increased plant-soil system nitrogen content might be attained via a decrease in nitrogen loss from the system, or an increase in nitrogen input to the system. The major forms of nitrogen loss are via leaching and gaseous processes, and are discussed in chapter 7. More significant may be increased nitrogen gain by the plant-soil system, which is also discussed in chapter 7, the mechanisms of which are discussed briefly here.

Dinitrogen fixation

Biological nitrogen fixation can be viewed as carbon (energy) limited (Gutschick, 1978; Sprent, 1993), and it has been hypothesised that increasing atmospheric CO_2 concentrations may result in increased nitrogen fixation due to a higher level of carbon availability (Lamborg & Hardy, 1983; Gifford *et al.,* 1996a). As the majority of nitrogen in the pre-industrial terrestrial biosphere originated from biological nitrogen fixation (Handley & Raven, 1992), and biological fixation appears to be stimulated by a high carbon availability relative to nitrogen availability (Havelka *et al.,* 1982; van Berkum, 1984) this seems a reasonable supposition (Gifford, 1992b).

There are three major relationship types between nitrogen fixing microorganisms (diazotrophs) and plants; symbiotic, associative and free living. There are many reports of growth under CO_2 enrichment increasing nitrogen fixation of the symbiotic relationship between *Rhizobium spp* and herbaceous legumes (eg. Shivashankar *et al.,* 1976; Latimore, 1984; Murphy, 1986; Masuda *et al.,* 1989; Reardon, 1990; Ryle & Powell, 1992), and of increases in N_2 fixation by similar relationships in woody plants (eg. Norby, 1987; Arnone & Gordon, 1990; Thomas *et al.,* 1991; Vogel & Curtis, 1995).

Associative relationships, which are host-diazotroph specific are common in grasses (van Berkum, 1984; Boddey, 1987). Bacteria, including *Azospirillum spp.* and *Azotobacter spp.* invade the root of the host, from which they obtain a supply of carbon (Boddey & Dobereiner, 1988). These relationships have been observed in both C_3 and C_4 grasses (Nelson *et al.,* 1975; Thompson *et al.,* 1984). Associative relationships have been observed to supply 30 to 40% of the nitrogen uptake of the plant (Boddey & Victoria, 1986), or have been observed to supply nitrogen at an annual rate of fixation of 2.0 to 4.5 g m^{-2} yr^{-1} (Boddey *et al.,* 1983; Boddey & Victoria, 1986).

Associative N_2 fixation has been enhanced by short term exposure to high CO_2 (900 $\mu L\ L^{-1}$) in the field and under hydroponic assay in the salt marsh grass *Spartina alterniflora* (Whiting *et al.*, 1986). Acetylene reduction, a semi-quantitative measure of N_2 fixation was increased by 27% shortly after exposure to elevated CO_2, which was attributed to an increase in carbon supply to the bacteria (Whiting *et al.*, 1986). Nitrogen fixation by the C_3 sedge *Scirpus olneyi* has also been noted in the field under CO_2 enrichment (Drake, 1992). As most of these occurrences of increasing N_2 fixation can be attributed to increases in photosynthate supply, or changes in the balance between carbon and nitrogen availability it seems likely that free living N_2 fixation may also increase in response to CO_2 enrichment.

Free-living diazotrophs include both autotrophs and heterotrophs (Havelka *et al.*, 1982). The free-living autotrophs include photosynthetic bacteria which require low O_2 levels for N_2 fixation, and the blue-green algae (cyanobacteria) (Havelka *et al.*, 1982). Blue-green algae require a high soil water content to avoid desiccation (Witty, 1979; Witty *et al.*, 1979), and are important in both temperate and tropical soils (Granhall, 1975; Havelka *et al.*, 1982). Free living heterotrophs known to fix N_2 include aerobes, facultative anaerobes and obligate anaerobes (Havelka *et al.*, 1982). Nitrogen fixation by blue-green algae has been estimated at between 1.3 and 4.8 g N m^{-2} yr^{-1} in a temperate wheat system (Witty *et al.*, 1979). However, little contribution of free living fixation has been noted in a wheat experiment assayed via $^{15}N_2$ methodologies (Bremer *et al.*, 1995). Fixation by free living diazotrophs can even be important under legume stands. Fixation by free living bacteria associated with the roots of *Medicago sativa* (lucerne) has been estimated at 3 mg N m^{-2} d^{-1} (Roper *et al.*, 1995), and microorganisms capable of fixing N_2 were found at 55 of 67 sites sampled in New South Wales (Thompson *et al.*, 1984). Thus, the capacity for free-living nitrogen fixation may be very widespread.

Illustrating the importance of carbon supply to N_2 fixation, organic substrates of a high C:N ratio favour heterotrophic diazotrophs (Havelka *et al.*, 1982), and the decomposition of plant material can support large rates of N_2 fixation (Roper, 1983, 1985; Dart, 1986). Root exudates and sloughed root material are also thought to stimulate N_2 fixation as they are low in nitrogen but form a readily available source of energy (van Berkum, 1984).

Thus, even ecosystems that do not contain a legume have the capacity to fix atmospheric N_2 at low rates. Growth under CO_2 enrichment may stimulate this fixation if carbon supply is increased, or if a higher surface soil moisture content is maintained. Such a low level of nitrogen input may be important in the longer term, especially in relation to the CO_2 responsiveness of the soil-plant system.

Dry deposition of nitrogen

Although rates of wet and dry deposition over the terrestrial land surface are largely a function of atmospheric concentrations of particulate and gaseous nitrogenous compounds, the plant can influence the rate of dry deposition of ammonia. At high ambient concentrations of ammonia plants may absorb it from the atmosphere (Hutchinson *et al.,* 1972), which may be either stomatal or cuticular absorption, or a combination of both (Denmead *et al.,* 1976; Sutton *et al.,* 1992). Plants have been observed to utilise ammonia as a sole source of nitrogen without affecting growth or development at concentrations in the order of 1 mg N m^{-3} (Faller, 1972), however these concentrations are much larger than normal atmospheric concentrations.

As with CO_2, there is a compensation point, or intercellular partial pressure of ammonia below which it will be evolved and lost from the leaf, and above which ammonia will be absorbed from the atmosphere and gained by the leaf (Farquhar *et al.,* 1980). The ammonia compensation point was found to be between 2 and 6 nbar (1.4 - 3.5 μg N m^{-3} at 26 & 33°C respectively) for a range of species experiencing adequate soil mineral nitrogen levels (Farquhar *et al.,* 1980). As atmospheric concentrations over land remote from significant anthropogenic sources range from 1 - 10 μg NH_3-N m^{-3} (Ayers & Gras, 1980; Wollenweber & Raven, 1993), atmospheric ammonia may often be a net source of nitrogen to the plant. Most research on ammonia exchange between leaves or canopies and the atmosphere has been carried out with plants which were relatively well fertilised with nitrogen. In a natural moorland environment, which the authors reported as relatively low in available nitrogen and being far from any (major) source of ammonia, ammonia was found to undergo dry deposition onto the leaves of the plants, as well as absorption through stomata, resulting in an effective canopy compensation point of zero (Sutton *et al.,* 1992). These moorland systems were estimated to receive an annual input of between 0.3 and 1 g m^{-2} of NH_3-N, under typical background atmospheric concentrations of 0.45 - 2.1 μg NH_3-N m^{-3} (Sutton *et al.,* 1992). Similarly, effective canopy compensation points at or near zero have been observed for unfertilised grasslands and coniferous forests (Sutton *et al.,* 1993a). This was not the case in systems with a higher nitrogen content (fertilised), where stomatal and net canopy compensation points were generally higher, estimated at 1-7 μg NH_3-N m^{-3} and 0-2.5 μg NH_3-N m^{-3} respectively (Sutton *et al.,* 1993b) although no details of foliar nitrogen concentrations were given in either of these studies.

Lolium multiflorum Lam. (Italian ryegrass) was observed to increase its absolute uptake of atmospheric ammonia in response to reduced mineral nitrogen supply (shoot %N 1.07-0.89% by mass) and to increased atmospheric ammonia concentration (Whitehead & Lockyer, 1987).

The response to atmospheric ammonia concentration was linear between 11.5 and 583 μg NH_3-N m^{-3}, resulting in a greater total plant dry weight at low-N and a greater total nitrogen content at both rates of nitrogen supply (Whitehead & Lockyer, 1987). Linearity of this relationship has been confirmed in the field at concentrations as low as 6 μg NH_3-N m^{-3} (Sommer & Jensen, 1991). Increases in ammonia sorption as plant nitrogen supply is reduced has also been demonstrated for *L. multiflorum* by Lockyer & Whitehead (1986), and implied by field data for wheat (Harper *et al.,* 1987) and barley (Schjoerring *et al.,* 1993a), although sorption does not appear to be sensitive to leaf nitrogen concentration when it exceeds 4.5% (Rogers & Aneja, 1980). Sorbed NH_3-N is combined into organic nitrogen (Lockyer & Whitehead, 1986; Whitehead & Lockyer, 1987). The sorbed nitrogen does not appear to be uniformly distributed throughout the plant, and tends to remain in leaf tissue (Lockyer & Whitehead, 1986), although in nitrogen stressed plants some translocation has been observed (Whitehead & Lockyer, 1987; Sommer & Jensen, 1991). Thus, atmospheric ammonia may be another source of nitrogen for ecosystems where low nitrogen supply limits productivity. This source may be more available to plants grown under CO_2 enrichment due to the lower leaf nitrogen concentration of such plants.

Observed nitrogen gain in non-legume systems

Nitrogen gain by grass-soil systems, as determined by nitrogen balance has been commonly observed. *Eleusine coracana* microcosms exhibited between a -3% and 12% change in total system nitrogen when the grass was grown on similar soils, but with varying histories, over a 16 week period (Moore,AW, 1963). The nitrogen loss was observed on the soil with the highest nitrogen concentration, and that experiment was a microcosm experiment, eliminating the possibility of sub-soil nitrogen transport to the surface soil (Moore,AW, 1963).

In the long term Broadbalk wheat experiment in England, nitrogen balance has shown an accumulation of 4.8 g N m^{-2} yr^{-1} above fertiliser inputs in a field receiving annual additions of fertiliser of 14.4 g N m^{-2} yr^{-1} (Powlson *et al.,* 1986). The nitrogen input was attributed to a combination of deposition and nitrogen fixation. In a long term field experiment at Coimbatore, South India, no loss of nitrogen from the soil-plant system was noted, after removal in produce was accounted for over 53 non-legume crops, implying that inputs from N_2 fixation and deposition balanced the losses of nitrogen from the system via gaseous processes and leaching (Mariakulandai & Thyagarajan, 1958; Dart, 1986). Field grown swards of annual ryegrass (*Lolium rigidum* Gaud.) at Merredin, Western Australia accumulated 6 g m^{-2} yr^{-1} of nitrogen between 1952 and 1954 in the surface 25 cm of soil, which was 67% of that accumulated by a

nodulated sward of *Medicago tribuloides* Desr. (Parker, 1957). The authors attributed this to biological nitrogen fixation, as they found no evidence of nitrogen transfer from the subsoil. This balance did not include the loss of nitrogen to the atmosphere when the swards were burnt in a grass fire in early 1954, and so may be an underestimate of total nitrogen gain. However no thought was given to the possibility of nitrogen deposition. In a previous report, nitrogen accumulation had been observed to increase in a cultivated soil relative to an adjacent undisturbed soil at a rate of 1.7 g m^{-2} yr^{-1} in the surface 10 cm (Parker, 1953). The author attributed this to biological fixation, as it was strongly correlated with soil carbon, with the caveat that some transfer of nitrogen from the subsoil was possible (Parker, 1953, 1957). These gains in nitrogen from the environment are not surprising when it is considered that in pre-industrial terms, most terrestrial nitrogen originated from biological N_2 fixation (page 1-8).

In summary, potential exists for nitrogen to be scavenged from the environment by plant-soil systems, especially those which have their productivity limited by low nitrogen supply. This may be enhanced in a high CO_2 world, for nitrogen fixation as carbon availability in the plant-soil system increases, and for dry nitrogen deposition as leaf nitrogen concentrations are lowered, potentially lowering the canopy compensation point for ammonia exchange. In the current study, nitrogen accumulation in microcosms growing under CO_2 enrichment was monitored, and ^{15}N enrichment and ^{15}N natural abundance techniques were used to elucidate potential fluxes of nitrogen between the microcosm and the environment.

Natural abundance of ^{15}N: A tool for the investigation of nitrogen cycling

The stable isotope of nitrogen, ^{15}N is present in the atmosphere at a fractional abundance of 0.3663 atom % (Mariotti, 1983). Both biological and physical processes cause isotopic fractionation, but to different extents (Shearer & Kohl, 1986; Handley & Raven, 1992).

The "heavy" isotope of nitrogen, ^{15}N, is discriminated against by nearly all enzymes using nitrogenous substrates, with the exception of nitrogenase (Handley & Raven, 1992), the key enzyme in nitrogen fixation. Handley and Raven (1992) compiled from the literature a selection of data for α from various organisms including higher plants, where α is defined as:

$$\alpha = \frac{rate\ constant\ ^{14}N}{rate\ constant\ ^{15}N}$$

Thus when $\alpha > 1$, ^{15}N is discriminated against, the product is depleted in ^{15}N and the substrate enriched. For nitrogen fixation ($N_2 \rightarrow$ organic N) values of α ranged from 0.991 to 1.0041. Other transformations of interest to this study had higher α values, such as denitrification

($NO_3^- \rightarrow N_2O$) 1.028 - 1.033, and the pathway of nitrification producing gaseous products ($NO_2^- \rightarrow N_2O$) 1.035 - 1.036.

Equilibrium constants are also affected by isotopic composition. The equilibrium isotope effect on the reaction $NH_4^+ + H^+ \leftrightarrow NH_3$ has been reported as 1.02, with NH_4^+ exhibiting the greatest enrichment at equilibrium (Hermes *et al.,* 1985). That is, at equilibrium, NH_4^+ will have a higher $\delta^{15}N$ than NH_3. Thus isotopic fractionation can also occur during ammonia volatilisation, enriching residual nitrogen in ^{15}N (Farquhar *et al.,* 1983; Shearer & Kohl, 1986).

On a global average basis total soil nitrogen has a greater fractional abundance of ^{15}N than is present in the atmosphere (Hoering & Ford, 1960; Cheng *et al.,* 1964; Shearer & Kohl, 1986; Handley & Raven, 1992). Assuming that the majority of nitrogen in terrestrial pools originates from biological N_2 fixation (page 1-8; Handley & Raven, 1992), the enrichment of the soil nitrogen pool with ^{15}N relative to the atmosphere has been attributed to the discrimination against ^{15}N in nitrogen loss processes, primarily denitrification (Hoering & Ford, 1960). This fractionation and resultant difference in $\delta^{15}N$ of nitrogen pools is used in this study to assist in the elucidation of nitrogen dynamics.

Allocation / distribution of carbon and nitrogen within the plant

Growth at high CO_2 is often observed to alter the distribution of carbon within the plant (eg. Stulen & den Hertog, 1993). It has been hypothesised that growth at high CO_2 will also lead to changes in nitrogen allocation between plant parts (Stitt, 1991), as observed in *Xanthium occidentale* Bertol. (Hocking & Meyer, 1985) and *Pinus sp.* seedlings (Griffin *et al.,* 1995). Alterations in distribution of carbon and nitrogen may indicate important changes in the functional response of the plant to CO_2 enrichment. They may also have important implications for ecosystem function, altering the proportion of carbon and nitrogen entering the leaf and root decomposition systems. Stulen & den Hertog (1993) hypothesised that many of the observed changes in carbon distribution (eg root carbon ratio, the proportion of total plant carbon in the root, which approximates root mass/weight ratio) were expressions of changes in water or nutrient availability under CO_2 enrichment, rather than physiological changes in carbon allocation. Carbon distribution also changes with plant age and hence size (Ballard *et al.,* 1936; Garnier & Freijsen, 1994), further confounding CO_2 effects. Studies that have used allometric relationships to elucidate allocation patterns have generally found no effect of CO_2 on carbon allocation (Bowler & Press, 1993; Baxter *et al.,* 1994a; Hunt,R *et al.,* 1995). A potentially more effective measure of comparing root and shoot function is the ratio of root length or area to leaf

area, which compares the areas associated with mineral nutrient and water capture to that involved with carbon capture (Körner & Renhardt, 1987). These relationships are investigated for *Danthonia richardsonii* in this study.

Leaf senescence and turnover

Rates of leaf initiation have sometimes been observed to increase slightly in response to CO_2 enrichment (Ackerly *et al.,* 1992). However, the effect of CO_2 enrichment on senescence is not consistent between species. It has variously been reported to delay senescence (eg. Carter & Peterson, 1983), have no effect (eg. Gunderson et al., 1993), or advance senescence (eg. Chang, 1975). In a sward study, CO_2 enrichment was observed to increase leaf litter without increasing standing live leaf (Navas *et al.,* 1995), potentially indicating an increased rate of leaf turnover at high CO_2. This has implications for the efficiency of nitrogen use for carbon accumulation, as senesced leaf often has the highest C:N ratio of any phytomass carbon pool in herbaceous systems. Ratios of senesced leaf to green leaf will be calculated in the microcosm study to infer rates of leaf turnover.

Transfer of root carbon and nitrogen to soil

There are two major mechanisms of carbon and nitrogen input into the soil - root death and rhizodeposition (Stanton, 1988). As up to 80-90% of plant biomass can occur below ground in grasslands (Stanton, 1988; Dormaar, 1992) modifications to the root system and soil environment may have a much larger impact on carbon and nitrogen accumulation in the soil under CO_2 enrichment than changes to the shoot. Direct measurement of root turnover and rhizodeposition was outside the scope of this study. *In vitro* decomposition assays were undertaken to assess changes in root decomposability (below). Changes in aggregated carbon and nitrogen input into the soil are inferred by increases in total soil carbon and nitrogen.

Decomposition of plant litter - Soil C & N accumulation

In the short term, the majority of nitrogen used for plant growth derives from recently decomposed plant litter (eg. Abbadie *et al.,* 1992). Decomposition rates of plant material show a correlation with its C:N ratio, which tends to be strong in the short term, but weaker in the longer term (Waksman & Tenney, 1928; Herman *et al.,* 1977; Melillo *et al.,* 1982; Berg, 1984; Fog, 1988; Aber *et al.,* 1989). As growth under CO_2 enrichment tends to increase the C:N ratio of plant litter (Coûteaux *et al.,* 1991; Cotrufo *et al.,* 1994; Boerner & Rebbeck, 1995), it has been hypothesised that decomposition rates will be slower in a high CO_2 world, limiting nutrient return to plant available forms and hence a continued biomass response to CO_2

enrichment (Melillo *et al.,* 1990). A complementary hypothesis of Díaz *et al.,* (1993) suggests that increases in microbial biomass under CO_2 enrichment may also immobilise nitrogen, reducing its availability for plant growth.

These hypotheses relating to decomposition and nutrient immobilisation have not been fully tested. Decomposition rates in the field are a function not only of litter quality, but also the environment, and the decomposer community (Swift *et al.,* 1979). As all three of these factors are possibly modified under CO_2 enrichment, studies at a higher order of complexity than simple *in vitro* decomposition assays are needed to address the question of decomposition, and the return of nutrients for plant growth under CO_2 enrichment. Two pools of soil carbon and nitrogen are defined, the "fast" pool, comprising microbial C or labile N, and the "slow" pool, the difference between total soil C and N and the fast pool, to test for differences in carbon and nitrogen accumulation in the more recalcitrant soil fractions. Litter quality, soil moisture content, and the quantities of microbial carbon and the associated fast and slow soil C and N pools will be assessed to provide more information on the possible long-term microcosm response to CO_2 enrichment.

Outline of thesis

A general outline of each chapter is given, along with the major hypotheses addressed.

Chapter 2. General methods

An outline of environmental monitoring and control equipment used in the Phytotron glasshouses throughout the study. Analytical methodologies are described.

Chapter 3. Between and within species variation in response of isolated plants to CO_2 with abundant nutrient supply

The overall objective of this study was to examine the responsiveness of a model grass ecosystem to CO_2, with the aim of inferring responses to CO_2 at the ecosystem level. As it was not possible to undertake multiple sward experiments with different grass species it was important to compare the CO_2 responsiveness of the chosen "model" grass *Danthonia richardsonii* Cashmore with other grass species. Those comparisons are made in this chapter for three ecotypes of *D. richardsonii* and seven other wild and domesticated grasses, grown as isolated plants with abundant supplies of nutrient and water.

Hypothesis: Isolated plants of the C_3 grass Danthonia richardsonii will exhibit similar biomass, leaf area and leaf nitrogen responses to CO_2 enrichment as other C_3 grasses when grown with abundant water and nutrient supply.

Chapter 4. Between and within species variation in response of isolated plants to CO_2 with growth limiting nitrogen supply

This chapter continues the comparison of the character of the CO_2 response of *Danthonia richardsonii* to that of other species. This chapter reports experiments where isolated plants were grown under levels of nitrogen supply which restricted growth.

Hypothesis: Isolated plants of the C_3 grass D. richardsonii will exhibit similar responses in biomass, total water use, leaf area, carbon distribution, and nitrogen concentration and distribution in response to CO_2 enrichment as other C_3 grasses when grown with growth-restricting levels of nitrogen supply.

Chapter 5. Acquisition and allocation of carbon and nitrogen by Danthonia richardsonii in response to restricted nitrogen supply and CO_2 enrichment

This chapter examines the physiological response at the whole plant level of *Danthonia richardsonii* to CO_2 enrichment under varying levels of nitrogen nutrition during the exponential phase of growth. Growth analysis techniques were employed to examine effects of CO_2 enrichment on leaf and root function. Allometric analysis was used to elucidate changes in carbon and nitrogen allocation, and these are compared with changes in measures of proportional distribution of carbon and nitrogen at harvest.

Hypothesis: Growth of isolated plants of D. richardsonii under CO_2 enrichment will result in changes in leaf function and in the allocation of nitrogen within the plant that are not the result of changes in plant size when grown under varying degrees of nitrogen stress. That is, growth at high CO_2 will result in changes in nitrogen use by the plant.

Chapter 6. Carbon accumulation, distribution and water use of D. richardsonii swards in response to CO_2 and nitrogen supply over four years of growth

The first of three chapters examining the response of an experimental model grass microcosm to CO_2 enrichment. In this chapter the general experimental procedure is outlined, and changes in carbon accumulation, carbon distribution and water use under CO_2 enrichment are discussed.

Hypothesis: Microcosms of D. richardsonii swards grown under CO_2 enrichment for four years will increase carbon accumulation, even when nitrogen supply severely limits productivity, and CO_2 enrichment will result in a lower rate of microcosm water use.

Chapter 7. Nitrogen accumulation and distribution in D. richardsonii swards in response to CO_2 and nitrogen supply over four years of growth

Nitrogen accumulation in the microcosms is discussed, along with possible sources of nitrogen loss to, and gain from the environment. Nitrogen distribution within the microcosm is also discussed.

Hypotheses: Growth of D. richardsonii microcosms under CO_2 enrichment will result in an increase in nitrogen sequestration from the environment, a decrease in the nitrogen concentration in the soil and plant carbon pools, and a greater proportion of nitrogen will be present in the high C:N ratio plant carbon pools.

Chapter 8. Decomposition and related soil parameters in Danthonia richardsonii swards in response to CO_2 and nitrogen supply over four years of growth

Factors affecting internal nutrient cycling, and hence the potential for a long term increase in microcosm carbon storage under CO_2 enrichment are discussed. These include litter quality, soil moisture content and soil and surface litter microbial biomass.

Hypotheses: Higher C:N ratios of plant litter produced under CO_2 enrichment will reduce its in vitro decomposability. This may not be expressed at the microcosm level, owing to compensations by changes in the microenvironment and in the decomposer population under CO_2 enrichment.

Chapter 9. Synthesis of findings

A general synthesis of results, and a short discussion of the implications of this work for carbon storage and productivity of grasslands and the terrestrial biosphere as a whole in a high CO_2 world.

Chapter 10. References

A list of works cited in this thesis.

Table 1.1 Estimated annual budget of anthropogenic carbon emissions averaged for the period 1980-1989 in Gt C yr^{-1} (1 Gt = 10^{15} g), with an estimated 90% confidence interval. From Schimel *et al.* (1995).

	Gt C yr^{-1}
CO_2 Sources	
(1) Emissions from fossil fuel combustion and cement production	5.5 ± 0.5
(2) Net emissions from changes in tropical land use	1.6 ± 1.0
(3) Total anthropogenic emissions (1) + (2)	7.1 ± 1.1
CO_2 Sinks	
(4) Atmospheric CO_2 concentration increase	3.2 ± 0.2
(5) Oceanic uptake	2.0 ± 0.8
(6) Northern Hemisphere forest regrowth	0.5 ± 0.5
(7) Additional terrestrial sinks [(1) + (2)] - [(4) + (5) + (6)]	1.4 ± 1.5

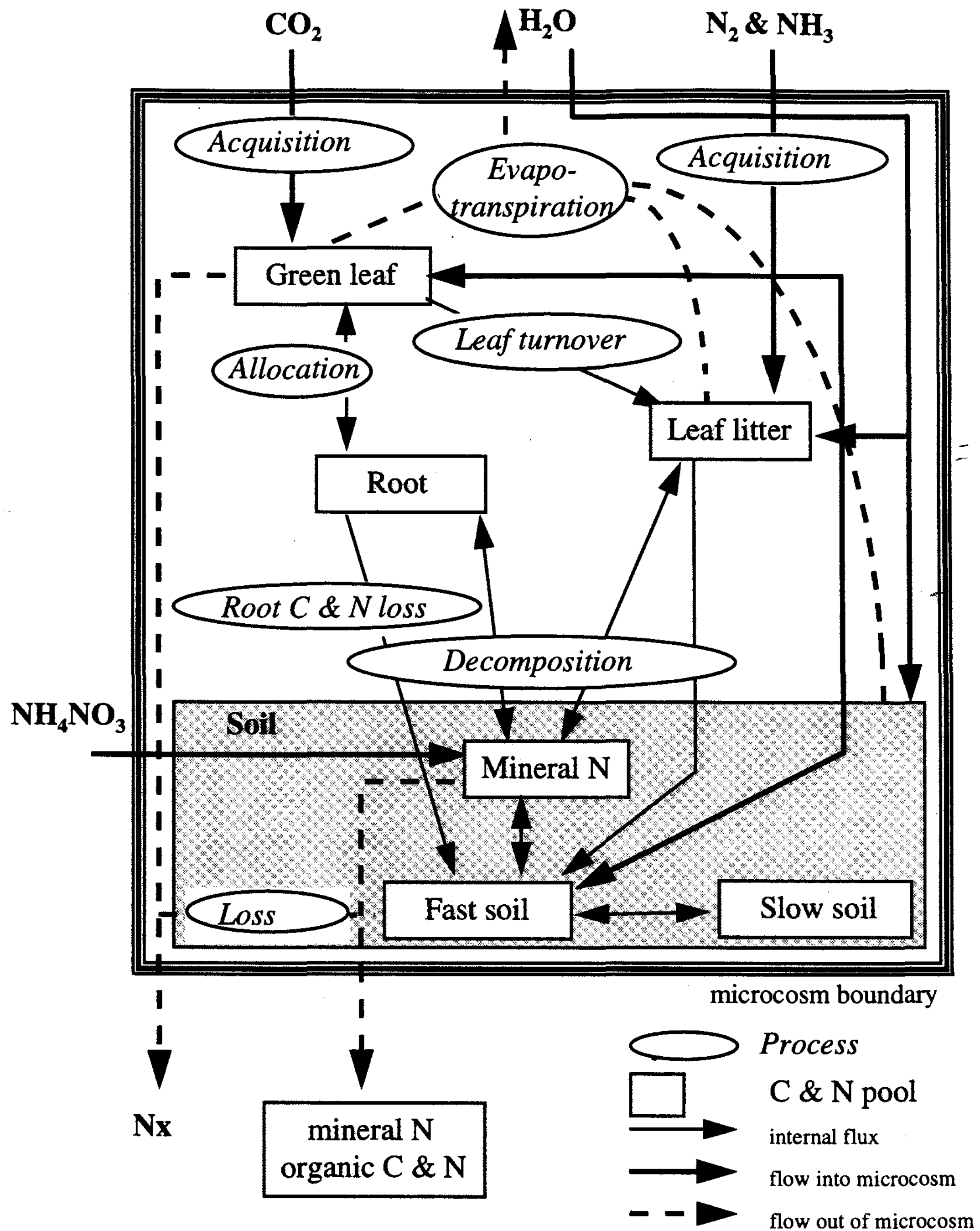
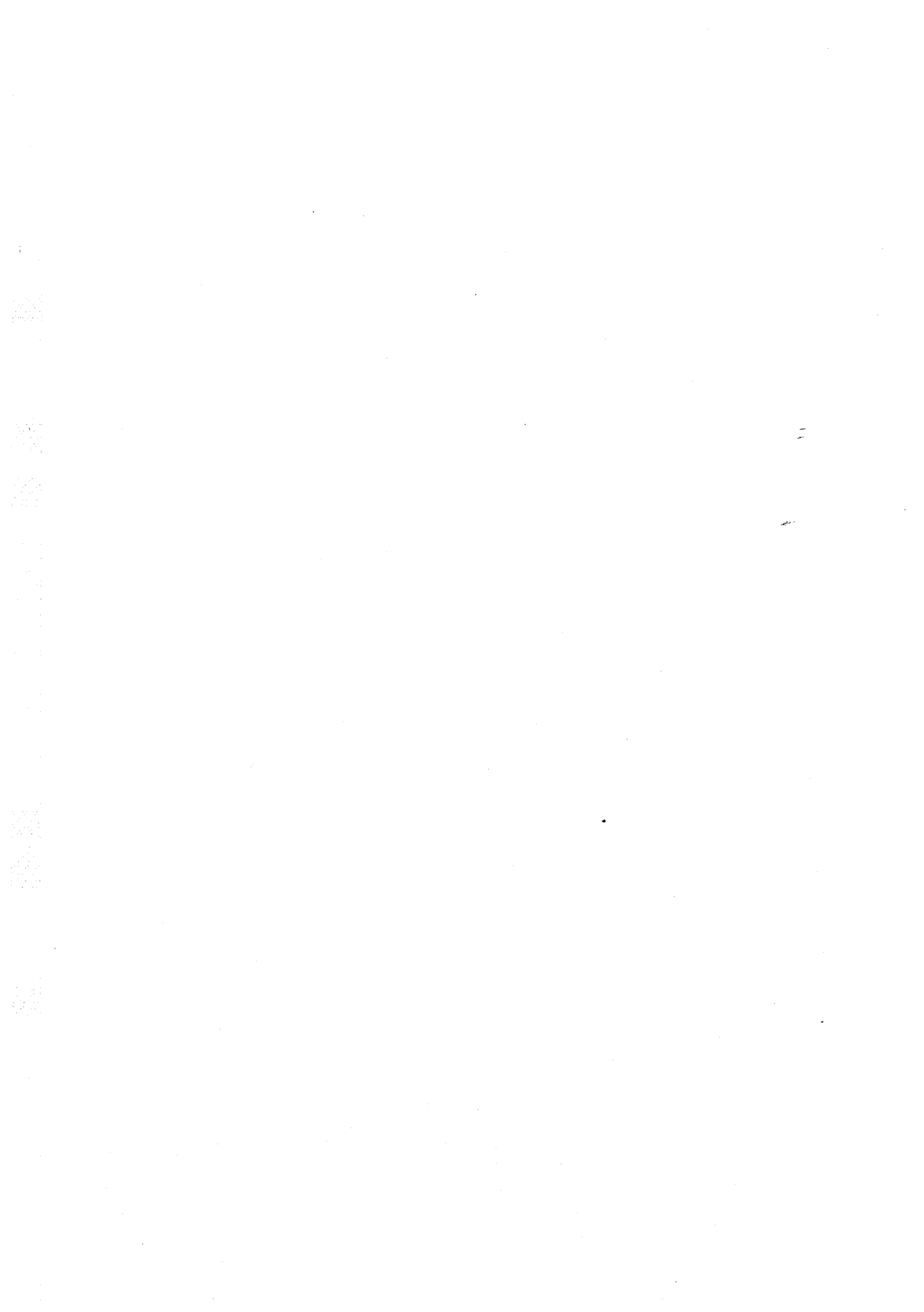


Diagram 1-1 A simple, conceptual model of microcosm function. Processes and pools sizes (not to scale) shown are addressed in this study. Those fluxes through the mineral N pool are of nitrogen only. Internal fluxes are generally inferred by changes in pool size. NH_4NO_3 and H_2O were controlled inputs in this study. Losses of carbon from the microcosm as respired CO_2 were not assessed. The soil pool which was measured throughout the experiment includes the mineral N, fast (microbial & labile) and slow ([total] - [microbial & labile]) pools which were measured or derived at day 1285 and 1469. Gaseous fluxes of nitrogen are those which are hypothesised to account for nitrogen loss (Nx represents oxides of nitrogen and N_2), as determined by ^{15}N budget, and nitrogen gain, as determined by mass balance.



Chapter 2. General methods

Glasshouse control

Only two Phytotron glasshouses (Morse & Evans, 1962) were available for the experiments described in this study, one of which was CO_2 enriched, the other maintained at near ambient CO_2 concentrations. Thus CO_2 levels were not replicated. Therefore, it was of critical importance to maintain uniform environmental conditions between glasshouses to minimise error associated with "glasshouse" effects. In addition to strict environmental control and monitoring[1], treatments were rotated between glasshouses every three months, and the benches of the microcosm experiment rotated within the glasshouse every month to average out any gradients within the glasshouses. Trolleys supporting isolated plant experiments were rotated within the glasshouses on a daily basis. Gradients within glasshouses were minimised by large fans mounted in front of the inlet air registers. Air flow through the glasshouses was 14 m^3 min^{-1} by day and 1.4 m^3 min^{-1} by night (Morse & Evans, 1962).

Control of glasshouse temperature, CO_2 concentration and dew point was independent of the monitoring system. That is, separate control and monitoring systems were used to minimise the possibility of undetected breakdowns in environmental control. All sensors (outlined below) were automatically logged approximately seven times every hour. Twenty-four hour averages of environmental parameters (day-time averages of CO_2 concentrations) were manually accumulated into a database system and human feedback on the control system was applied where necessary.

Glasshouse temperature was controlled on a sine wave by standard Phytotron microprocessor feedback controllers. A system of four aspirated, radiation shielded air temperature probes and three under-pot temperature probes were installed in each glasshouse to monitor air temperature. The human feedback loop was used to trim glasshouse temperature set-points to accommodate any small deviations between glasshouses in maximum, minimum and cumulated air temperatures.

Carbon dioxide concentration and dew point was monitored from a point in the center of each glasshouse. Carbon dioxide concentration was under continuous feedback control, with human

[1] Equipment for glasshouse environmental monitoring, CO_2 and dew point control was designed by Dr R.M. Gifford and CSIRO Plant Industry electronics staff. The author was involved with implementation, calibration, maintenance and database design.

trimming of set-points from time to time when necessary. In the enriched glasshouse, CO_2 was introduced into the air inlet duct prior to its entry to the glasshouse, at one of four flow rates (0, 4, 8, 12 L CO_2 min^{-1}). All CO_2 was passed through a column of a potassium permanganate formulation (Purafil II, Purafil Inc., Atlanta, Ga.) to remove possible hydrocarbon contaminants (Morison & Gifford, 1984). Carbon dioxide was scrubbed from the control glasshouse by continuously passing air from the glasshouse through two 20 L drums filled with soda lime (Drägersorb 800, Drägerwerk Aktiengesellschaft, Lübeck, Germany).

Dew point was increased when necessary by ultrasonic and high pressure spray humidifiers. A decrease in dew point was achieved by passing air over a condenser and running the condensed water down a drain. Both humidification and dehumidification were automatically controlled.

Evaporation was measured at four points in each glasshouse using lab-constructed wick evaporimeters. A wick drew water from a reservoir onto a horizontal filter paper which was exposed to the air. Water evaporated was determined gravimetrically.

Radiation was monitored using a tube solarimeter (Delta-T Devices Ltd, Cambridge, England) in each glasshouse, calibrated against a Kipp & Zonen solarimeter. Incident radiation below the glass was logged and was averaged over the two glasshouses before total daily incident radiation was calculated by the datalogger. Thus the radiation data presented are averaged over the glasshouses.

Overall, the level of environmental control attained was very good. Summaries of conditions during each experiment are given in the experimental chapters, and daily temperature, CO_2 and radiation levels over the experimental period are shown in Figure 6.1.

Analytical methods

Total carbon

Total plant mass is expressed as carbon content wherever possible in this study. This removes treatment bias associated with differing mineral contents, removes changes in biomass associated with different C allocation between structural and various non-structural carbohydrates having different carbon concentrations (Evans,GC, 1972), and reduces error associated with contamination of the root sample with the growth medium.

A LECO (CR-12) total carbon combustion analyser standardised against glucose was used to determine total carbon in the experiments discussed in chapters 3 and 4, and for earlier harvests

of the microcosm experiment (chapters 6-8). For carbon determination in the experiment of chapter 5, and for the latter harvests of the microcosm experiment a Europa ANCA-NT 20 - 20 stable isotope analyser was used. Secondary standards of green clover shoot were included for normalisation between batches on the Europa analyser.

Cross calibration for the sward experiment was achieved by analysing samples (soil and plant) with both methods (n = 505). A linear regression of total LECO %C on Europa %C was developed,

$$Europa\ \%C = 0.967 \times LECO\ \%C \quad r^2 = 0.998$$

and used to normalise all data to that of the Europa ANCA-NT 20 - 20 stable isotope analyser.

Total carbon in leachate

Wet dichromate digestion

Leachate carbon in the microcosm experiment was determined spectrophotometrically following dichromate digestion, modified from Walkley and Black (1934) prior to day 1104 (Table 6.5). Samples of leachate (20 mL) were freeze dried in glass scintillation vials. One mL of digestion mixture (100 mL conc. sulphuric acid + 50 mL 1 M potassium dichromate) was added to each vial, a Teflon seal placed in the vial lid, and the vial tightly sealed. Samples were digested at 120 °C for 2 hours. Digested sample solution (600 µL) was added to 1% barium chloride (700 µL), and absorbance was determined at 591 nm. Carbon concentration was determined against glucose standards. After day 1104 leachate carbon was determined on a Europa ANCA-NT 20 - 20 stable isotope analyser, as for leachate nitrogen (below).

Microbial biomass carbon

The carbon in the microbial biomass was estimated from the flush of ninhydrin reactive compounds following a ten day fumigation with chloroform (Amato & Ladd, 1988), as follows. The factor used to convert the ninhydrin-reactive N flush to microbial C was 21 (Amato & Ladd, 1988).

Fumigation and extraction - general method of soil extraction

Freshly harvested soil samples were sieved through a 1.5 mm mesh sieve to remove root fragments. For each assay a pair of samples (~15 g FW) were collected and stored in screw-capped extraction bottles at 2°C until analysis. An additional sample of soil was immediately weighed and dried for moisture content determination. When samples from 6 pots had accumulated (~3-4 days), one sample (unfumigated sample) of each pair was extracted with 40

mL of 2M potassium chloride by shaking at room temperature for 1 hour. The solution was filtered through double Whatman #42 filter papers (Whatman International Ltd. Maidstone, England) and an aliquot taken for NH_4^+ and NO_3^- determination by autoanalysis (page 2-25). The remaining filtrate was further sterilised by passing it through syringe mounted 0.45 μm filters (FP 030/2, Schleicher & Schuell GmbH, Dassel, Federal Republic of Germany) and frozen for later analysis.

The other sample of the pair (fumigated sample) was fumigated for 10 days at 27 °C in the dark with ethanol-free chloroform, in desiccators containing wet blotter paper. The ethanol-free chloroform was placed in a beaker containing anti-bumping granules. The desiccator was evacuated using a venturi pump on a mains pressure tap for 10 minutes from commencement of chloroform bubbling. After incubation residual chloroform was removed and samples re-evacuated for 15 min. The fumigated soil samples were then extracted with potassium chloride following the procedure outlined above.

Nitrogen determination

Total - Kjeldahl nitrogen

Kjeldahl nitrogen was determined in the experiments discussed in chapters 3 and 4, and for earlier harvests of the microcosm experiment (chapters 6-8) using a modified Kjeldahl method which is outlined below. For total nitrogen determination in the experiment of chapter 5, and the latter harvests of the microcosm experiment a Europa ANCA-NT 20 - 20 stable isotope analyser was used, secondary standards of green clover shoot being included for normalisation between runs.

Cross calibration for the sward experiment was achieved by analysing samples (soil and plant) with both methods (n = 477). A quadratic regression of total Kjeldahl %N on Europa %N was developed,

$$Europa\ \%N = 0.950 \times Kjeldahl\ \%N - 0.029 \times (Kjeldahl\ \%N)^2 \quad r^2 = 0.993$$

and used to normalise all data to that of the Europa ANCA-NT 20 - 20 stable isotope analyser. As the Kjeldahl assay does not efficiently recover nitrate, the Kjeldahl assay may have been expected to recover less N than the combustion method of the Europa - the opposite of what was observed. However, little nitrate is expected in plant tissue with nitrogen concentrations below 3% (by mass) (Garnier & Freijsen, 1994), a concentration rarely reached in the microcosm experiment.

Total nitrogen and carbon in leachate and demineralised water

Leachate was subsampled (800 mL) and concentrated by evaporation before analysis. Before evaporation the samples were acidified to pH 3.1 (hydrochloric acid) to inhibit microbial growth. The residual salts were analysed for nitrogen by Kjeldahl assays prior to day 1104. Samples collected after this day were analysed on the Europa ANCA-NT 20 - 20 stable isotope analyser for both nitrogen and carbon.

Kjeldahl nitrogen

Digestion - plant samples

Samples (~100 mg) were weighed into Tally-Ho cigarette paper (contains no nitrogen) and placed in a 75 mL digestion tube. Concentrated sulphuric acid (5 mL) was added to each digestion tube. After 1 hour at room temperature, 3 mL hydrogen peroxide was added to each tube and mixed. Digestion was completed by heating in a digestion block at 200 °C for 1 hour followed by 350 °C for 2 hours. After cooling, the digested sample mix was diluted to 75 mL.

Digestion - soil samples

Samples (~2 g) were weighed into Tally-Ho cigarette paper (contains no nitrogen) and placed in a 75 mL digestion tube. Concentrated sulphuric acid (5 mL) was added to each digestion tube. After 1 hour at room temperature, 4 mL hydrogen peroxide was added to each tube and mixed. Digestion was completed by heating in a digestion block at 200 °C for 1 hour followed by 350 °C for 4 hours. After cooling, the digested sample mix was diluted to 75 mL.

Distillation and titration

Sodium hydroxide (4 mL, 15N) was added to 25 mL of digested sample solution using a steam distillation apparatus, and the evolved NH_3 collected in 0.2% boric acid (5 mL), which contained an indicator (1:2 bromocresol green to methyl red by mass). The sample was then titrated with hydrochloric acid (0.01M) and nitrogen concentration determined against ammonium sulphate standards.

Mineral nitrogen

Mineral nitrogen (NH_4^+ & NO_3^-) was determined in the 2M potassium chloride soil extracts (page 2-23) using a TECHNICON TRAACS 800 Auto-Analyser (Anon., 1984; Markus *et al.*, 1985).

Mineral nitrogen concentration in demineralised water

Demineralised water samples were concentrated by evaporation before analysis (2 L $\rightarrow$ 50 mL). Before evaporation the samples were acidified to pH 3.1 (hydrochloric acid) to inhibit microbial growth. Samples were then analysed by autoanalysis as above.

Ninhydrin reactive N determination

The method was based on that of Amato & Ladd (1988) and Moore & Stein (1954). Acetate buffer (544 g sodium acetate trihydrate/400 mL distilled water/100 mL glacial acetic acid) was adjusted to a pH of 5.51 ± 0.03 (acetic acid/sodium hydroxide) and made to 1 L with distilled water. Depending upon expected concentration, 0.1 - 1 mL of the extract from the potassium chloride extraction (page 2-23) was made to 2 mL with 2 M potassium chloride. Two mL of freshly prepared ninhydrin reagent (2 g ninhydrin/0.2 g hydrindantin/50 mL methoxyethanol/50 mL acetate buffer, dark stored) was added and mixed well. After incubation in a boiling water bath for 15 min, samples were cooled in ice-water and diluted with 5 mL of 50% ethanol. Absorbance was determined at 570 nm and ninhydrin reactive N determined against leucine and ammonium sulphate standards.

Amino nitrogen

Amino-N in the potassium chloride extracts was determined as the difference between the total ninhydrin reactive N and ammonium-N in the unfumigated sample from the microbial carbon assay (Moore,S & Stein, 1954).

Potentially mineralisable N - index of microbial N

Potentially mineralisable N was determined following the method of Waring & Bremner (1964), as recommended by Keeney (1982). The flush of NH_4^+-N was determined after a 10 day anaerobic incubation at 40 °C. The potassium chloride extract from the unfumigated sample of the microbial C assay was used for initial NH_4^+ determination. Another sample of 15 g FW was collected from the sieved soil for anaerobic incubation in 20 mL of distilled water. After ten days, 20 mL of 4 M potassium chloride was added and extraction completed (page 2-23). Ammonium concentration was determined by autoanalysis (page 2-25). Nitrogen extracted by this method has a strong linear correlation with microbial nitrogen (Myrold, 1987; Stockdale & Rees, 1994).

Within plant carbon pools

Total non-structural carbohydrates (NSC)

Preliminary exploratory determinations of soluble carbohydrates and starch were made on a small number of samples (method based on D-Glc/UV method kit 716-251, Boehringer Mannheim GmbH, Mannheim, Germany). For senesced leaf, starch concentration was approximately 20% of total non-structural carbohydrate (the sum of glucose, fructose, sucrose, fructans and starch). For green leaf material, starch concentration was approximately 50% of total non-structural carbohydrate. Subsequently, all non-structural carbohydrates were analysed as a single pool by the following method.

Samples (~40 mg) were heated in a boiling water bath for thirty minutes, after which thermostable α-amylase (Megazyme Australia) in MOPS buffer (pH 7.0) was added and samples boiled for a further 5 minutes. Temperature was reduced to 50°C, sodium acetate buffer (pH 4.5) and amyloglucosidase (Megazyme Australia) added and samples incubated for a further one hour. Glucose equivalents were then determined by anthrone assay (Helbert & Brown, 1955), incubating for 13 minutes at 98°C. These conditions gave approximately equal readings of equal concentrations of glucose and fructose standards (within 3%), and an average 100% yield of glucose units from unmodified wheat starch (Sigma Chemical Co.).

The percent carbon concentration of soluble sugars is 40% for glucose and fructose, 42% for sucrose and about 43-44% for fructans. However, *Danthonia spp.* have been shown to accumulate only small quantities of fructans (Chatterton *et al.*, 1989). Hence glucose equivalents in the total-non structural carbohydrate assay were converted to carbon assuming a 40% carbon concentration. Structural carbon was calculated as the difference between total carbon and NSC-carbon.

Hemicellulose plus pectin

This fraction was determined as the dry mass lost from the residue of the NSC assay during the sodium hydroxide extraction in preparation for the cellulose assay. Sample preparation for the cellulose assay was by the method of R.C. Smith (personal communication). The residue from the total non-structural carbohydrate assay was dried, weighed and then incubated on a rotary end-over-end shaker at room temperature with 6 M sodium hydroxide and 0.5% sodium borohydride (to inhibit cellulose degradation) for 24 hours. The sample was spun (microfuge, 14000 rpm, 10 min) and the supernatant discarded. This process was repeated four times, solubilising hemicellulose and some pectin. The sample was then incubated for 24 hours with 6

M sodium hydroxide, 0.5% sodium borohydride and 4% boric acid at room temperature on an end-over-end shaker, solubilising de-acetylated hemicellulose and de-esterified pectin. The sample was then spun (microfuge, 14000 rpm, 10 min) and the supernatant discarded. The sample was then rinsed three times by mixing with distilled water which was slightly acidified with a few drops of 50% acetic acid to neutralise the sodium borohydride. The sample was dried and weighed. The mass lost from the sample during this extraction includes hemicellulose, pectin, and any protein and lipid solubilised during this procedure.

Hemicellulose's are a diverse group of polysaccharides (Budavari, 1989) which constitute 25-50% (DW) of primary and about 30% (DW) of secondary cell walls (Salisbury & Ross, 1991). A wide range of sugar residues are present in hemicellulose, the most abundant being D-xylose units, having a carbon concentration of 40% (Budavari, 1989).

Pectin's are polysaccharides present in the primary cell wall (10-35% of DW) and middle lamella (Salisbury & Ross, 1991), consisting of a wide range of sugar monomers. The major sugar residue of pectin is D-galactose, which has a C concentration of 40% (Budavari, 1989).

This fraction also includes some protein and lipid which was not removed in the non-structural carbohydrate procedure. The carbon concentration of the hemicellulose-pectin fraction was assumed to be 40%. This may underestimate hemicellulose-pectin carbon, as carbon concentration is higher in the polysaccharide due to the loss of a H_2O unit in each linkage between the hexose monomers. The presence of significant proportions of lipid in this fraction would also increase its actual carbon concentration.

Cellulose

Cellulose was determined by a modified Updegraff method (Updegraff, 1969). The residue from the hemicellulose-pectin extraction was suspended in acetic-nitric reagent (glacial acetic acid/distilled water/conc. nitric acid, 8:2:1) and heated in a boiling water bath for 30 min to hydrolyse non-cellulosic polysaccharides. The sample was spun (2500 g, 5 min), rinsed with water and then acetone and dried. The supernatant was discarded. The residue was dissolved in 67% sulphuric acid, shaking for 1 hour at 25°C. Samples were then diluted and glucose determined via the anthrone assay (Helbert & Brown, 1955) against glucose standards. Average yield of glucose from cellulose standards (Whatman International Ltd. Maidstone, England) was 99.6%.

The primary cell wall comprises 9-25% (DW) cellulose, and the secondary cell wall 41-45% (DW) cellulose (Salisbury & Ross, 1991). Cellulose consists of β linked glucose monomers,

$(C_6H_{10}O_5)_n$ and is approximately 44.4% carbon (Budavari, 1989). However, as the assay separates the glucose monomers, and they are spectrophotometrically determined against a glucose standard, the carbon concentration of glucose (40%) (Budavari, 1989) is used to express cellulose as glucose-carbon equivalents of total structural C.

Lignin

Lignin was determined following the acetyl bromide method of Iiyama & Wallis (1990). Residue (~45 mg DW) from the non-structural carbohydrate assay was digested in 6 mL of a mixture of 73% glacial acetic acid, 23% acetyl bromide, and 2.8% perchloric acid at 70 °C in a fume hood for 1.5 hours. The digested solution was diluted (200 µL + 10 mL) into a 30:70 mix of 2 M sodium hydroxide and glacial acetic acid. Absorbance was determined at 280 nm. Secondary standards (*Pinus / Eucalypts* wood mix) were calibrated against a bagasse[2] lignin (from sugarcane) primary standard.

The lignin content of monocotyledon tissue varies from about 1.2% to 26% of dry weight (Lewis & Yamamoto, 1990). Lignin is a diverse plant polymer consisting of three major subunits, *p*-hydroxyphenyl, guaiacyl, and syringyl monomers (Lewis & Yamamoto, 1990), having carbon concentrations of 76.0, 68.3, and 62.7% respectively. Monocot lignin composition tends to a 50:50 ratio of guaiacyl and syringyl monomers by maturity (Lewis & Yamamoto, 1990). Thus the carbon concentration of lignin was assumed to be the average of these two monomers (65.5%).

Lignin may be slightly overestimated by this method, as in other methods, if cinnamic acid residues are linked to the lignin, or due to the presence of aromatic amino acids which adsorb at 280 nm (Morrison, 1972; Iiyama & Wallis, 1990), both of which are often present in grasses (Iiyama & Wallis, 1990; Lewis & Yamamoto, 1990). However this error is usually small as cinnamic acid concentration in herbaceous plants is generally less than 10% of that of lignin (Higuchi *et al.,* 1967; Iiyama & Wallis, 1990).

Extractable polyphenolics

Total extractable polyphenolics were determined by the Folin-Denis method, modified from Anderson & Ingram (1993) which detects hydrolysable tannins (not thought to be found in monocotyledons (Swain, 1979)), condensed tannins and non-tannin polyphenolics. Tannic acid (Aldrich Chemical Company Inc.) was used as a standard, and results are expressed as tannic acid equivalents. The carbon concentration of tannic acid is approximately 54% (Budavari,

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[2] Kindly supplied by Dr F.A. Wallis, CSIRO Division of Forest Products.

1989), and this value was used to express results on a structural C basis. This method is only indicative of total polyphenolics, with extraction of 30 to 95% of total polyphenolics reported (Swain, 1979). The method is subject to error if relative concentrations of the various phenolic compounds change between treatment (Cipollini *et al.,* 1993). Senesced material was oven dried in this study - rather than freeze dried - which may reduce the recovery of polyphenolics (Lindroth & Pajutee, 1987). It is assumed that this effect was uniform across treatments.

Approximately 150 mg of sample was incubated at 80°C for 1 hour in 4 mL of 50% methanol. The extract was quantitatively filtered, using 50% methanol to rinse the sediment, and made to 10 mL with distilled water. A 1 mL aliquot was mixed with 2.5 mL Folin-Denis reagent (100 g L^{-1} sodium tungstate, 20 g L^{-1} phosphomolybdic acid, 50 mL L^{-1} orthophosphoric acid) and 2 mL sodium carbonate (17%), and made to 10 mL with H_2O. After 20 minutes at room temperature, absorbance was determined at 760 nm, and total extractable polyphenolics determined against tannic acid standards.

Arbuscular mycorrhizal infection

Arbuscular mycorrhizal (AM) infection was assessed by the line intersect method of Newman (1966), and results were expressed as percent of root length infected. Prior to measurement, roots were stained following the method of Gazey et al. (1992). Roots were incubated in 10% potassium hydroxide at 65°C until clear (~5 hours), rinsed with 10% hydrochloric acid and then stained by incubation at 65°C for 30 min in lactoglycerol blue (1.3 g trypan blue/650 mL 90% lactic acid/600 mL glycerol/800 mL distilled water). The stained roots were stored in lactic glycerol (100 mL lactic acid/200 mL glycerol/100 mL distilled water) prior to counting under a light microscope.

Chapter 3. Between and within species variation in response of isolated plants to CO_2 with abundant nutrient supply

Introduction

The CO_2 response of a wide range of herbaceous plants has been examined, including many Gramineae (eg. Kimball, 1983). The literature suggests that there is a broad range of responsiveness to CO_2 within Gramineae (eg. Hunt *et al.,* 1991; Greer *et al.,* 1993; Watson & Graves, 1993). To my knowledge there are no reports of the responses of *Danthonia richardsonii* Cashmore to CO_2 enrichment. As it was not possible to undertake many long-term sward experiments with a wide range of species, it was important to assess how the response of *D. richardsonii* to CO_2 enrichment compared to the response of other grasses. In this chapter the response of three ecotypes of *D. richardsonii* is compared with seven other wild and domesticated C_3 grasses under conditions of ample nutrient supply. In the following chapter the response *D. richardsonii* to CO_2 under conditions of growth limiting nitrogen supply is compared with a number of other species.

Materials and methods

Seeds of the ten genotypes were imbibed on filter paper. Full names and abbreviations used in the text and figures are given in Table 3.1. The sterilised potting mix was 75% potting compost and 25% coarse river sand with a pH of 6.5. Three grams of Plantacote 4M (15:10:16:2 N:P:K:S + trace elements; Aglukon Spezialdünger GmbH / Schering Ag) slow release fertiliser was added to each pot on pot fill with 1.4 L (2.8 kg) of potting mix. On germination (17/9/91: day 0) five seeds were transplanted into each pot. Pots were immediately assigned to CO_2 treatments (352 $\mu L\,L^{-1}$ 'control', 707 $\mu L\,L^{-1}$ 'enriched', in the same glasshouses as the microcosm experiment, chapters 6-8). On emergence seedlings were thinned to one plant per pot. Pots were watered to drip-through twice daily. After the first harvest the remaining pots were transplanted into 10 L pots to maintain an unrestricted rooting volume. Nutrients were then supplemented by watering every morning with full strength #2 Hoagland solution (Hewitt, 1966) until drip-through. Additional water was supplied twice daily until drip through. Environmental conditions experienced during growth are outlined in Table 3.2. Destructive harvests were undertaken on day 42 ("awn peep" of the most advanced species, *D. richardsonii* (3)) and day 71 (seed set on at least one tiller of those species which entered the reproductive phase of growth during the experiment). *A. scabrum* (1), *F. rubra* and *Poa spp.* remained vegetative throughout the experiment.

At the first harvest plants were fractioned into leaf lamina, stem + leaf sheath, and root. At the second harvest roots were not recovered. Plant material was oven dried at 70 °C. Leaf material from the first harvest was analysed for total carbon and Kjeldahl nitrogen concentrations.

Plants were grown as isolated, spaced plants on trolleys which were rotated daily within each glasshouse. The positioning was assumed to be random and the analysis blocked on time where there was data from both harvests. Data were analysed by ANOVA (Genstat 5 Committee, 1993). Natural log transformations were applied where necessary to remove heteroscedasticity. Differences between treatment means were compared with protected LSD tests where appropriate (P_L). Enhancement ratios ($R_{e/c}$) were calculated as:

$$R_{e/c} = \frac{absolute\ enriched\ response}{absolute\ control\ response}$$

to compare responses between species.

Results

Plant mass

Growth at high CO_2 increased total dry mass accumulation to the first harvest (Figure 3.1; $P<0.001$) with an average $R_{e/c}$ of 1.94. A large species effect on total dry matter accumulation to the first harvest was evident ($P<0.001$). There was no significant interaction between species and CO_2 for total dry mass, although $R_{e/c}$ varied from 1.71 for *Agropyron spp.* to 2.57 for *P. pratensis*.

Total above ground dry mass was determined at both harvests (Figure 3.2). There was an interaction between harvest and CO_2 on total above ground dry mass ($P<0.001$), with $R_{e/c}$ greater at the first harvest than the second (1.91 and 1.88 respectively). All species responded to CO_2 enrichment when averaged over harvest ($P_L<0.001$), however they differed in the extent of the response ($P<0.01$). *Agropyron scabrum* (1) exhibited the lowest response with an $R_{e/c}$ of 1.40 and *P. sieberana* the highest with an $R_{e/c}$ of 2.79. The $R_{e/c}$ of *D. richardsonii* was 1.97, 2.34 and 1.89 for ecotype (1), (2) and (3) respectively.

Tiller number and mass

At harvest the CO_2 effect on tiller number differed between species (Figure 3.2; $P<0.05$). All species except *D. richardsonii* (1), *D. linkii,* and *A. scabrum* (1) had a significant increase in tiller number in response to CO_2 enrichment when averaged over harvest ($P_L<0.05$). Average tiller dry mass ([total plant dry mass]/[no. of tillers]) was increased in all species by growth at

high CO_2 at the first harvest (Figure 3.1; $P<0.001$). The range in $R_{e/c}$ for average tiller mass was from 1.20 for *M. stipoides* (1) to 1.66 for *P. sieberana*, although the interaction between species and CO_2 was not significant.

Leaf characteristics

Growth at high CO_2 significantly increased leaf area per plant when averaged over species and harvest (Figure 3.2; $P<0.001$). This response was species dependant ($P<0.001$). All species increased leaf area per plant in response to CO_2 enrichment with $R_{e/c}$ ranging from 1.2 for *A. scabrum* (2) to an $R_{e/c}$ of 3.4 for *P. sieberana*, although the increase in *Agropyron spp.* was not statistically significant.

The response of leaf nitrogen concentration (per unit carbon) at the first harvest to CO_2 enrichment varied between species (Figure 3.3; $P<0.001$). All species except *P. sieberana* exhibited a decrease in leaf N concentration at high CO_2 ($P_L<0.05$), ranging from an $R_{e/c}$ of 0.66 for *A. scabrum* (1) to 0.91 for *F. rubra*. Leaf nitrogen surface density (mg N cm^{-2} leaf area) was not altered by growth CO_2 concentration except in *P. sieberana* where it increased (Figure 3.3; $R_{e/c}$ 1.48; $P_L<0.05$). *P. sieberana* leaf from the second harvest was analysed, and there were no CO_2 effects on leaf nitrogen concentration or leaf nitrogen surface density.

Discussion

The grass species grown in this experiment exhibited many of the "classic" responses to CO_2 enrichment, and the responses of *Danthonia richardsonii* were similar to those of the other species. Growth at high CO_2 increased total plant dry mass at the first harvest, and total shoot mass at both harvests in all species. Leaf area was generally increased by CO_2 enrichment, as was tiller number, although there were some species which did not respond. There were no statistical differences between species in the CO_2 response of total dry mass at day 42. However there were species effects on shoot dry mass response to CO_2 enrichment when averaged over harvest. Leaf nitrogen concentration generally decreased under CO_2 enrichment. These points will now be discussed in more detail.

The stimulation of total plant dry mass at day 42 (Figure 3.1) was much larger than that generally reported in the literature, although the response was within the range observed by Kimball (1983). This effect was also noted at day 72 as an increase in total shoot mass (Figure 3.2). No species effect on the CO_2 response of dry mass at day 42 was evident, while dry mass in the control CO_2 treatment varied by a factor of 9.6. This is contrary to the hypothesis of

Poorter (1993), *viz.* that plants that have a slow growth rate would show less of a response to CO_2 enrichment than those with a fast growth rate. Literature reviews such as that of Poorter (1993) where comparisons are made between plant growth characteristics and CO_2 response must be treated with caution. There are many environmental factors that can impact on CO_2 responsiveness, and large differences have been noted in responses of the same species between experiments. An example of this is the total dry mass response of *Festuca rubra* to CO_2 enrichment. In this experiment, total dry mass of *F. rubra* responded strongly to CO_2 enrichment (Figure 3.1; $R_{e/c}$=2.17), while in the study of Hunt *et al.* (1991) it showed no response to CO_2 enrichment. It is not known why the response of *F. rubra* varied so much between experiments. The plants were of a similar age at harvest, and were grown under similar temperatures. The differences may be related to genotype, or the lack of CO_2 enrichment early in growth in the experiment of Hunt *et al.* (1991), as those plants were not allocated to CO_2 treatments until 7 days after germination, which was 12% of the experimental period. Plants respond to CO_2 enrichment from very early in growth (chapter 5; Masle et al., 1993; Barrett & Gifford, 1995b), and the lack of exposure over the first few days of growth may greatly affect growth response in the longer term.

Another important factor to consider when making these inter-species comparisons, including those of this study, is that the CO_2 responsiveness of standing biomass varies throughout the life-cycle of the plant (Loehle, 1995; Gifford *et al.,* 1996a). This is demonstrated by the lower CO_2 response of shoot dry mass at day 71 than at day 42 in this experiment (Figure 3.2). Thus, comparisons which are not made at some physiologically comparable stage of growth, such as physiological maturity, may be misleading.

The dry mass response was a combination of increased tiller dry mass (Figure 3.1) and increased tiller number (Figure 3.2). However, tiller numbers of *D. richardsonii* (1), *D. linkii* and *A. scabrum* (1) did not increase in response to CO_2 enrichment (Figure 3.2). Thus an increase in tiller number was not a prerequisite for a CO_2 response in dry mass. This contrasts with data for rice, where increases in dry matter accumulation were attained only via increases in tiller number (Baker *et al.,* 1990). Increased tiller mass may result from a more rapid physiological development towards maturity. This was not noted in this experiment, as the number of tillers past 'awn peep' as a proportion of total tillers was not affected by CO_2 enrichment (not presented).

Leaf area per plant was increased under CO_2 enrichment (Figure 3.2), although not significantly so in *Agropyron spp.* Leaf area generally responds to CO_2 enrichment in plants not experiencing

nutrient or water stress (eg. Samarakoon & Gifford, 1995; Samarakoon *et al.,* 1995). The lack of leaf area response of *Agropyron spp.* to CO_2 enrichment may be related to the tillering response. As tiller number did not increase under CO_2 enrichment in this species, leaf area increase would have been limited to increases in individual leaf size or increases in leaf number per tiller. Under these high nutrient conditions maximal leaf size and leaf number per tiller may have been attained in the control-CO_2 plants, thereby limiting the CO_2 response.

Leaf nitrogen concentration decreased in all species, and leaf nitrogen surface density was not affected by CO_2 enrichment at day 42, except for *P. sieberana* (Figure 3.3). Decreases in leaf nitrogen concentration (per unit mass or carbon) are usually observed under CO_2 enrichment (Arp & Berendse, 1993). Increases in leaf nitrogen concentration under CO_2 enrichment have been noted for *Carex bogelowii* (Oberbauer *et al.,* 1986), and in some species growing near natural CO_2 vents in Italy (Körner & Miglietta, 1994). These responses are not well understood, and those from the site in Italy will be further discussed in chapter 7.

The reason for the increase in leaf nitrogen concentration and leaf nitrogen surface density in *P. sieberana* is not known, although it is possibly due to an excessive supply of nitrogen. Increases in leaf nitrogen surface density in other species have been observed when grown with high levels of nitrogen supply (eg. Sage *et al.,* 1989), including *D. richardsonii* (2) (Figure 5.3). Leaf nitrate concentrations were not determined in this experiment, but with leaf nitrogen concentrations in the order of 4.5% (dry mass), accumulation of nitrate in leaf may be expected (Garnier & Freijsen, 1994), and the effect may only have been noted in *P. sieberana* as it was the smallest species at harvest. Leaf nitrogen concentration of *P. sieberana* was unaffected by CO_2 enrichment at day 71. The leaf area and leaf nitrogen concentration increases in response to CO_2 enrichment ($R_{e/c}$=3.4 & 1.5 respectively) in *P. sieberana* may have contributed to this species large increase in dry mass under CO_2 enrichment ($R_{e/c}$=2.8).

Conclusions

All three ecotypes of *Danthonia richardsonii* showed similar relative responses in dry matter accumulation to CO_2 enrichment under abundant nutrient supply, which were similar to the other species tested. Hence the use of *D. richardsonii* as a model grass is supported. While the dry mass response to CO_2 enrichment was similar between species, there were other differences between species in their response to CO_2 enrichment. The responses of other growth characteristics of *D. richardsonii* were similar to those of the most of the other grasses tested. However, three species did not increase tiller number in response to CO_2 enrichment, including *D. richardsonii* (1). *Agropyron spp.* showed little response in leaf area, while leaf nitrogen

concentration and leaf nitrogen surface density of *P. sieberana* were increased under CO_2 enrichment. Thus, while all species showed similar responses in dry mass to CO_2, the characteristics of this response differed. Thus at high CO_2 competitive relationships may be altered between species. However, this is not a topic of research in the present study.

Table 3.1 Species, abbreviations used in figures and collection sites (where available). Supplied by [1]Dr Richard Groves CSIRO Div. of Plant Industry, [2]Mr D. Eddy, NSW Dept. of Agriculture, [3]Dr's P. Larkin and P. Banks, CSIRO Div. of Plant Industry and [4]Dr R. Whalley, University of New England.

Species	Abbreviation	Site of origin	Site code
Danthonia richardsonii Cashmore (1)	*D. rich.* 1	Armidale, NSW[1]	ARB
Danthonia richardsonii (2)	*D. rich.* 2	Cowra, NSW[1]	065
Danthonia richardsonii (3)	*D. rich.* 3	Kingston, NSW[1]	SK
Danthonia linkii Kunth. var. *linkii*	*D. linkii*	Tamworth, NSW[2]	
Agropyron scabrum (Labill.) Beauv. (1)	*A. scab.* 1	Mt Franklin NSW[3]	
Agropyron scabrum (2)	*A. scab.* 2	Utah, USA[3]	
Festuca rubra L.	*F. rubra*	Commercial seed	
Microlaena (Labill.) R. Br. var. *stipoides* (1)	*M. stip.* 1	Boliva Hill, Glen Innes NSW[4]	Wallies Site 27
Poa pratensis L.	*P. prat.*	Commercial seed	
Poa sieberana Spreng.	*P. sieb.*	Armidale, NSW[4]	Box Hill Drive

Table 3.2 Environmental conditions in the glasshouses over the period of the experiment. Absolute values are followed by one standard deviation in parenthesis where appropriate. Glasshouse temperature was increased from 17 °C to 20 °C on day 10 resulting in the large standard deviation for temperature. Radiation data are averages for both CO_2 treatments.

	Control	**Enriched**
CO_2 (avg μL L^{-1})	352 (9.3)	707 (17.6)
Dew Point (avg °C)	11.3 (1.7)	11.1 (1.8)
Temperature (avg °C)	19.7 (1.2)	19.8 (1.2)
Thermal Time (°C, base 0)	1397	1404
Total Evaporation (mm)	1016	970
Radiation (avg MJ m^{-2})	14.9	
Total Radiation (MJ m^{-2})	1076	

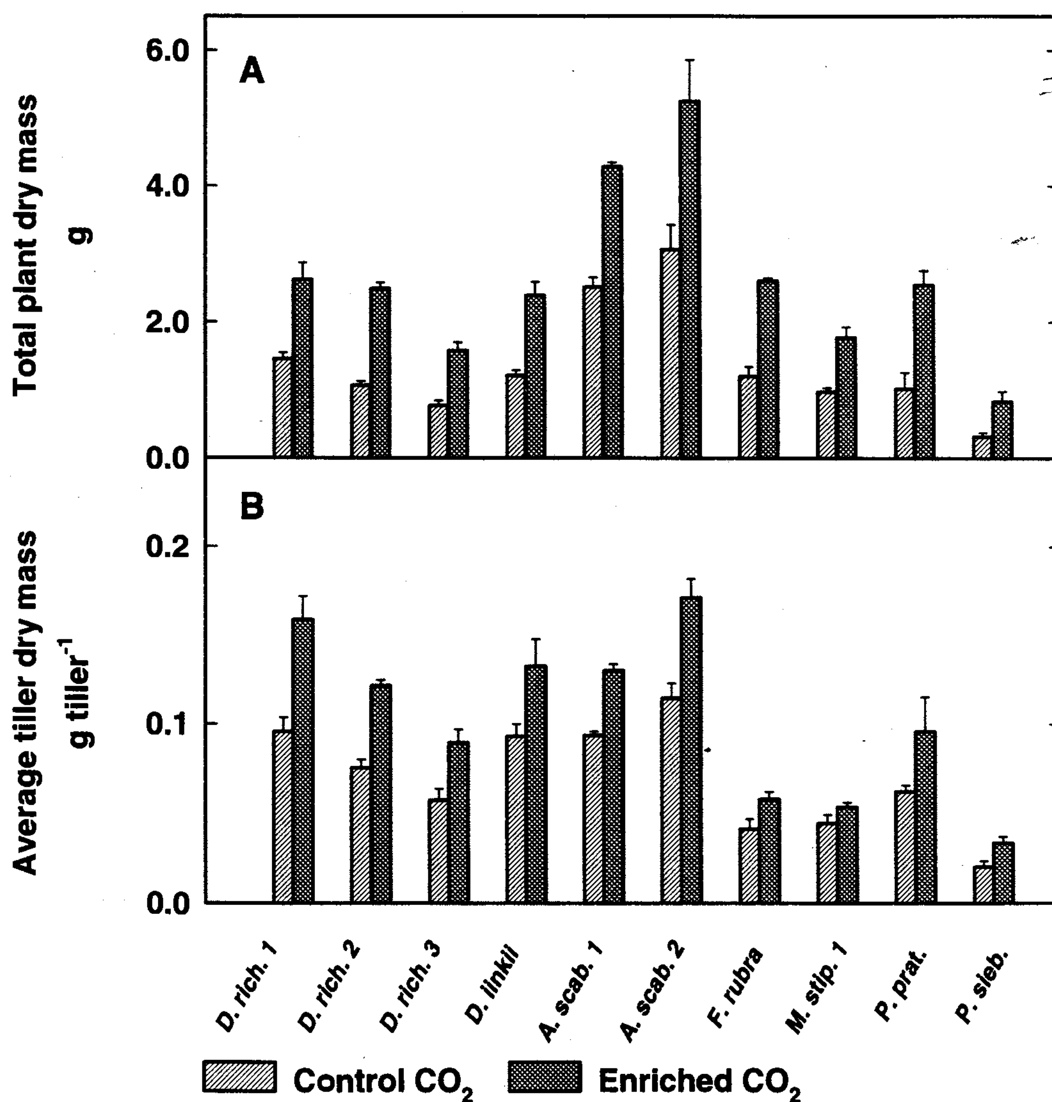

Figure 3.1 (A) Total plant dry mass and (B) average tiller dry mass at day 42. Error bars show one standard error of the mean and are present on all bars. See Table 3.1 for full species names.

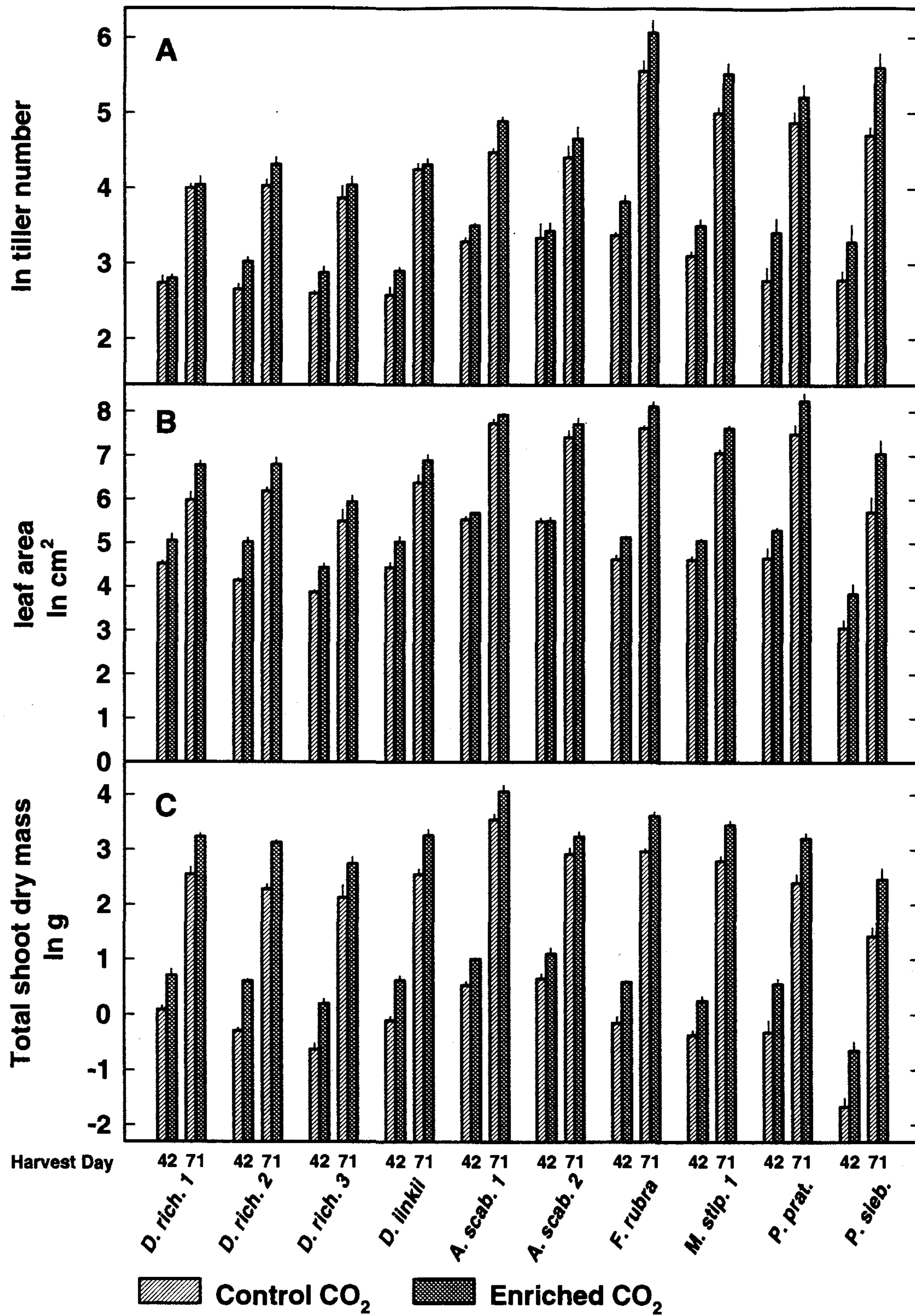

Figure 3.2 (A) Tiller number, (B) leaf area and (C) total shoot dry mass at both harvests. Error bars show one standard error of the mean and are present on all bars. See Table 3.1 for full species names.

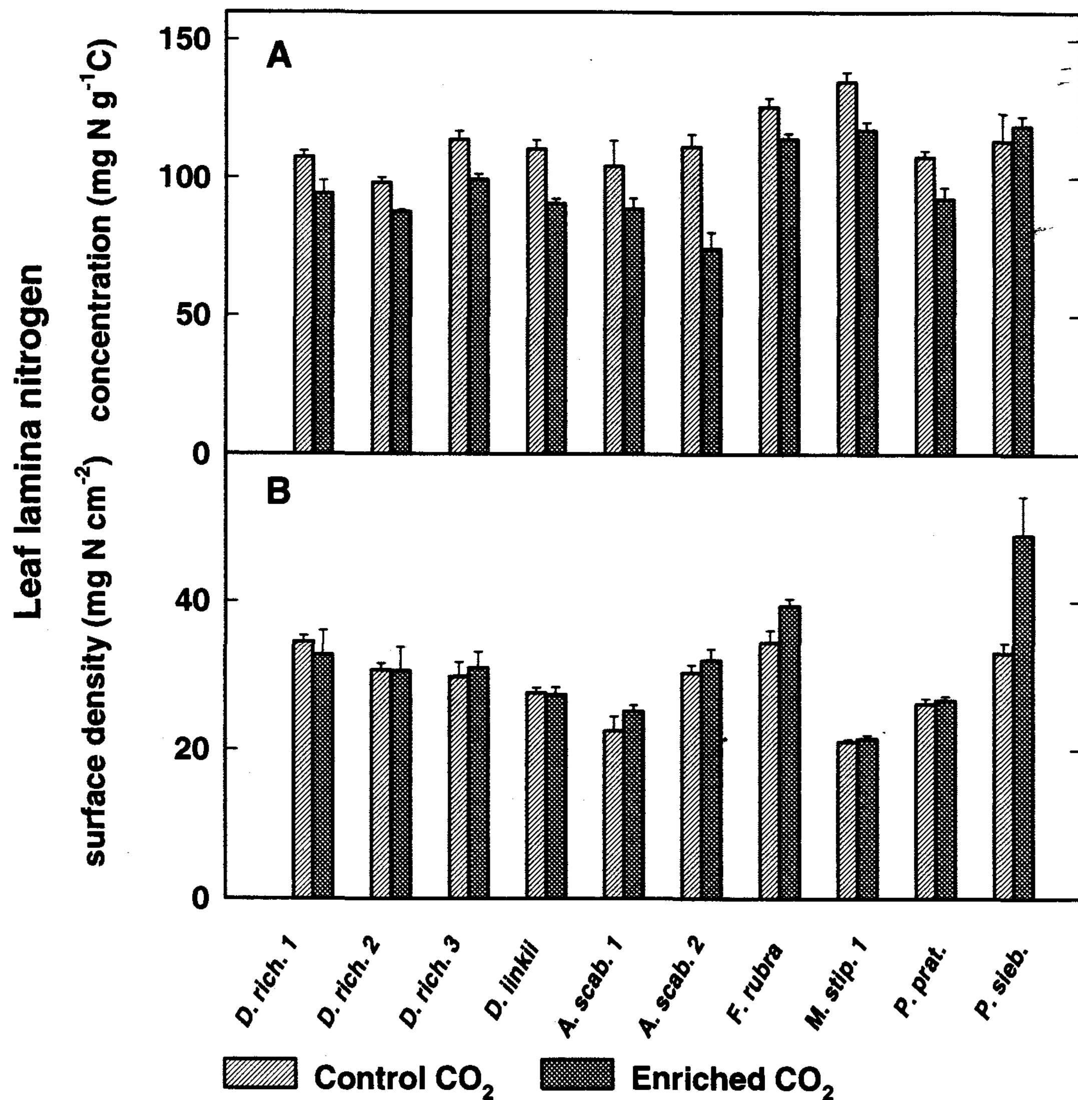

Figure 3.3 (A) Leaf lamina nitrogen concentration and (B) leaf nitrogen surface density at day 42. Error bars show one standard error of the mean and are present on all bars. See Table 3.1 for full species names.

Chapter 4. Between and within species variation in response of isolated plants to CO_2 with growth limiting nitrogen supply

Introduction

In chapter 3 we saw that when isolated plants of *Danthonia richardsonii* were grown with abundant supplies of nutrient and water all ecotypes showed similar increases in dry mass gain in response to CO_2 enrichment. These responses were also similar to those of the other species tested. However, there were some ecotype/species differences in the response of other growth characteristics to CO_2 enrichment. This included a response in tiller number to CO_2 enrichment in ecotypes (2) and (3) of *D. richardsonii*, but not in ecotype (1). While *D. richardsonii* exhibited a similar increase in dry mass in response to CO_2 enrichment as other grasses in that experiment, it is not known whether it will also show a similar response under nutrient stress. Thus, in this chapter, the general growth response of isolated plants of two ecotypes of *D. richardsonii* are compared with those of two ecotypes of *Microlaena stipoides* and two species of *Vulpia* under CO_2 enrichment with restricted nitrogen supply. In addition, the effect of growth under CO_2 enrichment on whole plant water use is examined.

Materials and Methods

Three separate single harvest experiments are reported here, with ecotypes (1) and (2) of *Danthonia richardsonii* (experiment 1, day 0: 18/08/92, harvested day 71), *Vulpia bromoides* and *Vulpia ciliata*[3] (experiment 2, day 0: 18/08/92, harvested day 76), and two ecotypes of *M. stipoides*[4] (experiment 3, day 0: 17/05/93, harvested day 65). Seeds were imbibed on filter paper. Pots were lined with plastic bags to eliminate nitrogen loss from leaching and filled with 6 kg of fine silica sand to a volume of 4.3 L. Upon germination four seeds were planted into each pot. Pots were immediately assigned to CO_2 (~360 $\mu L\,L^{-1}$ 'control', ~720 $\mu L\,L^{-1}$ 'enriched'; Table 4.1) and N treatments (below).

Water was applied regularly as a fine spray of demineralised water until seedling emergence. Upon emergence seedlings were thinned to one plant per pot and 150 g of alkathene beads (ICI Plastics, Melbourne Aust.) were added to each pot to reduce evaporative water loss. Daily

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[3] *Vulpia spp.* seed kindly provided by Dr C. Jones, Botany Department, University of New England. Collected on the New England Tablelands, NSW.

[4] *Microlaena spp.* seed kindly provided by Dr R.B. Whalley, Botany Department, University of New England. Ecotypes 1 and 2 were collected from sites 27 and 2 respectively (collectors sites) on the New England Tablelands, NSW.

water additions for each treatment were determined by weighing a subset of pots and calculating the water needed to return each treatment to pot capacity (28% θ_v). Water was applied as demineralised water using a calibrated piston pump. All pots were individually weighed weekly and water added to return them to pot capacity. Pot water content was maintained above 22% θ_v, except on two occasions when it fell to approximately 19.5% θ_v in the control high-nitrogen *V. bromoides* treatment. Evaporative losses were measured from a number of pots which were not seeded, and these were used as blanks in transpiration calculations. Small differences in evaporative demand between glasshouses were corrected for by multiplying the transpiration of the enriched treatment (T_e) by the ratio of evaporimeter evaporation in the control glasshouse (E_c) to that of the enriched glasshouse (E_e) within a weekly measurement period, ie.,

$$T_E = T_e \cdot \left(\frac{E_c}{E_e} \right)$$

where T_E is the corrected transpiration of the enriched plants. *Microlaena spp.* pot surfaces were covered with alkathene beads and aluminium foil to reduce water loss and algal growth. Evaporation from the soil surface was negligible and no blanks were used in transpiration calculations for that experiment. Total non-structural carbohydrate concentrations of leaf lamina and root were determined for *Microlaena spp.* only.

Nutrient was supplied twice weekly in solution as nitrate at rates of 0.4 or 1.6 mg N pot^{-1} day^{-1}, hereafter low- and high-N (Table 4.2). The nutrient solution was based on a solution used regularly for other grasses (P. Hocking, personal communication). Linear programming techniques were used to alter the balance of constituent compounds to attain the desired nitrogen concentration, while maintaining the concentrations in solution of the other macronutrients. The supply of nutrients other than nitrogen was considered not to be growth limiting. Soil solution pH was monitored in extra pots and was greater than 6.0 at harvest. Total potential salt concentration (neglecting uptake) in the pots at harvest was approximately 3 times full strength Hoagland solution (Hewitt, 1966). It was assumed that this did not inhibit growth.

At harvest plants were fractioned into leaf lamina, sheath + stem, and root (leaf + sheath and root for *Vulpia spp.*) and freeze dried. *D. richardsonii* and *Vulpia spp.* had six replicate plants per treatment, spaced evenly over two trolleys within each glasshouse. Replicate pots within a trolley were bulked before chemical analyses were performed on the resultant 2 replicate blocks. *M. stipoides* had six replicate blocks in each glasshouse, containing one plant from each treatment within CO_2 level. Pairs of blocks were bulked together for chemical analysis,

resulting in 3 replicate blocks, or 24 experimental units. Trolleys were rotated within each glasshouse daily. Trolleys and CO_2 levels were swapped between glasshouses once within each experiment. Carbon and nitrogen (Kjeldahl) concentrations were measured in each of the plant fractions. For statistical analysis by ANOVA (Genstat 5 Committee, 1993) the design was assumed to be completely random. Natural log transformations were applied where necessary to remove heteroscedasticity. Protected LSD tests were performed where appropriate (P_L).

Results

Carbon

Growth at high CO_2 increased total plant carbon in each experiment when averaged over N level (Figure 4.1; P<0.01). Only *Microlaena spp.* exhibited an interaction between CO_2 and N level, with the response greater at high-N than low-N ($R_{e/c}$=1.49 & 1.35 respectively; P<0.05). There were no interactions within experiments between CO_2 and "ecotype" (species in *Vulpia spp.* experiment).

Non-structural carbohydrate concentration of Microlaena stipoides

Non-structural carbohydrate (NSC) concentrations of *Microlaena spp.* were increased in leaf tissue in response to CO_2 enrichment (Table 4.3; P<0.001). Non-structural carbohydrate concentrations of root tissue were not altered by growth under CO_2 enrichment (Table 4.3).

Tiller number

Tiller numbers at harvest (Figure 4.2) were not changed by CO_2 enrichment in *Danthonia spp.* or *V. ciliata*, while they were increased in *Vulpia bromoides* (P<0.01) and *Microlaena spp.* (P<0.01). Tiller number past 'awn peep' was not affected by CO_2 enrichment (not presented), and *Vulpia spp.* remained vegetative throughout the experiment.

Green leaf lamina area

Leaf area was increased by CO_2 enrichment in *M. stipoides* and *V. ciliata* (P<0.05), but not in *V. bromoides* or *D. richardsonii* (Figure 4.3).

Nitrogen

Total plant nitrogen increased under CO_2 enrichment in *M. stipoides* when averaged over N level, but not in the other species (Figure 4.4). Leaf nitrogen concentrations (per unit carbon) were reduced by CO_2 enrichment when averaged over N level in all species (Figure 4.5; P<0.001). In *M. stipoides* the reduction at high CO_2 was greater at the high rate of N supply

($P<0.001$). Leaf nitrogen surface density was reduced by CO_2 enrichment in *Vulpia spp.* and *M. stipoides* (Figure 4.6; $P<0.05$), and a similar trend was noted in *D. richardsonii.*

Root nitrogen concentration (per unit carbon) was reduced by growth at high CO_2 when averaged over N level in *D. richardsonii* and *Vulpia spp.* (Figure 4.7; $P<0.05$), while in *M. stipoides* root nitrogen concentration was *increased.* There was an interaction between CO_2 and N level in *M. stipoides* ($P<0.001$), with the CO_2 effect greater at high-N. When expressed on a structural carbon basis the increase was only observed at high-N (Table 4.3).

Distribution of carbon and nitrogen

Root carbon ratio (g root C g^{-1} total plant C) was unaffected by CO_2 enrichment in *D. richardsonii,* while it was increased in *Vulpia spp.* ($R_{e/c}$=1.14) and *M. stipoides* ($R_{e/c}$=1.14) when averaged over N level ($P<0.05$; Figure 4.8). Root nitrogen ratio (g root N g^{-1} total plant N; Figure 4.9) was increased at high CO_2 in all experiments (*D. richardsonii* $R_{e/c}$=1.12, *Vulpia spp.* $R_{e/c}$=1.24, *M. stipoides* $R_{e/c}$=1.54; $P<0.05$). In *M. stipoides* the CO_2 effect was greater at high-N than at low-N ($R_{e/c}$=1.78 & 1.36 respectively; $P<0.05$) and ecotype (2) showed a larger response to CO_2 than ecotype (1) ($R_{e/c}$=1.65 & 1.42 respectively; $P<0.05$).

Water

Total transpiration of high CO_2 grown plants was lower than in the control plants when averaged over N level in all experiments (Figure 4.10; $P<0.01$). Transpiration efficiency (TE, g C kg^{-1} H_2O; Figure 4.11) was increased at high CO_2 when averaged over N level in all experiments ($R_{e/c}$=1.69; $P<0.001$).

Discussion

Within species, responses to CO_2 increase were similar. The responses between species to CO_2 increase were also broadly similar. All species increased carbon accumulation in response to CO_2 enrichment at both levels of N supply. All species exhibited an increase, or no change in root carbon ratio. *Microlaena stipoides* accumulated more nitrogen per plant under CO_2 enrichment, while no increase was noted in the other species. All species increased root nitrogen ratio, and leaf nitrogen concentrations were reduced under CO_2 enrichment. This response was very strong in *Microlaena stipoides,* and coupled with an increase in total plant nitrogen resulted in an *increase* in root nitrogen concentration under CO_2 enrichment. Total transpiration was lower in all species under CO_2 enrichment, and transpiration efficiency was increased. These points will now be discussed in more detail.

Growth at low-N only reduced the response in total plant carbon to CO_2 enrichment in *M. stipoides*, although the response was still significant and similar trends were noted in the other species (Figure 4.1). A lower increase in total dry matter accumulation in response to CO_2 enrichment under nitrogen limitation is often observed (eg. Larigauderie *et al.*, 1988; Bowler & Press, 1993). However, that reduction in response is not necessarily universal, increases in the CO_2 response are sometimes reported when nitrogen is limiting growth (eg. Bowler & Press, 1993; Idso & Idso, 1994). Thus, in this study, growth at high CO_2 increased carbon accumulation in all species even when growth was strongly limited by nitrogen supply.

The response of tiller number at harvest to CO_2 varied between species (Figure 4.2). Tiller number at harvest was not changed by CO_2 enrichment in *Danthonia spp.* or *Vulpia ciliata*, while they were increased in *Vulpia bromoides* and *Microlaena spp.* Thus, as when nutrient supply was not limiting growth (chapter 3), the response in total plant carbon to CO_2 enrichment in Gramineae is not necessarily via an increase in tiller number.

Leaf area was increased under CO_2 enrichment in *Microlaena spp.* and *Vulpia spp.*, but not in *Danthonia spp*, even at the higher rate of N supply (Figure 4.3). Increases in leaf area are common under CO_2 enrichment in plants grown with ample nutrients (eg. chapter 3). As this experiment involved only a single harvest, it is not known if there was a transient response in leaf area to CO_2 in *Danthonia spp.* Leaf area generally responds to CO_2 enrichment in plants not experiencing nutrient or water stress, although this response may be transitory when water is not limiting growth (Samarakoon & Gifford, 1995; Samarakoon *et al.*, 1995). A transient response in leaf area of *Danthonia spp.* to CO_2 enrichment may have occurred, as maximal main tiller leaf size was increased under CO_2 enrichment at high-N (eg. $R_{e/c}$=1.32 for leaf 5, P_L<0.001, not presented). The lack of a sustained leaf area increase at high CO_2 is probably linked with the non-response of tiller number to CO_2 enrichment. However, if leaf area did not increase greatly under CO_2 enrichment, the increase in total plant carbon implies that photosynthesis was not strongly down-regulated under CO_2 enrichment. Thus, it is apparent that while carbon and dry mass accumulation responses to CO_2 enrichment are often related to increases in leaf area, this is not always the case, and carbon accumulation can be greater under CO_2 enrichment without increases in leaf area.

Total plant nitrogen increased greatly in response to increased N supply (Figure 4.4). There was little CO_2 effect on nitrogen accumulation, except in *Microlaena spp.* where total plant nitrogen was increased at high CO_2. When N supply is severely restricting growth, as in this experiment,

it could be expected that there be no effect of CO_2 on nitrogen uptake, as roots of both CO_2 treatments fully exploit the available nitrogen. Thus the increase in total plant nitrogen under CO_2 enrichment of *Microlaena spp.* is unexpected. However, approximately 70% of applied nitrogen was present in the plants at harvest, so there was scope for treatment to effect nutrient uptake. This may have been simply the result of a larger root system at high CO_2 (not presented, but see total plant carbon and root carbon ratio, Figure 4.1 & Figure 4.8). Alternately, an intrinsic difference in nitrogen uptake rate between CO_2 treatments may be responsible for the increased nitrogen uptake, as noted under certain conditions in *Plantago major* spp. *pleiosperma* Pilger, *Urtica dioica* L., *Eichhornia crassipes* (Mart.) Solms (Stulen *et al.*, 1994), and *P. taeda* (Larigauderie *et al.*, 1994). This could not be adequately assessed with data from a single harvest. Effects of CO_2 on nitrogen uptake by roots of *D. richardsonii* are investigated in the following chapter.

Leaf nitrogen concentration decreased under CO_2 enrichment in all species (Figure 4.5), as did leaf nitrogen surface density, although the reduction in leaf nitrogen surface density was not significant in *D. richardsonii* (Figure 4.6). Reductions in leaf nitrogen concentrations are a common response to CO_2 enrichment (eg. Figure 3.3; Arp & Berendse, 1993), and are potentially related to a different allocation of resources within the plant under CO_2 enrichment as photosynthetic nitrogen use efficiency is increased (Stitt, 1991). Non-structural carbohydrate concentrations were measured in *Microlaena spp.*, and were increased in leaf under CO_2 enrichment, as is commonly observed (eg. Table 5.3, chapter 6; Wong, 1990). When expressed on a structural carbon basis, leaf nitrogen concentrations were still lower under CO_2 enrichment (Table 5.3).

Root nitrogen concentrations were lower under CO_2 enrichment in *Danthonia spp.* and *Vulpia spp.*, but were *higher* in *Microlaena spp.*, at least at the high rate of N supply (Table 4.3, Figure 4.7). Although root nitrogen concentration is generally decreased under CO_2 enrichment, reports of increases are not uncommon (Arp & Berendse, 1993) and may be related to changes in nitrogen allocation under CO_2 enrichment.

Root carbon ratio was not changed (*Danthonia spp.*) or increased under CO_2 enrichment (Figure 4.8). Root carbon ratio is often reported to increase in response to CO_2 enrichment, and are often thought to be related to CO_2 induced reductions in water or nutrient availability (Stulen & den Hertog, 1993). This was not the case in this experiment, as water was abundant, and the changes in root carbon ratio were not larger at the low rate of N supply. Root carbon ratio changes throughout the natural development of the plant (Ballard *et al.*, 1936), and hence

with plant size. As plant size (total plant carbon) was changed by CO_2 enrichment, changes in root carbon ratio may be confounded with the natural progression of plant development. In chapter 5 this is further investigated with *D. richardsonii*. Thus, such changes in carbon distribution may be a response to other environmental factors or to ontogenetic drift, rather than due to the direct effect of CO_2 increase.

Root nitrogen ratio (proportion of plant nitrogen present in root) was increased under CO_2 enrichment in all species (Figure 4.9). The shift in root nitrogen ratio was greater than that observed for carbon, resulting in reduced nitrogen investment in processes involved with carbon gain, and increased investment in process involved with the acquisition of nutrients and water. This effect will be further examined in *Danthonia* in chapter 5.

Growth under CO_2 enrichment reduced total water use per plant (Figure 4.10), and increased transpiration efficiency (Figure 4.11). Transpiration efficiency is almost always increased under CO_2 enrichment, as CO_2 reduces stomatal conductance (Morison, 1993; Idso & Idso, 1994), an example of CO_2 improving the efficiency of resource use. The reduction in stomatal conductance under CO_2 enrichment is sometimes offset by increases in leaf area, resulting in no change in total plant water use under CO_2 enrichment (Samarakoon & Gifford, 1995; Samarakoon *et al.*, 1995). This was not the case in any species in this experiment, as the increases in leaf area of *Microlaena spp.* and *V. ciliata* were not large enough to offset the assumed decrease in stomatal conductance at high CO_2.

Conclusions

Growth at high CO_2 increased carbon accumulation in all genotypes, even when nitrogen was limiting growth. An increase in tiller number or leaf area was not a prerequisite for this increased carbon accumulation under CO_2 enrichment. Leaf nitrogen concentration was lower under CO_2 enrichment in all genotypes. Root nitrogen concentrations were lower at high CO_2 in *Danthonia spp.* and *Vulpia spp.*, but increased in *Microlaena spp.* Root nitrogen ratio increased under CO_2 enrichment to a greater extent than root carbon ratio, suggesting a greater allocation of nitrogen to tissue involved with nutrient capture under CO_2 enrichment. This will be examined further in the next chapter. *Danthonia richardsonii* exhibits broadly similar responses to CO_2 enrichment as other grass species, either when nutrient supply is abundant or when nitrogen supply limits growth, supporting its use as a model grass species for growth in microcosms in this study.

Table 4.1 Environmental conditions in the glasshouses over the period of the experiments. Absolute values are followed by one standard deviation in parenthesis where appropriate. Radiation data are averages for both CO_2 treatments.

	Control	**Enriched**
Experiment 1		
D. richardsonii		
CO_2 (avg µL L^{-1})	360 (13)	725 (11)
Dew Point (avg °C)	9.8 (0.1)	9.8 (0.3)
Temperature (avg °C)	20.0 (0.3)	20.0 (0.4)
Thermal Time (°C, base 0)	1437	1437
Total Evaporation (mm)	1013	1041
Radiation (avg MJ m^{-2})	10.4 (3.9)	
Total Radiation (MJ m^{-2})	752	
Experiment 2		
Vulpia spp.		
CO_2 (avg µL L^{-1})	359 (13)	723 (12)
Dew Point (avg °C)	9.8 (0.2)	9.8 (0.3)
Temperature (avg °C)	19.9 (0.3)	19.9 (0.4)
Thermal Time (°C, base 0)	1535	1535
Total Evaporation (mm)	1055	1084
Radiation (avg MJ m^{-2})	10.6 (4.1)	
Total Radiation (MJ m^{-2})	817	
Experiment 3		
M. stipoides		
CO_2 (avg µL L^{-1})	362 (17)	714 (32)
Dew Point (avg °C)	11.2 (0.99)	11.2 (0.97)
Temperature (avg °C)	20.1 (0.58)	20 (0.46)
Thermal Time (°C, base 0)	1329	1322
Total Evaporation (mm)	293	287
Radiation (avg MJ m^{-2})	4.9 (2.0)	
Total Radiation (MJ m^{-2})	326	

Table 4.2. Composition of nutrient solution

	Concentration in nutrient solution (mg L^{-1})	
Nutrient	**Low-N**	**High-N**
N	70.1	280.2
P	31.0	31.0
K	449.7	449.7
S	115.2	115.2
Mg	48.6	48.6
Ca	200.4	250.5
Cl	443.4	0.30
Fe	5.0	5.00
Mn	0.50	0.50
B	0.40	0.40
Zn	0.12	0.12
Cu	0.040	0.04
Mo	0.020	0.02
Co	0.020	0.02
I (µg L^{-1})	0.99	0.99

Table 4.3 Non-structural carbohydrate concentration and nitrogen concentration per unit structural carbon of *Microlaena spp.* averaged over ecotype. Non-structural carbohydrate concentrations were not determined for *Danthonia spp.* or *Vulpia spp.* *P* levels for CO_2 and N are for the main effect. $P<0.001$ represented by ***, $P<0.01$ by **, $P<0.05$ by * and not significant by ns.

		N level			*P* level		LSD *P*<0.05
		Low N	**High N**	**CO_2**	**N**	**CO_2*N**	**CO_2*N**
Non-structural carbohydrate concentration mg NSC-C g^{-1} Structural C							
Green Leaf	Con CO_2	147	181	***	***	ns	31
	Enr CO_2	208	267				
Root	Con CO_2	43	68	ns	**	ns	22
	Enr CO_2	48	77				
Nitrogen concentration per unit structural carbon mg N g^{-1} Structural C							
Green Leaf	Con CO_2	75	107	***	***	*	7
	Enr CO_2	62	84				
Root	Con CO_2	21	40	***	***	***	3
	Enr CO_2	20	53				

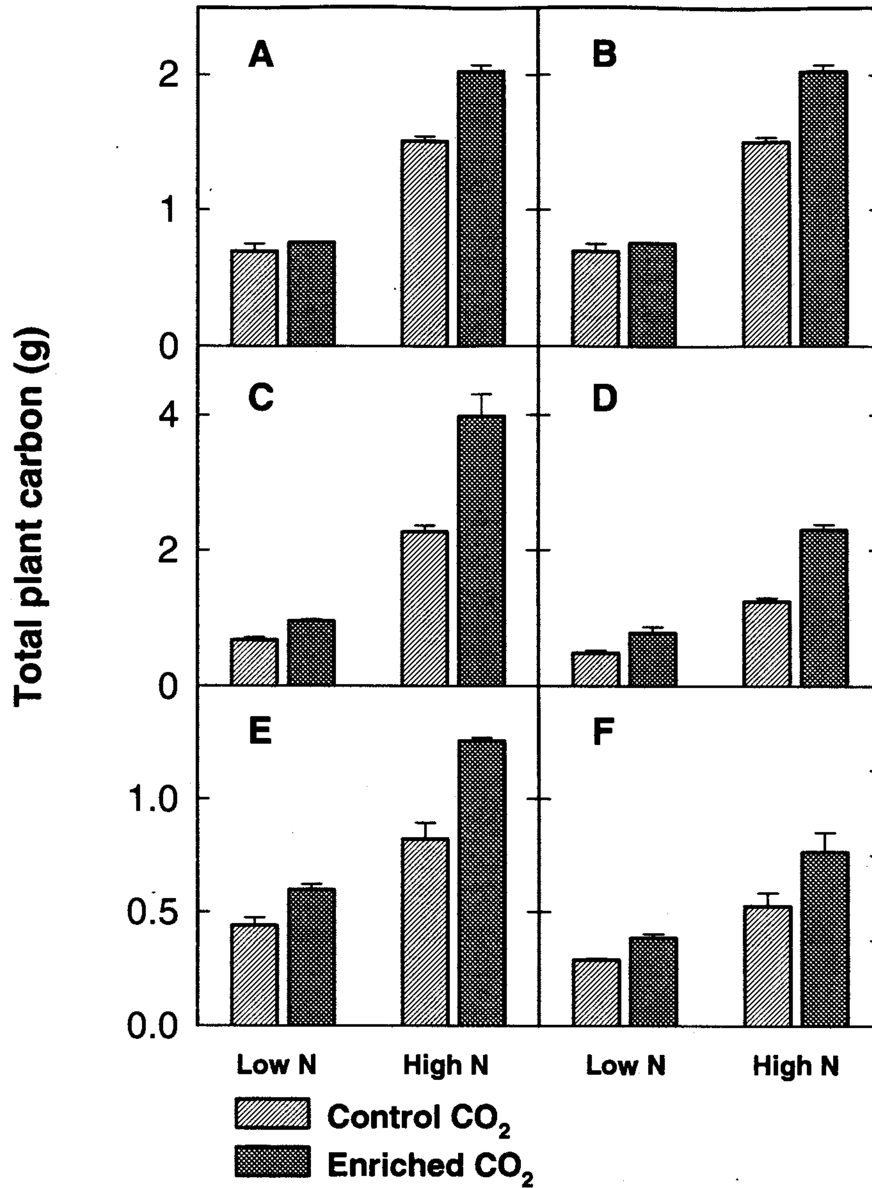


Figure 4.1 Total plant carbon. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

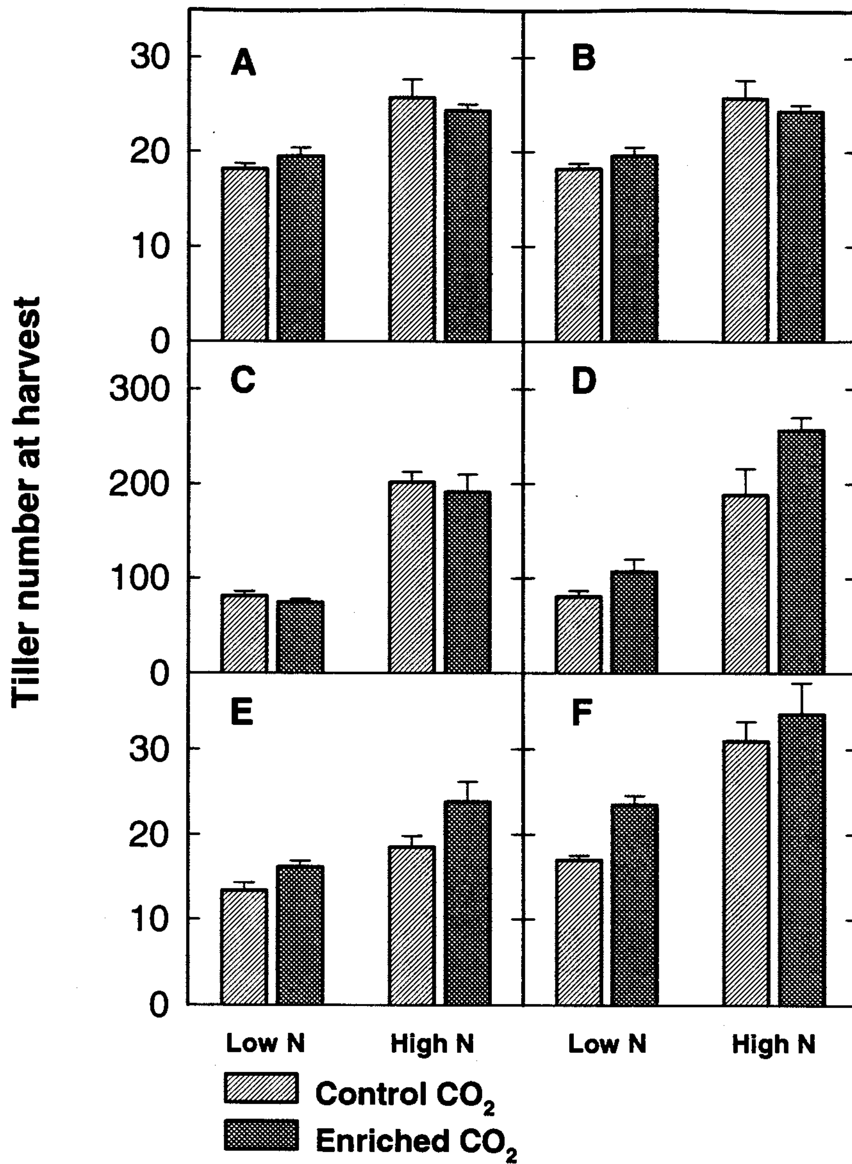


Figure 4.2 Tiller numbers at harvest. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

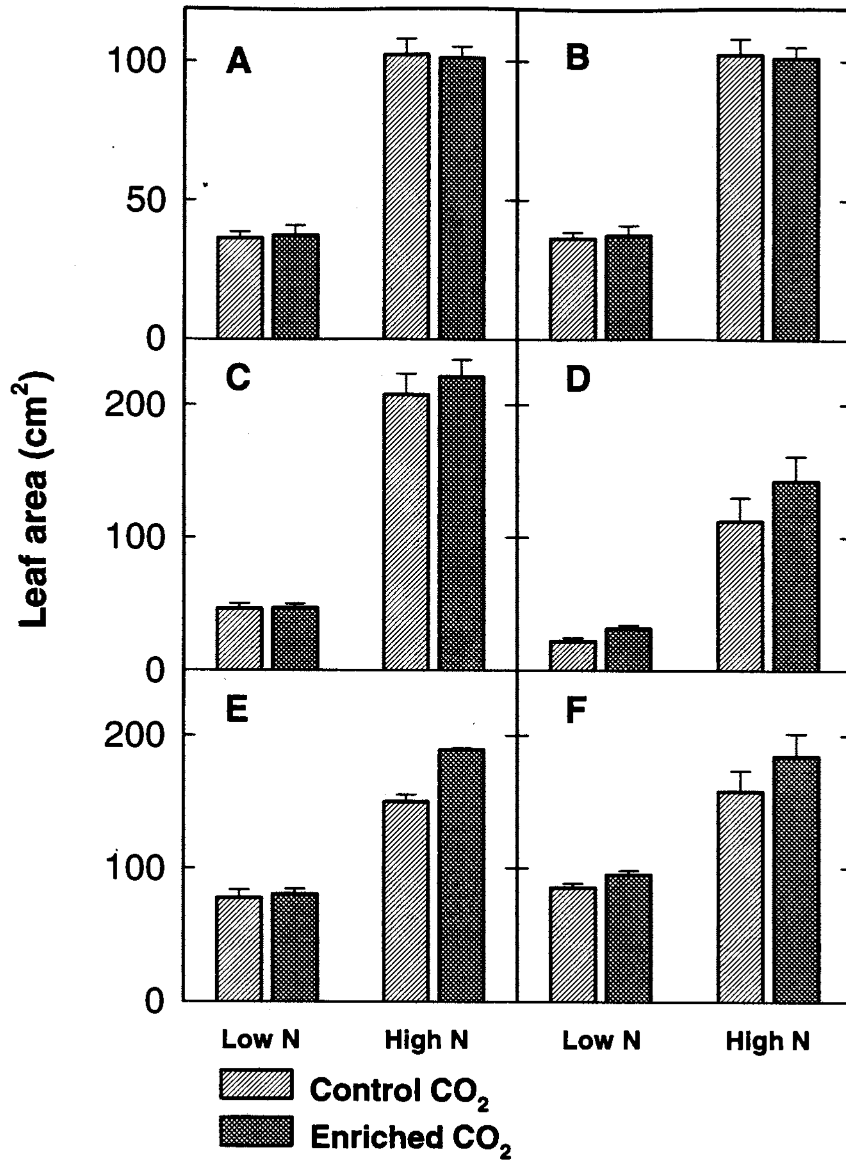


Figure 4.3 Green leaf area. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

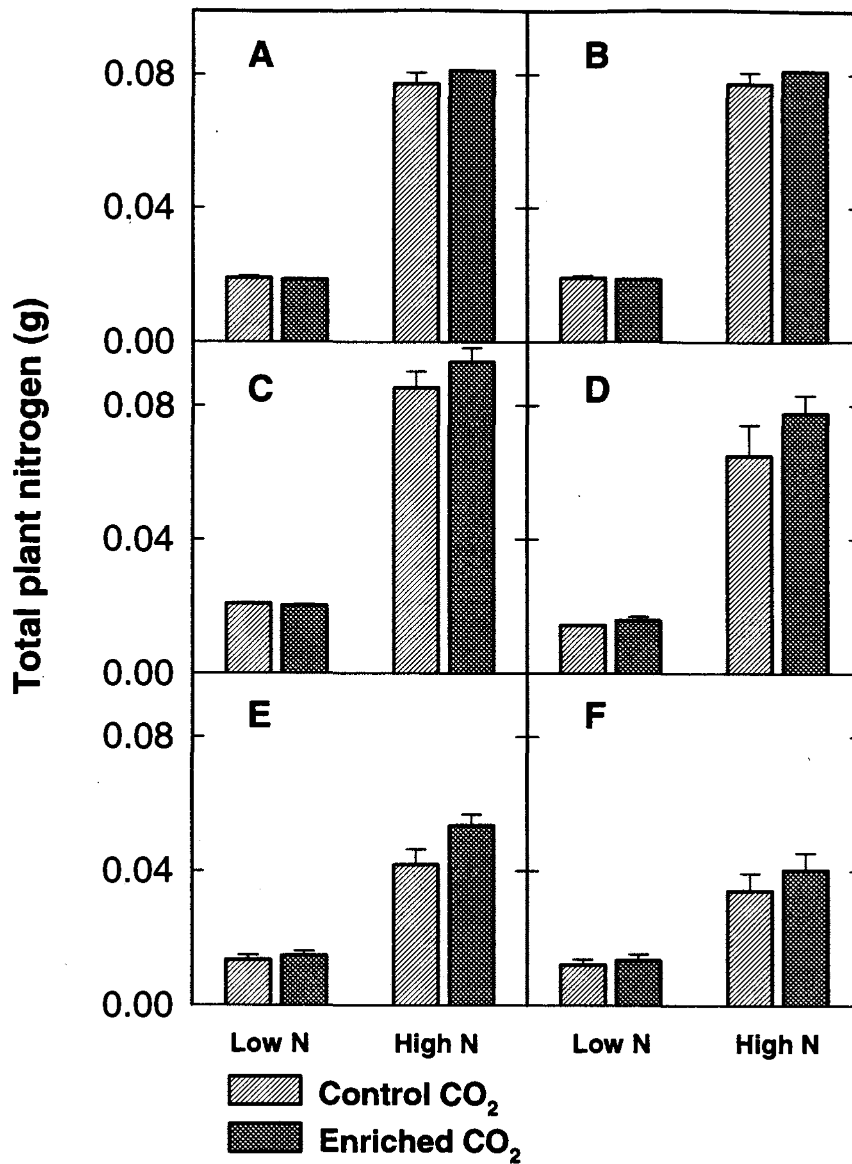


Figure 4.4 Total plant nitrogen. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

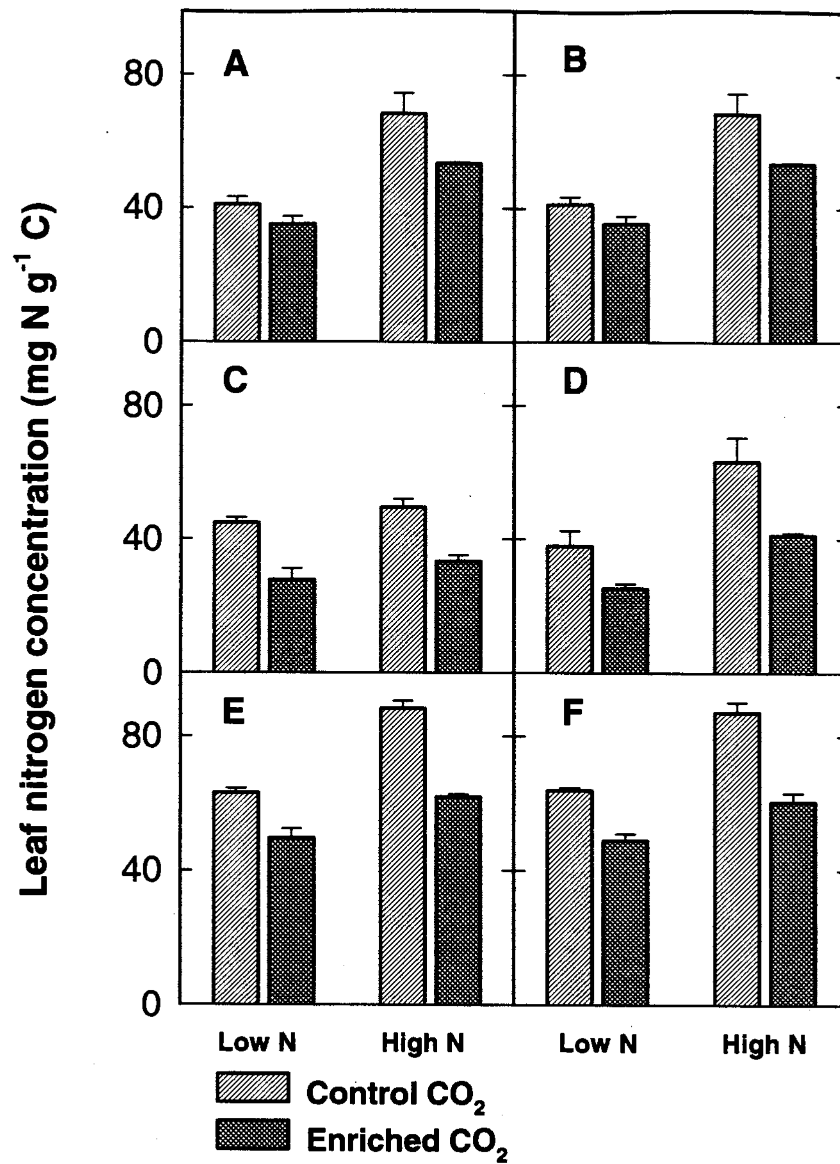


Figure 4.5 Leaf nitrogen concentration. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

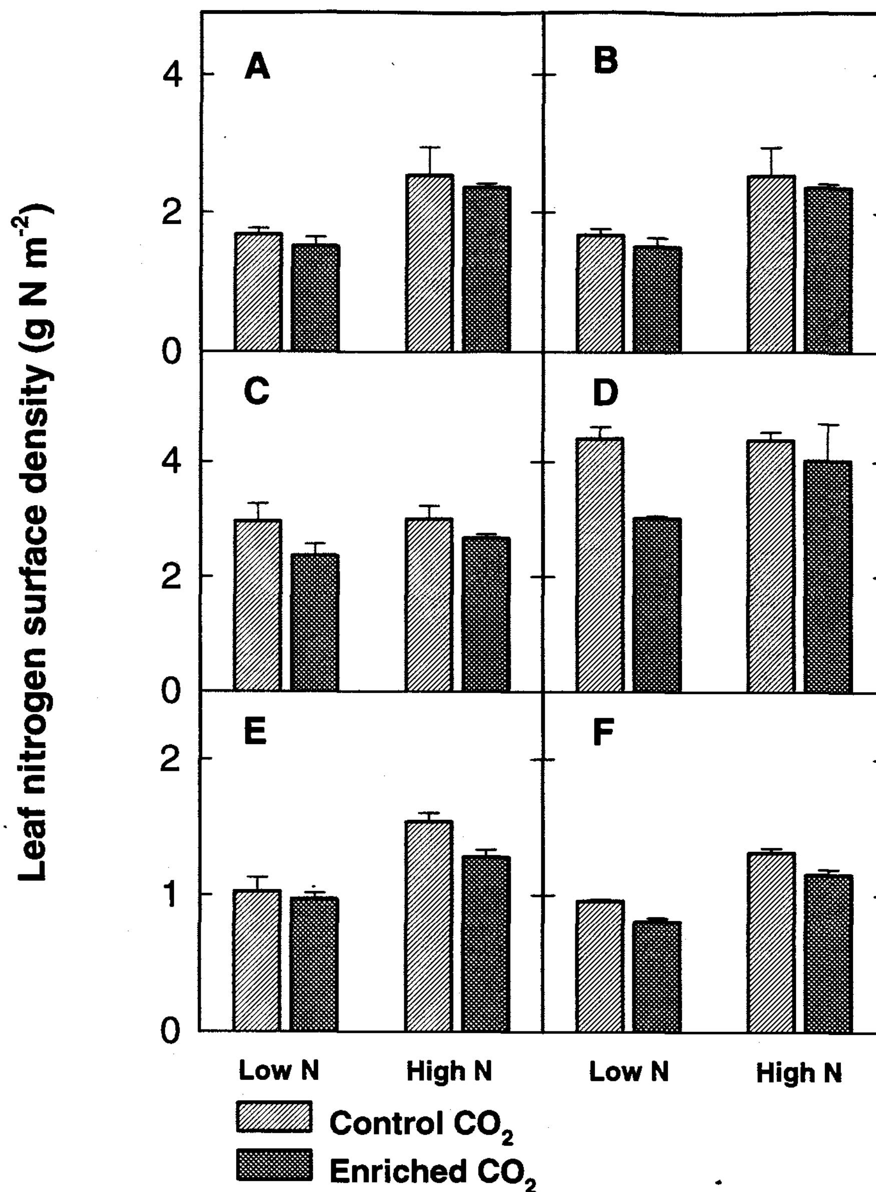


Figure 4.6 Leaf nitrogen surface density. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

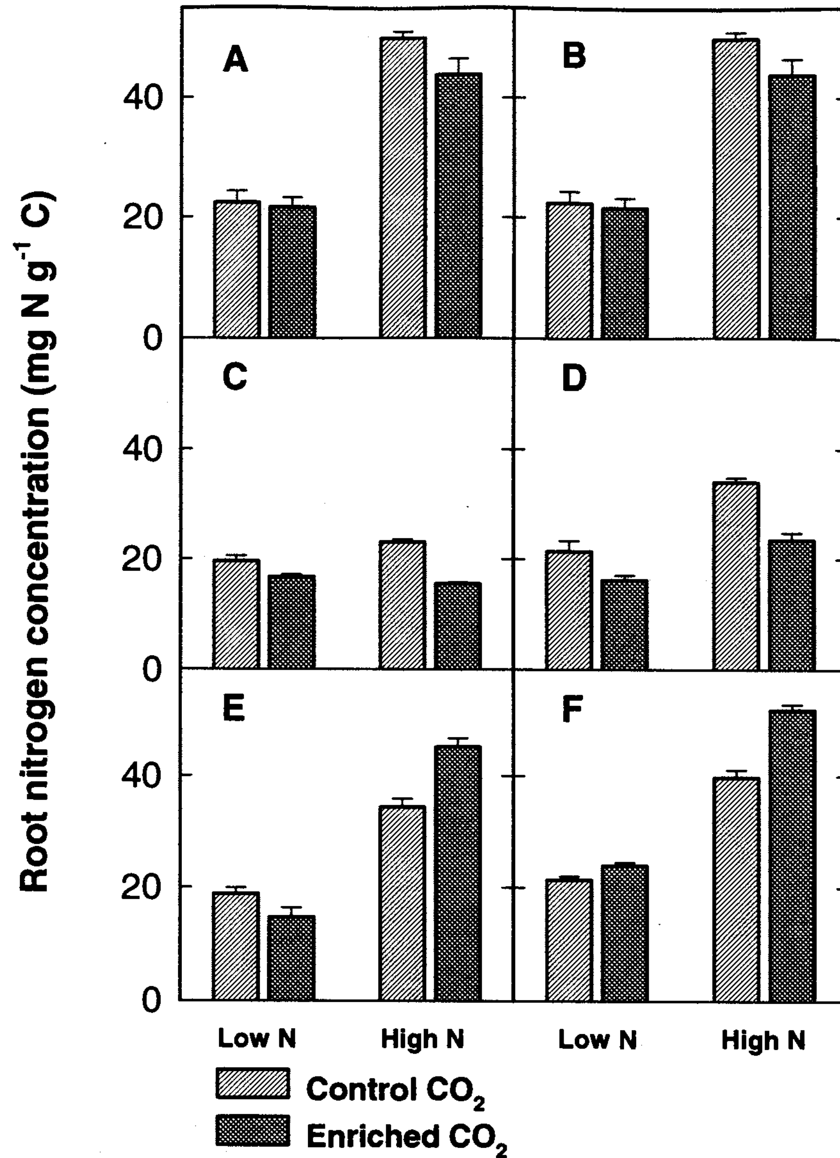


Figure 4.7 Root nitrogen concentration. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

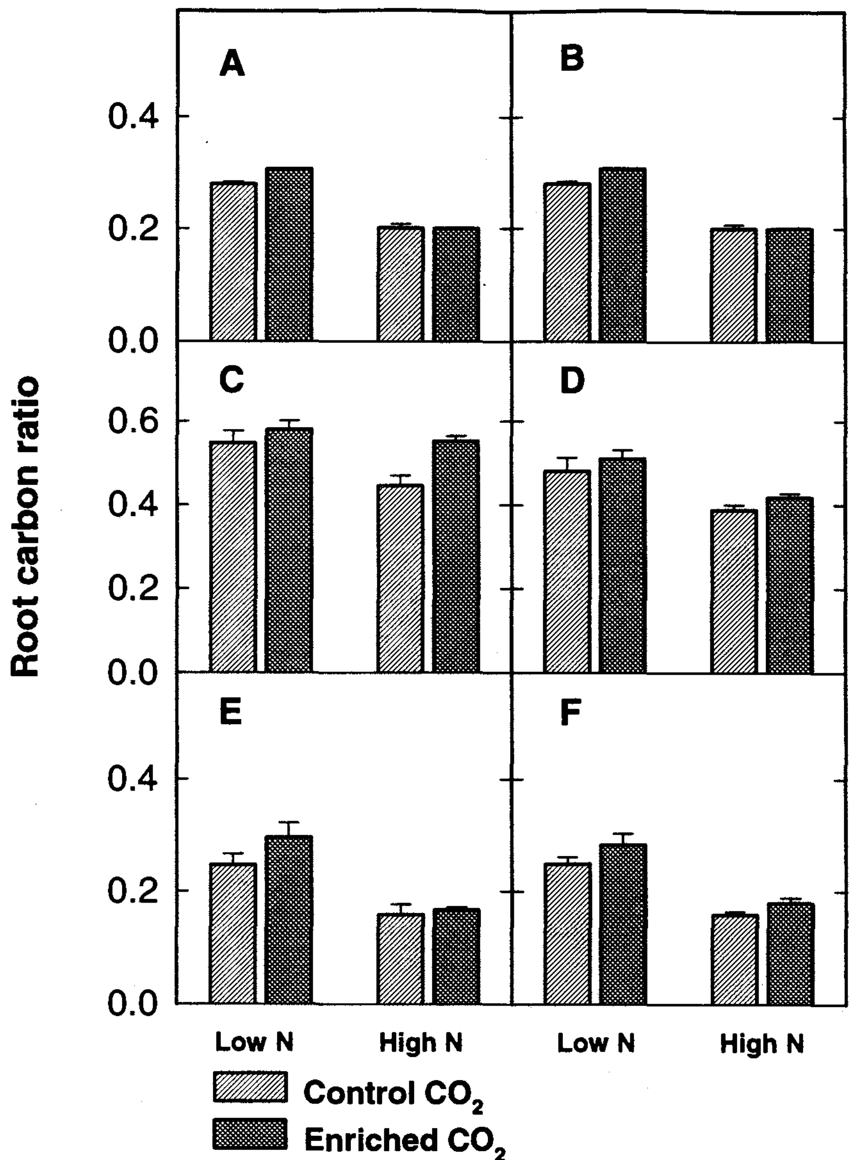


Figure 4.8 Root carbon ratio. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

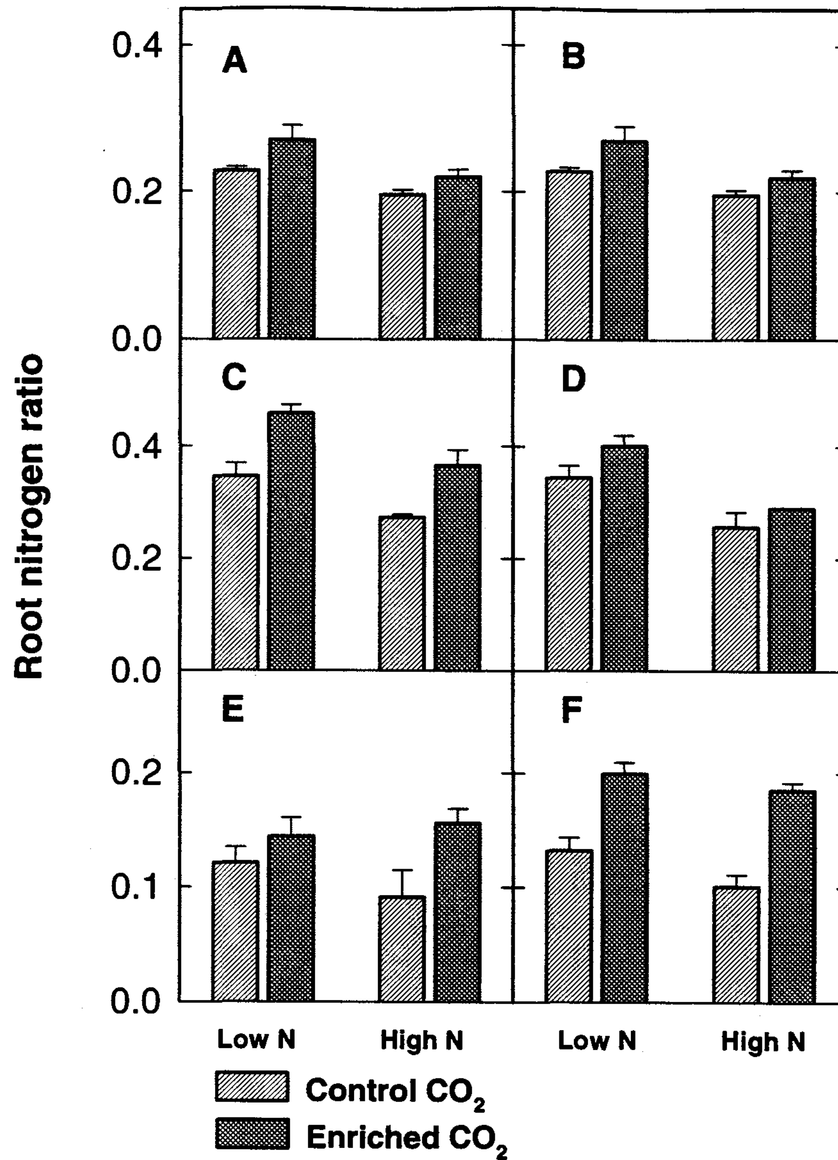


Figure 4.9 Root nitrogen ratio. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

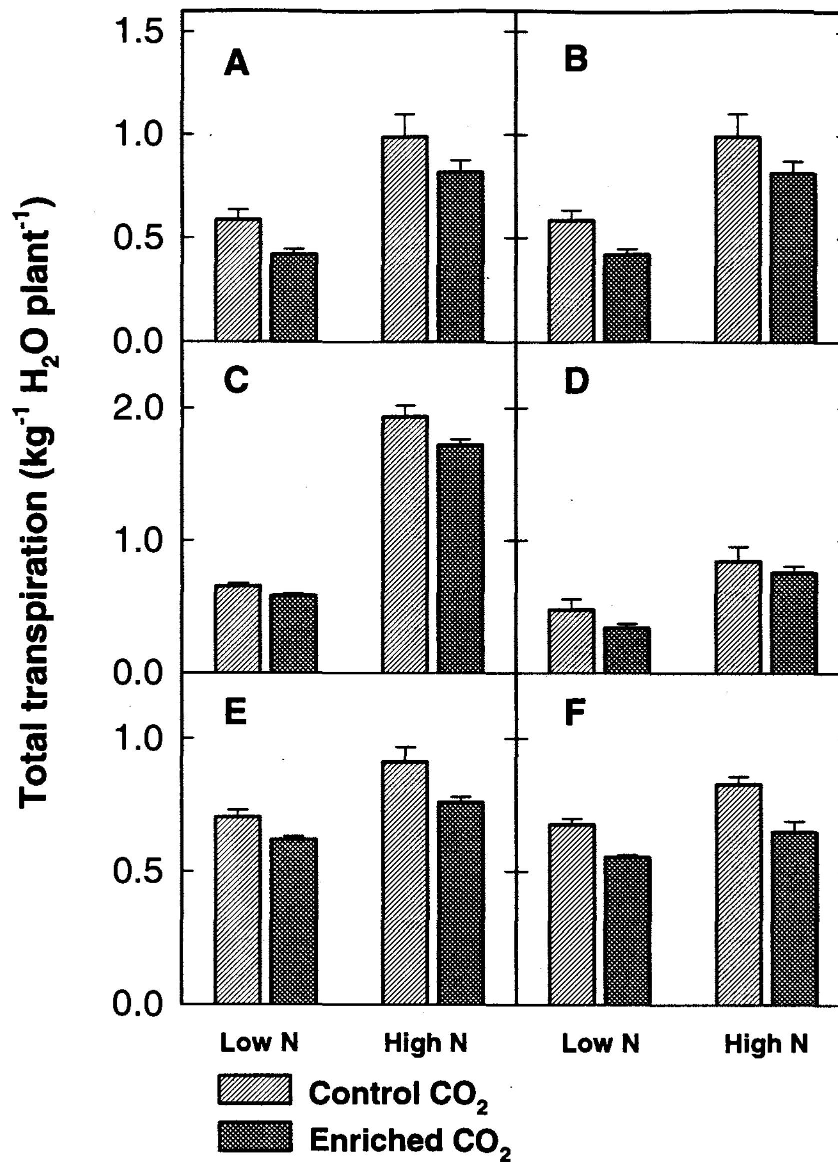


Figure 4.10 Total transpiration per plant over the experimental period. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.

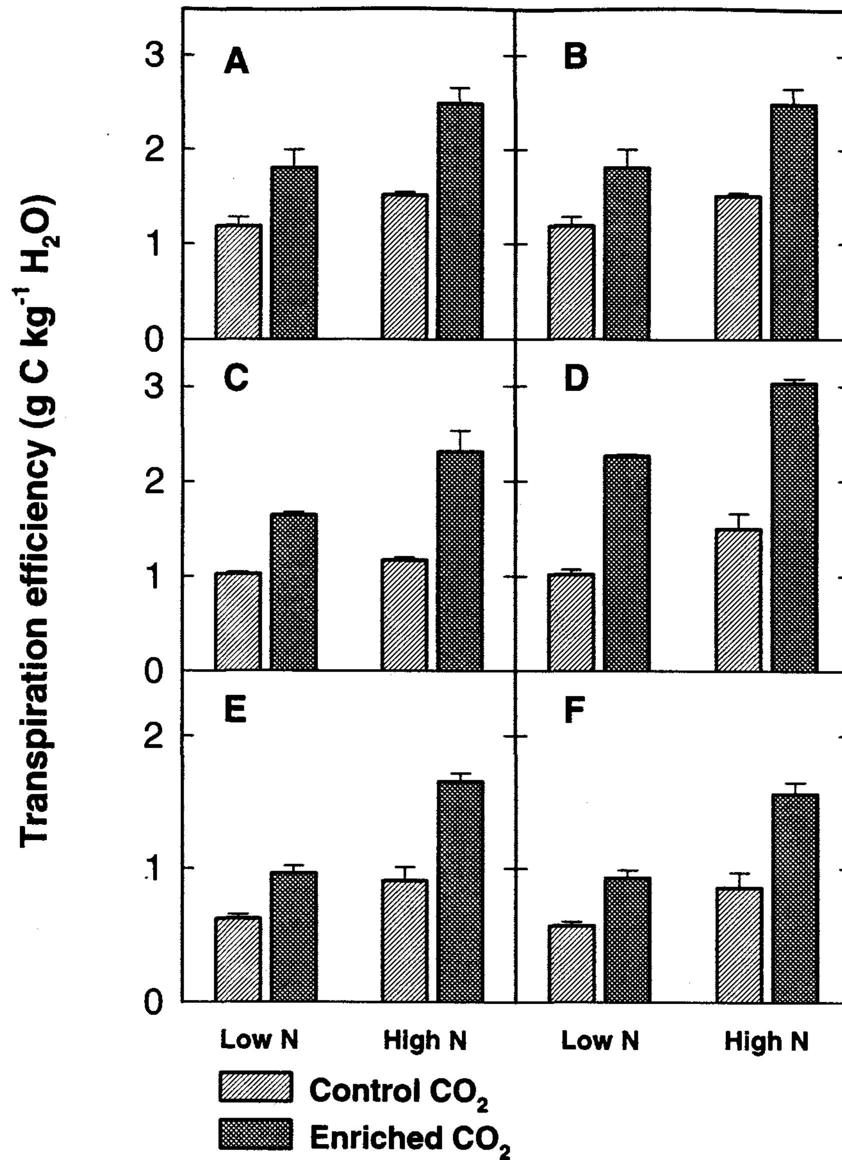
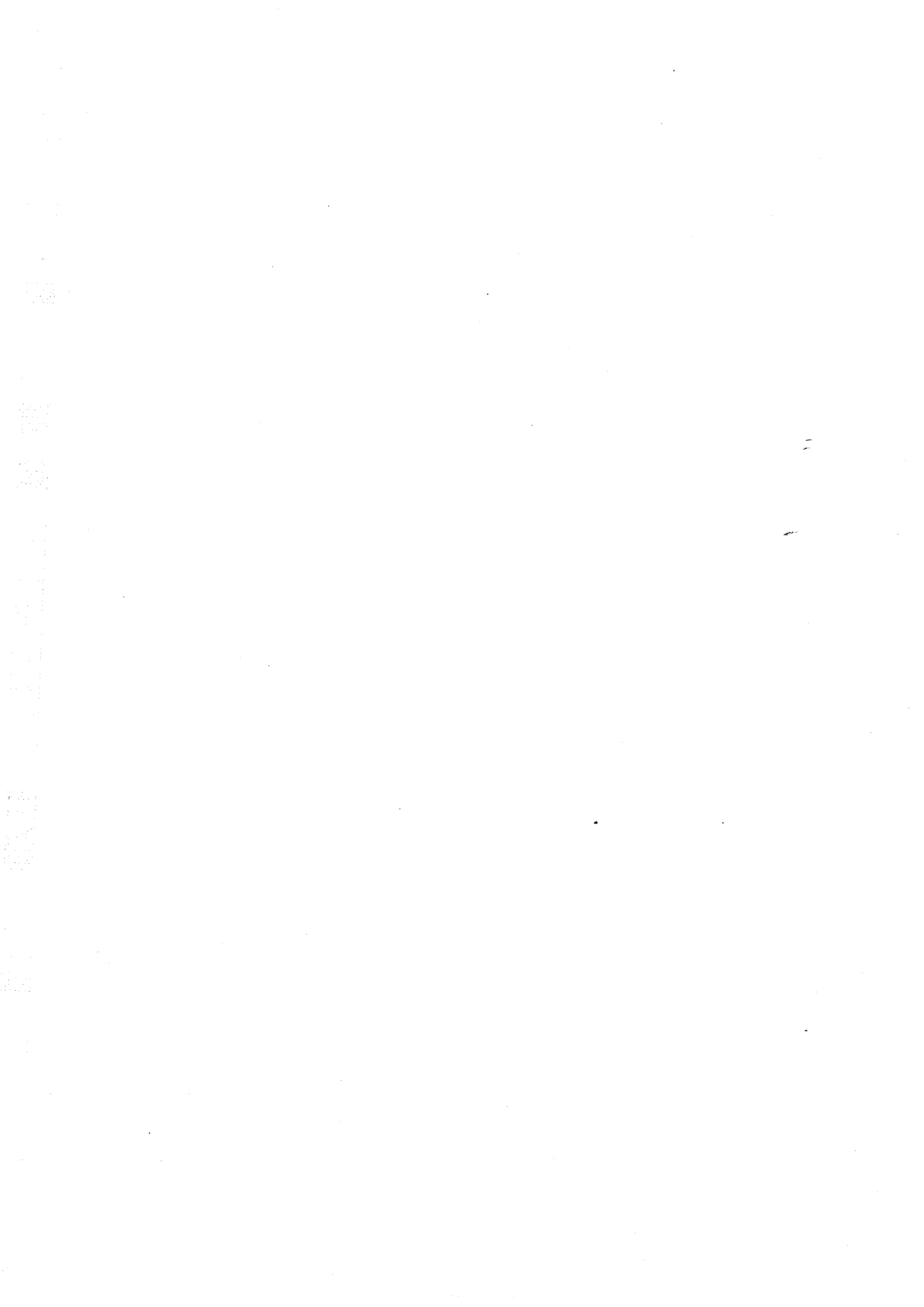


Figure 4.11 Transpiration efficiency. *D. richardsonii* 1 (A) & 2 (B), *V. bromoides* (C), *V. ciliata* (D) and *M. stipoides* 1 (E) & 2 (F). Error bars represent one standard error of the treatment mean and are present on all bars.



Chapter 5. Acquisition and allocation of carbon and nitrogen by *Danthonia richardsonii* in response to restricted nitrogen supply and CO_2 enrichment

Introduction

In the previous chapters we have seen that *Danthonia richardsonii* Cashmore shows similar responses to CO_2 enrichment as other Gramineae when grown under conditions of abundant nutrient supply, and when nitrogen supply restricts growth. Growth at high CO_2 is often accompanied by a down-regulation of photosynthesis, sometimes associated with a reduction in nitrogen concentration of photosynthetic tissue (Stitt, 1991; Arp & Berendse, 1993; Bowes, 1993). The decrease in nitrogen concentration in green leaf may result in an increase in the proportion of total plant nitrogen invested in root, as observed in *Danthonia spp., Microlaena spp.* and *Vulpia spp.* in the previous chapter, and in *Xanthium occidentale* Bertol. (Hocking & Meyer, 1985) and *Pinus sp.* seedlings (Griffin *et al.,* 1995). Little is known about the generality of the change in nitrogen distribution by growth at high CO_2, and how these changes in distribution may be associated with changes in plant morphology and function.

Detailed data on root morphology and function were not collected from the experiments of the previous chapters. However, increases in root weight ratio (approximates root carbon ratio) in response to high CO_2 are often reported when plants are nutrient or water stressed (Stulen & den Hertog, 1993), and such changes were observed in the previous chapter. This may result in a greater root surface area, allowing a more thorough exploration of the soil for nutrient and water capture (Allen *et al.,* 1992). Another indicator of a change in the functional balance between root and leaf is the ratio of root length to leaf surface area (Körner & Renhardt, 1987). This ratio was sensitive to nitrogen supply in a number of perennial grasses (Boot & Mensink, 1990) and is examined here for CO_2 sensitivity as the root surface area to leaf area ratio. This relationship may be expected to be more sensitive to CO_2 enrichment than specific uptake rates of nitrogen, as CO_2 enrichment does not have a direct effect on root function, as it does on leaf function. Wild plants also tend to increase absorptive area, rather than specific uptake rates in response to nutrient stress (Chapin, 1980).

The growth response to CO_2 in well watered plants primarily results from increased carboxylation efficiency at high CO_2 (Bowes, 1993). Plant nitrogen productivity (NP_P, Ingestad, 1979) can be used to conceptualise the effects of increased carboxylation efficiency and

changes in nitrogen dynamics on carbon gain by the plant. Although many factors influence NP_P (Lambers *et al.,* 1990) a simple approach proposed by Garnier & Vancaeyzeele (1994) has been adopted in this study, where NP_P is the product of leaf nitrogen productivity (NP_L) and leaf nitrogen ratio. Plant nitrogen productivity is related to relative growth rate (RGR) as the product of NP_P and whole plant nitrogen concentration (Ingestad, 1982). Root function, as related to the acquisition of nitrogen, is often described as specific absorption rate, defined as the rate of nitrogen absorption per unit root weight (Hunt,R, 1982). In this study net nitrogen absorption rate (NNAR) is defined as the rate of increase in plant nitrogen (N_P) per unit root surface area (A_R), ie.,

$$NNAR = \frac{1}{A_R} \cdot \frac{dN_P}{dt}$$

Many characteristics of plants change with age and increasing plant size (Ballard *et al.,* 1936; Evans,GC, 1972; Farrar & Williams, 1991). It has been hypothesised that some of the common responses to CO_2 enrichment may result solely from changes in plant size, such as the decrease in total plant nitrogen concentration (Coleman,JS *et al.,* 1993). Relative growth rate evaluated over fixed periods from germination is commonly insensitive to CO_2 enrichment (Watson,J & Graves, 1993), but declines with plant size as plants move out of the exponential phase of growth (Ballard *et al.,* 1936; Evans,LT, 1993; Gifford *et al.,* 1996a). Although the response of plants to CO_2 enrichment with respect to time is important in its own right, it is important to separate real CO_2 effects on allocation and function from those related to changes in ontogeny or plant size in understanding the physiological response of the plant to CO_2 increase (Loehle, 1995). Thus allometric relationships were examined to test for actual differences in carbon and nitrogen *allocation* between CO_2 treatments (Troughton, 1955; Hunt,R, 1978; Farrar & Williams, 1991), rather than changes in *distribution* (eg. root carbon ratio) at harvest due to changes in plant size.

This chapter addresses the following hypotheses related to growth over the first 37 days from seed imbibition. *a)* Growth at high CO_2 alters characteristics of leaf carbon acquisition, involving increases in net assimilation rate and leaf nitrogen productivity; these changes are independent of plant size. *b)* Growth at high CO_2 increases root surface area relative to leaf surface area, and increases nitrogen and possibly carbon allocation to root to maintain a balance between leaf and root function; these changes are independent of changes in plant size. *c)* Characteristics of nitrogen uptake by root (NNAR) will not be changed by growth at high CO_2.

Materials and methods

Seeds of *D. richardsonii* (ecotype 2, chapter 3) were imbibed on rinsed blotting paper. Three germinated seeds were placed in each 81 mm, 0.70 L cylindrical pot filled with 900g of fine silica sand and saturated with demineralised water. Pots were immediately randomly assigned to treatments and placed in one of two glasshouses of the Canberra Phytotron at either 362 ('control') or 748 $\mu L\ L^{-1}$ ('enriched') atmospheric CO_2 concentrations with other environmental conditions as summarised in Table 5.1. Plants were thinned to 1 per pot on emergence. The experimental treatments were 4 harvests * 3 N levels * 2 CO_2 levels, with 3 replicates consisting of 9 plants per replicate (ie. 648 plants). Plants were rotated within glasshouses daily, and treatments were swapped between glasshouses once during the experiment.

The three nitrogen treatments were 0.05, 0.2 or 0.5 mg N $pot^{-1}\ day^{-1}$, supplied five days a week as 10 mL of nutrient solution (hereafter low-, mid- and high-N), the composition of which is shown in Table 5.2. Twenty additional pots in each CO_2 treatment were supplied with Hoagland solution (#2; Hewitt, 1966) at a rate equivalent to 6 mg N $pot^{-1}\ day^{-1}$. Four of these pots from each CO_2 level were harvested for comparative purposes at each harvest. Nutrient application commenced on day three. Pots were placed on saucers and watered twice daily. Water was not permitted to overflow from the saucers.

Destructive harvests were carried out on days 16, 23, 30 and 37 after imbibition. Green areas, root length and projected root area were determined with a DELTA-T Mk 2 area measurement system and DELTA-T DIAS v1.11 software (Delta-T Devices, Cambridge). Root length and projected area was determined on unstained samples, as the roots were needed for elemental analysis. This underestimated actual root length (~30%) and projected root area (~50%). Roots from three extra plants from each treatment at each harvest were stained with methyl violet (Webb, 1990) and calibrations developed between unstained and stained root length, and unstained and stained projected root area. To minimise errors associated with such a calibration at least three observations of root length and area were made for each root sample, with root orientation altered between observations and the observational mean used for further analysis. Data presented are corrected to stained root lengths and areas. Root projected area was converted to surface area by assuming the roots were cylindrical. Root data was not collected from the Hoagland treatment.

Plant material was partitioned into leaf lamina, leaf sheath (hereafter leaf and sheath respectively), root, and senesced leaf fractions and freeze dried. Senesced leaf carbon and

nitrogen accounted for, at maximum only 0.23% and 0.14% of total plant C and total plant nitrogen respectively, and was excluded from all analyses. Plants within replicates were combined for dry mass determination, but data are expressed per plant. Total non-structural carbohydrates were determined for day 37 only, owing to insufficient sample mass at the earlier harvests.

Primary data analyses were by ANOVA with GENSTAT 5.3 (Genstat 5 Committee, 1993). Statistical analyses were on within-replicate means and the design was assumed to be completely random. The Hoagland treatment was analysed as a separate experiment. Logarithmic transformations were applied where necessary to remove heteroscedasticity. Differences between treatment means were tested by protected LSD methods (P_L) where appropriate. The growth analysis parameters relative growth rate (page 5-91; Evans,GC, 1972), leaf nitrogen productivity (page 1-5) and plant nitrogen productivity (page 1-5; Keay *et al.*, 1970; Ingestad, 1979) and net nitrogen absorption rate (page 5-66) were calculated using the software package HPCURVES (Hunt,R & Parsons, 1974). Linear functions were used for the relationship between $ln(x)$ and time for all parameters x. These data were analysed by t-test using the fitted values and their standard errors as calculated by HPCURVES.

Allometric analysis was used to elucidate allocation patterns by removing plant size effects (Troughton, 1955; Hunt,R, 1978; Farrar & Williams, 1991). The function

$$y = bx^k,$$

linearised to:

$$\ln y = \ln b + k \ln x$$

was fitted to the data, where x and y are the attributes of the plant fractions. The allometric constant (k, the slope of the regression) gives the ratio of the logarithmic increases of x and y. For k less than unity, y is increasing at a slower rate than x. The interpretation of the intercept (b) is more difficult. A difference in b may result from an earlier difference in k, or, if k differs, b may differ between treatments if the fitted lines converge on a similar point (Troughton, 1955). Thus differences in b were generally disregarded if there was a significant difference in k. Multiple step-wise linear regression (Genstat 5 Committee, 1993) was used to determine significant differences in b and k produced by treatments. Linear regression is strictly not the correct statistical model, as in this case both the dependent and the independent variable have an associated variance (Snedecor & Cochran, 1980), probably of a similar magnitude. The method was checked by regressing the previously independent variable on the previously dependant variable. Both methods produced significance in equivalent terms. As the true

relationship must lay between these two fits, linear regression was considered reliable in this study.

Results

Growth, distribution and allocation

Unless otherwise specified, all probabilities for allometric parameters are given for the main effect of either CO_2 or nitrogen level. All allometric models accounted for more than 98% of the variance without the inclusion of an interaction between CO_2 and nitrogen level.

Green leaf nitrogen concentrations of the high-N ambient-CO_2 treatment were about 5% (by mass) prior to day 30, and those of the Hoagland experiment remained above 4.5% throughout the experiment. Green leaf nitrogen concentrations of this ecotype of *D. richardsonii* grown under conditions that were assumed to be non-growth limiting were approximately 4.2% after 49 days growth (chapter 3). This suggests that nitrogen was saturating growth in this experiment at the highest rate of N supply until day 30, and throughout the Hoagland experiment. This is supported by the lack of response in total plant carbon, leaf and root area to the nitrogen increment between mid- and high-N prior to day 30, and the lack of response between the high-N treatment and the Hoagland grown plants prior to day 37. Thus, the Hoagland experiment can be considered a high nitrogen control. As the Hoagland experiment was not structured like the other treatments it was analysed separately, and the results are included for comparative purposes.

Total plant carbon (C_P) was increased by high CO_2 when averaged over all treatments (Figure 5.1; $P<0.001$), and there was an interaction with N supply ($P<0.01$). The CO_2 effect was significant at all N levels ($P_L<0.01$). The relative effect of CO_2 was similar at low- and mid-N ($R_{e/c}=1.37$ & 1.38 respectively), while it was much higher ($R_{e/c}=2.00$) at the high rate of N supply. Growth under CO_2 enrichment increased carbon accumulation in the Hoagland experiment ($R_{e/c}=1.28$; $P<0.001$).

Non-structural carbohydrate content was increased in leaf ($P<0.001$) and sheath ($P<0.05$) by CO_2 enrichment at day 37, but in root was increased by CO_2 only at high-N ($P<0.05$; Table 5.3). Structural carbon at day 37 was increased by CO_2 enrichment, from 34.9 mg C to 42.9 mg C at mid-N and 47.2 mg C to 88.9 mg C at high-N ($P<0.001$; not determined at low-N).

Total plant nitrogen (N_P) increased under CO_2 enrichment (Figure 5.2; $P<0.001$), although this increase was dependant on N supply rate ($P<0.001$) and was only significant at high-N

(P_L<0.05). Growth at high CO_2 significantly reduced k for the relationship between total plant nitrogen and total plant carbon (Table 5.4 & Figure 5.2; P<0.001), including that of the Hoagland experiment (P<0.001).

Leaf nitrogen concentration (per unit carbon) decreased at high CO_2 when averaged over all treatments (Figure 5.3; P<0.001) with an $R_{e/c}$ of 0.77. Leaf nitrogen concentration was reduced under CO_2 enrichment in the Hoagland experiment when averaged over harvest (P<0.001), although there was no effect on day 16. The allometric constant (k) for the relationship between leaf nitrogen and leaf carbon was not affected by growth CO_2 concentration (Table 5.6) while the intercept (b) was lower when averaged over N level (Table 5.7; P<0.001). Although no interaction between CO_2 and N was evident, b was little different at high N. This does not affect the outcome, as there was a non-significant decrease in k at high N. Thus, total leaf nitrogen was lower at high CO_2 regardless of total plant carbon content. A similar effect was noted in the Hoagland treatment, although the reduction at high CO_2 was in k (Table 5.6; P<0.01). As b was also lower under CO_2 enrichment (Table 5.7) the end result was the same - a lower leaf nitrogen concentration independently of plant size. Leaf nitrogen surface density was reduced by CO_2 when averaged over all treatments (Figure 5.3; P<0.01). This effect was dependant on both N level and harvest (P<0.06), such that the reduction was significant (P_L<0.05) except at high-N prior to day 37. In the Hoagland experiment, CO_2 increased leaf nitrogen surface density when averaged over harvest (P<0.001), and there was no significant interaction with harvest. Growth at high CO_2 reduced k for the allometric relationship between total leaf nitrogen and leaf area (Table 5.6; P<0.001), with a similar effect for the Hoagland experiment (P<0.05).

Sheath nitrogen concentration (per unit carbon) was reduced by high CO_2 when averaged over all other treatments (P<0.001, not presented) with an $R_{e/c}$ of 0.87, and an $R_{e/c}$ of 0.94 in the Hoagland experiment when averaged over harvest (P<0.001). These decreases were apparent when expressed on a structural C basis at day 37 (Table 5.3; P<0.001). The allometric constant for the relationship between total sheath nitrogen and total plant carbon was lower under CO_2 enrichment (Table 5.4; P<0.001). This was not the case in the Hoagland treatment.

Root nitrogen concentration (per unit carbon) were decreased at high CO_2 (Figure 5.3; P<0.001) with an $R_{e/c}$ of 0.84 when averaged over all treatments. The CO_2 effect varied with N supply rate (P<0.05). A reduction in root nitrogen concentration was apparent at high CO_2 at all rates of N supply (P_L<0.05), and the effect increased as N supply rate increased. However, there was no effect of CO_2 on root nitrogen concentration in Hoagland treatment, apart from on day

30 when it was reduced (Figure 5.3; *P*<0.01). The reason for this 'dip' in nitrogen concentration on day 30 is not known. When expressed on a structural C basis root nitrogen concentration decreased in response to CO_2 (Table 5.3; *P*<0.001). The allometric relationship between total root nitrogen and total plant carbon was not affected by CO_2 level in either experiment (Table 5.4 & Table 5.5).

Leaf area (A_L) was increased by growth at high CO_2 only at the highest rate of N supply (Figure 5.4; *P*<0.001) and in the Hoagland experiment (*P*<0.01). Growth at high CO_2 increased root length (not presented; *P*<0.001) and root surface area (A_R; Figure 5.4; $R_{e/c}$=1.43) when averaged over all treatments (*P*<0.001). Root length and area were not determined in the Hoagland treatment. Growth under CO_2 enrichment reduced the intercept (*b*), of the allometric relationship between leaf area and total plant carbon (Table 5.7 & Figure 5.4; *P*<0.001). The same effect was noted in the Hoagland experiment (*P*<0.05). No treatment had a significant effect on the allometric relationship between root surface area and total plant carbon (Figure 5.4, Table 5.6& Table 5.7). Specific leaf area was lower under CO_2 enrichment when averaged over all other treatments (*P*<0.001) with an $R_{e/c}$ of 0.81, as was specific root surface area when averaged over other treatments (*P*<0.001) with an $R_{e/c}$ of 0.83 (Table 5.8). Specific leaf area in the Hoagland experiment was also reduced under CO_2 enrichment when averaged over harvest (*P*<0.001). Both specific leaf area and specific root area (C mass basis) declined over the experiment (*P*<0.001; not presented). The intercept of the allometric relationship between leaf area and total leaf carbon showed non-significant reductions at high CO_2, at least at the lower rates of nitrogen supply (Table 5.7), indicating a lower specific leaf area under CO_2 enrichment, independently of plant size. However, those trends were not evident for the allometric relationship between root surface area and total root carbon (Table 5.7).

The ratio between leaf area and root surface area decreased in response to CO_2 when averaged over all treatments (Figure 5.5; *P*<0.001). As the rate of N supply increased leaf to root surface area ratio increased (*P*<0.001). This was reflected in the allometric relationship between root surface area and leaf area (Figure 5.5, Table 5.6 & Table 5.7), with a significant reduction in *b* at high CO_2, although there tended to be little effect at high-N.

Leaf carbon ratio was reduced slightly by high CO_2 when averaged over all other treatments (Figure 5.6; *P*<0.001) with an $R_{e/c}$ of 0.97, and increased with increasing N supply (*P*<0.001). Sheath carbon ratio harvest (Figure 5.6) was unchanged by CO_2 enrichment. Root carbon ratio (Figure 5.6) increased at high CO_2 when averaged over the other treatments (*P*<0.001) with an

$R_{e/c}$ of 1.06. Distribution of total plant carbon in the various pools in the Hoagland experiment were not affected by CO_2 enrichment. Allometric relationships between leaf, sheath and root carbon and total plant carbon, or root carbon and total shoot carbon were not affected by growth CO_2 level in any nitrogen treatment (Table 5.4 & Table 5.5).

Total root nitrogen (N_R) increased by growth at high CO_2 (not presented; $P<0.001$), and the CO_2 effect increased as the rate of N supply increased ($P<0.001$) from an $R_{e/c}$ of 1.18 at low-N (ns) to 1.24 and 1.50 respectively at mid- and high-N ($P_L<0.01$). Root nitrogen ratio was, on average higher in plants grown at high CO_2 (Figure 5.7; $P<0.001$) The increase was dependant on the rate of N supply ($P<0.05$) and was significant only at the low and mid rate of N supply ($P_L<0.01$). The intercept (b) for the relationship between total root and total shoot nitrogen was higher (less negative), at least at the lower rates of N supply under CO_2 enrichment ($P<0.001$), indicating an increased allocation of total plant nitrogen to root (Table 5.5). This was reflected in a higher b for the relationship between root nitrogen and total plant nitrogen (Table 5.5; $P<0.001$). The allometric relationships between shoot and root nitrogen were not altered in the Hoagland experiment (Table 5.4 & Table 5.5).

The effect of CO_2 on total leaf nitrogen (N_L) was dependant on N supply ($P<0.001$; not presented). At low-N growth at high CO_2 reduced total leaf nitrogen with an $R_{e/c}$ of 0.89 (ns), while at high-N it increased with an $R_{e/c}$ of 1.31 ($P_L<0.05$, not presented). Leaf nitrogen ratio was reduced high CO_2 when averaged over all treatments (Figure 5.7; $P<0.001$) with an $R_{e/c}$ of 0.92. The allometric relationship between total leaf and total plant nitrogen was not statistically affected by growth CO_2 level in either experiment (Table 5.4 & Table 5.5), although the non-significant reductions in b and lesser reductions in k carry biological significance in accounting for the statistical increase in nitrogen allocation to root.

Total sheath nitrogen (N_S) was unchanged at low- & mid-N by growth at high CO_2, while at high-N it was increased ($P<0.01$; not presented). Sheath nitrogen ratio was increased at high CO_2 (Figure 5.7; $P<0.001$) with an $R_{e/c}$ of 1.09. The allometric relations between total sheath nitrogen and total plant nitrogen were not affected by growth CO_2 level (Table 5.4 & Table 5.5).

Functional growth analysis parameters

Relative growth rate (RGR) after day 16 was not statistically affected by CO_2 level at any rate of N supply (Table 5.9). RGR may not have been constant over the period of determination at low-N (Figure 5.1), although a non-linear curve-fit could not be statistically justified. Visual examination shows that any non-linearity did not affect conclusions on the non-response of

RGR to CO_2, the curves at low-N being essentially parallel. Growth at high CO_2 increased net assimilation rate at all N levels (NAR; Figure 5.8). Leaf and plant nitrogen productivity (NP_L & NP_P respectively) were generally increased under CO_2 enrichment at all N levels, although the effect of CO_2 on NP_P was small at low-N (Figure 5.8). Growth CO_2 level had no effect on net nitrogen absorption rate (NNAR; Figure 5.8). The CO_2 effect on these functional parameters did not appear to be related to plant size, but this was not statistically tested (Figure 5.9).

Discussion

The three hypotheses were supported by these results for isolated *Danthonia richardsonii* plants. Growth under CO_2 enrichment increased carbon accumulation at all levels of N supply. This was not primary due to increases in leaf area, as allometric relationships showed a reduction in leaf area under CO_2 enrichment at a given total plant carbon - a reduction in leaf area ratio. The increase in carbon accumulation could be attributed to increases in net assimilation rate (per unit leaf area, NAR) and leaf nitrogen productivity (NP_L). This resulted in a differing pattern of resource allocation under CO_2 enrichment, with a shift away from allocation to tissue involved with carbon capture towards that involved with water and nutrient capture. This was expressed as a reduction in nitrogen allocation to leaf and a corresponding increase in nitrogen allocation to root, and an increase in root surface area relative to leaf surface area under CO_2 enrichment. Root function, expressed as net nitrogen absorption rate (NNAR) was not increased in response to CO_2. Many of the CO_2 effects on allometric relationships visually appeared small at high-N, although no statistical interactions between CO_2 and nitrogen were evident, indicative of a lack of resolution of the technique. The main effects are discussed, which are the most pertinent for this study, as the higher N input levels experienced levels of N availability higher than those generally observed both in the field, and in the microcosm experiment, as indicated by the high leaf nitrogen concentrations (cf. Figure 5.3 & Figure 7.6) (Field & Mooney, 1986; Garnier & Freijsen, 1994). Most changes in allometric relationships owing to CO_2 enrichment were observed in b, the intercept, rather than in k, the allometric constant. As all plants started from seed, this implies that the k must have differed before the first harvest, suggesting that CO_2 effects over the first few days of growth are very important. These observations are now discussed more fully.

The increase in total plant carbon under CO_2 enrichment was larger, both in absolute and relative terms at the high rate of N supply (Figure 5.1). However, at the lowest rate of N supply the CO_2 effect was still significant. These results are similar to those observed for *Bromus mollis* (Larigauderie *et al.,* 1988), but contrast with *Xanthium occidentale* Bertol. (Hocking &

Meyer, 1985), which exhibited the greatest relative growth enhancement at the lowest rate of N supply. The relative increase of total plant carbon due to CO_2 enrichment of 100% at the highest rate of N supply in the main experimental treatments was greater than the average biomass increase of 51% for immature plants in the review of Kimball (1983), or of 37% from the study of Poorter (1993). However, the percentage response to CO_2 enrichment for isolated plants is very dependent on the time of harvest (Gifford & Morison, 1993; Barrett & Gifford, 1995b; Loehle, 1995), as it is accentuated during exponential increase of plant size (Gifford *et al.*, 1996a).

Relative growth rate (RGR) was statistically unaffected by CO_2 level, although RGR tended to be higher at high-N and in the Hoagland experiment (Table 5.9). An increase in relative growth rate is a pre-requisite for an increase in plant carbon accumulation. However the resolving power of RGR is low (Norby & O'Neill, 1991), and small differences in relative growth rate can result in significant differences in absolute growth over time (Evans,LT, 1993; Gifford *et al.*, 1996a). A statistical CO_2 effect on relative growth rate is generally only observed very early in growth, if it is captured at all (Garbutt *et al.*, 1990; Watson,J & Graves, 1993; Baxter *et al.*, 1994a; Stulen *et al.*, 1994). Relative growth rate normally declines with increasing plant size (Evans,LT, 1993) as growth moves out of the exponential phase. From the mathematics of RGR, it must approach zero as phytomass increases, as small incremental rises in phytomass are divided by an increasingly large phytomass. Hence, it may be expected that RGR would be reduced more rapidly under CO_2 enrichment, as high CO_2 grown plants accumulate phytomass more rapidly. Gifford *et al.* (1996) demonstrated this with a simple model. In fact, when simulated RGR was plotted as a function of plant phytomass, RGR was *increased* by CO_2 enrichment for a much greater period than when plotted as a function of time, indicating a sustained physiological response to CO_2 enrichment (not presented). Biological variability, and constraints on the size of experiments generally preclude the use of other than exponential relationships between plant mass and time over the period of plant growth commonly measured, and transient increases in RGR are often missed as harvests do not occur early enough. Thus, as the plants in this experiment all originated from similar seed, relative growth rate must have been higher under CO_2 enrichment prior to the first harvest to allow for the observed increase in carbon accumulation (Figure 5.1). This was the case (Table 5.9), and the measured RGR over the experimental period approximated the average RGR needed to move from total plant carbon at day 16 to that at day 37. Thus, the small increases in relative growth rate under CO_2 enrichment in the high-N and Hoagland experiment were lost in experimental variability.

It has been hypothesised that when plants are not water stressed, the main CO_2 response will be attained via an increase in carboxylation efficiency, and the related decrease in energy and carbon loss through the photorespiratory recovery pathway (Bowes, 1991; Conroy & Hocking, 1993). In this experiment net assimilation rate (NAR), a measure of carbon accumulation per unit leaf area, and leaf nitrogen productivity (NP_L), a measure of carbon accumulation per unit leaf nitrogen, were stimulated by growth at high CO_2 (Figure 5.8). Increases in net assimilation rate are often observed in C_3 grasses, such as *Agrostis capillaris* L., *Arrhenatherum elatius* (L.) Beauv., *Festuca ovina* L., *Festuca rubra* L., *Nardus stricta* L., and *Poa alpina* L. (Bowler & Press, 1993; Baxter *et al.,* 1994a; Hunt,R *et al.,* 1995). As net assimilation rate can be considered a surrogate for whole plant net photosynthesis (net of both respiratory and exudate C loss), it is apparent that complete photosynthetic down-regulation on a whole plant basis did not occur at any N level in response to CO_2 enrichment. That is, net assimilation rate was higher under CO_2 enrichment, implying that leaf photosynthetic rates were higher in the high CO_2 grown plants at their growth CO_2 concentration. Although photosynthetic down-regulation in response to CO_2 enrichment is commonly observed (Bowes, 1991), it is by no means universal and many species show incomplete down-regulation in response to CO_2 enrichment (eg. Ryle *et al.,* 1992). Increases in NAR were not fully expressed as increased RGR due to decreases in leaf area ratio (not presented as a function of time, but see discussion on leaf area below). Increases in NP_L were not fully expressed as increased plant nitrogen productivity (NP_P) due to reductions in leaf nitrogen ratio (below & Figure 5.7), and increased NP_P was not fully expressed as increased RGR due to decreases in whole plant nitrogen concentration (not presented as a function of time, but see discussion on total plant nitrogen below). However, these changes were not large enough to negate the CO_2 effect on carbon accumulation (Figure 5.1).

Total plant nitrogen was increased by CO_2 enrichment only at high-N and in the Hoagland experiment (Figure 5.2). Nitrogen concentration (per unit carbon) at harvest was decreased in all tissues by CO_2 enrichment (Figure 5.3; sheath not presented). This was not only a result of increased carbon accumulation under CO_2 enrichment, as the allometric constant for the relationship between total plant nitrogen and total plant carbon was lower under CO_2 enrichment, with accompanying lower intercepts (Table 5.4 & Table 5.5). Thus, in this experiment, the reduction in total plant nitrogen concentration in response to CO_2 enrichment was a physiological response to CO_2 enrichment, rather than a repercussion of differing plant size as hypothesised by Coleman *et al.* (1993). Decreased nitrogen concentration in plant tissues grown at high CO_2 is a common phenomenon (expressed at a common time), although it

appears to be dependant on species and the growth environment (Arp & Berendse, 1993). Ryle *et al.* (1992) observed that nitrogen concentrations in expanded leaf laminae, calculated on a total mass basis, were reduced by CO_2 enrichment in *Lolium perenne* L. cv. Melle, while the nitrogen concentration in the root and leaf sheath was not changed. Wild radish (*Raphanus sativus* x *raphanistrum*) exhibited lower leaf nitrogen concentrations, and higher root nitrogen concentrations when grown under CO_2 enrichment (Chu *et al.,* 1992). Plants in those studies all experienced conditions of nitrogen abundance or surfeit, as the control CO_2 treatments (~350 $\mu L\ L^{-1}$) had leaf nitrogen concentrations of about 5% (by mass) or greater. This may commonly be the case in controlled environment studies, limiting the applicability of results to the field. Under nitrogen restriction, seedlings of *Quercus alba* L. (Norby *et al.,* 1986) and *T. aestivum* (Hocking & Meyer, 1991) exhibited depressions in the nitrogen content of all tissues when grown at high CO_2.

It has been hypothesised that decreases in nitrogen concentration may simply be the result of dilution by increased concentrations of non-structural carbohydrates (eg. Loehle 1995), such as in *Poa alpina* and *Agrostis capillaris*, where no changes in the nitrogen concentration of leaf, sheath or root were noted when expressed on a structural carbon basis (Baxter *et al.,* 1994b). However, leaf nitrogen concentrations in excess of 6% (structural dry mass) indicate that those plants experienced very high levels of nitrogen availability. This was not the case in this study at the lower rates of N supply. Non-structural carbohydrates were higher under CO_2 enrichment (Table 5.3), but nitrogen concentrations were still strongly reduced by CO_2 enrichment when expressed on a structural C basis (Table 5.3). A reduction in nitrogen concentration appears the most common response under conditions when nitrogen restriction is limiting growth (Arp & Berendse, 1993). It has been argued that leaf nitrogen surface density (g N m^{-2} leaf) is a more appropriate physiological measure to relate leaf nitrogen to photosynthetic capacity (Field & Mooney, 1986; Evans,JR, 1989). From this experiment, it is apparent that leaf nitrogen surface density is sensitive to both N supply and CO_2 concentration (Figure 5.3). At lower rates of N supply, growth at high CO_2 reduced leaf nitrogen surface density, while at high rates of N supply it was increased. As leaf nitrogen concentration was 5-6% (by mass) or greater in the treatments where leaf nitrogen surface density was increased by CO_2 enrichment - concentrations which are higher than those generally observed in the field (Field & Mooney, 1986; Garnier & Freijsen, 1994) - the measure may be confounded by luxury uptake of nitrogen and its storage as nitrate (Garnier & Freijsen, 1994). Thus this increase in leaf nitrogen surface density at high-N supply rates may differ if it were expressed on an organic nitrogen basis, and hence the results at high N may have little ecological significance.

Leaf area at any given time was increased by CO_2 enrichment only at the highest rate of N supply (Figure 5.4). Leaf area was also observed to be unresponsive to CO_2 at low-N in *Bromus mollis* (Larigauderie *et al.*, 1988), *Agrostis capillaris, Nardus stricta* (Bowler & Press, 1993) and in *Danthonia richardsonii* and *Vulpia bromoides* in the previous chapter (Figure 4.3). Thus, total plant carbon was increased without an increase in leaf area. This is demonstrated as a lower intercept (*b*) in the high CO_2 treatments for the allometric relationship between leaf area and total plant carbon (Figure 5.4 & Table 5.5), although the effect was small at high-N. Thus, at any common total plant carbon, plants grown under CO_2 enrichment had a lower leaf area than those grown under the control CO_2 level, at least at the lower levels of N supply. As the reduction in *b* for the allometric relationship between leaf area and total plant carbon under CO_2 enrichment was not accompanied by changes in the allometric relations of carbon allocation (below), the reduction in leaf area at mid- and high-N was compensated for by the non-significant decrease in *b* for specific leaf area (Table 5.5). A reduction in specific leaf area, independent of plant size has also been observed in cotton (Barrett & Gifford, 1995b).

Root length (not presented) and root surface area at harvest were increased by CO_2 enrichment (Figure 5.4), while specific root surface area was reduced (Table 5.8). Little data is present in the literature on the effect of CO_2 on root area development, although root length increased in response to CO_2 in *Sanguisorba minor, Lotus corniculatus, Plantago media, Gossypium hirsutum* (L.), *Glycine max* (L.), and in *Sorghum bicolor* (L.) Moench, a C_4 (Chaudhuri *et al.*, 1986; Del Castillo *et al.*, 1989; Allen *et al.*, 1992; Rogers *et al.*, 1992; Ferris & Taylor, 1993), but not in *Anthyllis vulneraria* L. or *Populus grandidentata* (Ferris & Taylor, 1993; Curtis *et al.*, 1994). Increases in root area or root length may have the effect of increasing the volume of soil explored by the root system, or enabling the root system to explore a given volume more thoroughly. Increases in root length density were noted for *S. bicolor, G. max* and *G. hirsutum* when grown in large volumes of soil (Chaudhuri *et al.*, 1986; Del Castillo *et al.*, 1989), indicating a more thorough exploration of the same soil volume, while the total volume explored was similar between CO_2 treatments. Apart from transitory effects early in growth due to increases in the rate of root area development, this may be the most realistic result, as the total volume available for exploration by the root system is often physically constrained, even in the field. Whichever response occurs diffusional limitations to nutrient uptake should be reduced by growth at high CO_2.

Allometric relations between root surface area and total plant or root carbon were not changed by CO_2 enrichment (Figure 5.4, Table 5.6 & Table 5.7). Thus, the observed increases in root surface area, and decreases in specific root surface area at high CO_2 were the result of increases

in total plant carbon. As the allometric relationship between leaf area and total plant carbon indicated a *lower* leaf area under CO_2 enrichment, while that for root surface area was unchanged, there was an alteration in the functional balance between carbon absorbing and nutrient absorbing area under CO_2 enrichment. This is shown as an increase in the root surface area to leaf surface area ratio at harvest under CO_2 enrichment (Figure 5.5). This was not related to changes in plant size, as the intercept for the allometric relationship between root surface and leaf surface area was increased under CO_2 enrichment, although the effect tended to be lower as N supply increased (Table 5.7). A decrease in this ratio was also noted as the rate of N supply increased (Figure 5.5), and the allometric constant was reduced by increasing N supply (Table 5.6). Nitrogen sensitivity of this functional relationship has been noted as a reduction in the leaf area to root length ratio with decreasing N supply in a number of perennial grasses (Boot & Mensink, 1990). This relationship is more robust than one based on dry matter partitioning as it is a direct relationship between the surface areas involved with nutrient and water capture, to those with carbon and light capture (Körner & Renhardt, 1987), and is not confounded by changes in the density of the tissues involved, or by carbohydrate storage.

Root carbon ratio at harvest increased slightly at high CO_2 at the expense of leaf carbon ratio when nitrogen was growth limiting (Figure 5.6). This effect is commonly observed under nutrient limitation (Stulen & den Hertog, 1993). However, allometric relationships between root carbon and shoot carbon, between root, leaf, or sheath carbon and total plant carbon (Table 5.4 & Table 5.5), and between root carbon and root surface area (Table 5.6 & Table 5.7) were not altered by growth under CO_2 enrichment. Thus changes in carbon distribution and specific root area (Table 5.8) in this experiment were fully explained by changes in plant size. These findings support those for the grasses *Agrostis capillaris* L., *Arrhenatherum elatius* (L.) Beauv., *Festuca ovina* L., *Festuca rubra* L., *Nardus stricta* L., *Poa annua* L. and *Poa alpina* L. (Bowler & Press, 1993; Baxter *et al.*, 1994a; Hunt,R *et al.*, 1995). In this study growth under CO_2 enrichment did not change carbon allocation between plant parts *per se*, but rather the changes in carbon distribution were the result of differences in plant size between the CO_2 levels. This change in root carbon ratio as plant size increased was not evident in *D. richardsonii* in chapter 4 (cf. Figure 4.1 & Figure 4.8). These plants were much older and larger than those in this experiment, and later in growth root carbon ratio may vary little with time (Ballard *et al.*, 1936; Garnier & Freijsen, 1994).

Leaf nitrogen ratio was reduced in the high CO_2 grown plants, while root nitrogen ratio was increased (Figure 5.7). This effect was greater than the shift in carbon distribution (Figure 5.6)

and was greater at the lower rates of N supply. The observation of a shift in nitrogen distribution, which differs from that of carbon distribution is supported by changes in the allometric relationship between shoot nitrogen and root nitrogen, and individual plant nitrogen pools and total plant nitrogen under CO_2 enrichment (Table 5.4 & Table 5.5). Allometric relationships between root nitrogen and total plant carbon were not altered under CO_2 enrichment (Table 5.4 & Table 5.5). This relationship was reflected as the increased allocation of the total plant nitrogen to root (Table 5.4 & Table 5.5), although the effect was lower at high-N. Thus the reduction in root nitrogen concentration under CO_2 enrichment (Figure 5.3) was a plant size related effect, supporting the hypothesis that growth at high CO_2 would result in a differing allocation of nitrogen, involving lower nitrogen investment in photosynthetic tissue and an increase in nitrogen investment in tissue associated with the capture of other resources.

Changes in nitrogen allocation at harvest which are not a result of changes in carbon allocation have been observed in *Pinus taeda* L. seedlings, *Pinus ponderosa* Dougl. ex Laws. seedlings (Griffin *et al.*, 1995), *X. occidentale* (Hocking & Meyer, 1985), and a tropical plant assemblage (Körner & Arnone, 1992). This reduction in nitrogen allocation to leaf may result from a reduction in nitrogen allocation to nitrogenous compounds involved with photosynthesis. Total soluble protein and Rubisco contents have been observed to decline in response to CO_2 enrichment in *D. richardsonii* (Lutze, Evans,JR & Gifford, unpublished). Rubisco and other Calvin cycle enzymes (Sage *et al.*, 1989; Besford, 1990; Rowland-Bamford *et al.*, 1991; Stitt, 1991; Bowes, 1993; Nie,GY *et al.*, 1995) and chlorophyll (Sage *et al.*, 1989; Xu *et al.*, 1994) contents have often decreased in a range of plant species in response to CO_2 enrichment.

Net nitrogen absorption rate (per unit root surface area) was not affected by growth at high CO_2 (Figure 5.8). Most data in the literature on the effect of CO_2 on root absorption rate of nitrogen are expressed on a root dry mass basis (specific absorption rate), and hence are potentially confounded by changes in non-structural carbohydrate concentration, tissue density or changes in specific root area. However, specific absorption rate was not affected by CO_2 level in this experiment (not presented). Specific absorption rate of wild radish (Chu *et al.*, 1992) and *Pinus virginiana* Mill. (Luxmoore *et al.*, 1986) was not altered by CO_2 enrichment while *Abutilon theophrasti* and *Amaranthus retroflexus*, a C_4, showed an initial depression in specific absorption rate by CO_2 enrichment, which disappeared after 60 days growth (Coleman,JS & Bazzaz, 1992). Stulen *et al.* (1994) found that SAR was stimulated by CO_2 enrichment prior to 10 days of growth in *Plantago major* spp. *pleiosperma* Pilger and *Urtica dioica* L., while *Eichhornia crassipes* (Mart.) Solms showed a positive response to CO_2 at a high nutrient level but not at a low nutrient level. Larigauderie *et al.* (1994) noted that nitrogen uptake rate on a

root length basis by *P. taeda* was decreased by CO_2 enrichment at low-N, but increased by CO_2 at high-N. Thus, diverse results on the effect of CO_2 enrichment on root function have been reported. Some of those data may be confounded by changes in NSC concentrations or tissue density. In this study, the performance of each unit of root area in nitrogen acquisition was not affected by CO_2 enrichment, and nitrogen concentrations in root, when compared at equal plant carbon contents were not altered by growth CO_2 level.

Plant nitrogen productivity was increased by high CO_2 at all N supply rates as a result of increased leaf nitrogen productivity (Figure 5.8), although the response was small at low-N. Plant nitrogen productivity increased at high CO_2 in *Betula pendula* Roth. (Pettersson *et al.*, 1993). Baxter *et al.* (1994b) observed no significant effects of CO_2 on nitrogen productivity of *A. capillaris* or *P. alpina*, although leaf nitrogen concentrations of 5% or greater indicated the possibility of luxury nitrogen consumption and a proportion of this nitrogen may have been present as nitrate (Garnier & Freijsen, 1994), a non-metabolically active form of nitrogen. This may also have contributed to the small effects on nitrogen productivities in the Hoagland treatment.

These differences in functional characteristics between CO_2 levels are not changes associated with differing plant size (Figure 5.9), but are real physiological effects of growth under CO_2 enrichment - the driving force behind the growth and allocation response. The increase in leaf nitrogen productivity in this study did not result in a directly proportional increase in plant nitrogen productivity and hence carbon gain, as allocation of nitrogen to leaf was reduced. The corresponding increase in nitrogen allocation to root (as a function of total plant nitrogen) and root area increase under CO_2 enrichment, with respect to time, resulted in a greater capture of nitrogen at the highest rates of N supply (Figure 5.2), reflected in the lower effect of CO_2 on the allometric relationships at high-N. The increased nitrogen capture formed a positive feedback on growth, further increasing absolute carbon gain. The response of *D. richardsonii* to the greater nitrogen allocation to root was to maintain root surface area (with respect to total plant carbon), rather than increase uptake capacity. This response is similar to the general response of wild plants from low fertility habitats to nutrient stress, where the area available for nutrient absorption is increased rather than nutrient absorptive capacity (Chapin, 1980).

The observed change in nitrogen allocation and resultant decrease in leaf nitrogen concentration does not support the hypothesis that all changes in leaf nitrogen are solely due to carbohydrate dilution (eg. Loehle, 1995), or that changes in whole plant nitrogen concentration simply reflect increases in plant size (Coleman,JS *et al.,* 1993). Thus, these data show that

growth under CO_2 enrichment can produce real changes in the physiology of nitrogen use, other than apparent changes due to differences in plant size or non-structural carbohydrate content.

Table 5.1 Environmental conditions over the experimental period. Standard deviations in parenthesis where appropriate.

	Ambient	**Enriched**
CO_2 (avg $\mu L\ L^{-1}$)	362 (9)	748 (37)
Dew Point (avg °C)	12.9 (1)	13.0 (1)
Temperature (avg °C)	20.9 (1.5)	20.9 (1.5)
Thermal Time (°C, base 0)	1712	1711
Total Evaporation (mm)	382	408
Radiation (avg MJ m^{-2})	9.9 (3.5)	
Total Radiation (MJ m^{-2})	823	

Table 5.2 Composition of nutrient solution.

	Concentration in nutrient solution (mg L^{-1})		
	Low N	**Mid N**	**High N**
N	7.0	28.0	70.1
P	31.0	31.0	31.0
K	449.7	449.7	449.7
S	112.2	112.2	112.2
Mg	48.6	48.6	48.6
Ca	200.4	200.4	200.4
Cl	602.7	549.5	443.1
Fe	50	50	5
Mn	0.5	0.5	0.5
B	0.4	0.4	0.4
Zn	0.12	0.12	0.12
Cu	0.04	0.04	0.04
Mo	0.02	0.02	0.02
Co	0.02	0.02	0.02
I (μg L^{-1})	0.99	0.99	0.99

Table 5.3. Non-structural carbohydrate (NSC) concentration and structural N concentration 37 days after imbibition. $P<0.001$ represented by ∗∗∗, $P<0.01$ by ∗∗, $P<0.05$ by ∗ and not significant by ns. Non-structural carbohydrates were not determined on the Hoagland plants.

		Nitrogen level			Probabilities			LSD *P*<0.05
		Low	**Mid**	**High**	**CO_2**	**N**	**C∗N**	**C∗N**
Non-structural carbohydrate concentration mg C g^{-1} structural C								
leaf	Con CO_2	171	227	215	∗∗∗	∗∗∗	∗∗	48
	Enr CO_2	249	295	412				
sheath	Con CO_2	nd	127	114	∗	ns	ns	34
	Enr CO_2	116	157	137				
root	Con CO_2	82	73	72	ns	ns	ns	18
	Enr CO_2	75	81	100				
Nitrogen concentration per unit structural carbon mg N g^{-1} structural C								
leaf	Con CO_2	48	61	97	∗∗∗	∗∗∗	∗	11
	Enr CO_2	36	48	59				
sheath	Con CO_2	nd	48	78	∗∗∗	∗∗∗	∗	9
	Enr CO_2	33	42	54				
root	Con CO_2	27	41	70	∗∗∗	∗∗∗	∗	8
	Enr CO_2	23	31	46				

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Table 5.4 Allometric relationships involving carbon or nitrogen allocation. Summary of values of the allometric constant (k) for the full model, where C and N are the total C and N contents of the plant ($_P$), leaf ($_L$), shoot ($_{SH}$), sheath ($_S$), or root ($_R$), and A is the leaf ($_L$) or root ($_R$) surface area. Mean is followed by the standard error. Probabilities (Prob.) are those for model improvement when the term is included in the model by stepwise-multiple linear regression, P<0.001 and P<0.01 represented by *** and ** respectively, ns represents non-significance and nd is not determined. There were no significant interactions between CO_2 and N level. Hoagland shows k for the pots supplied with Hoagland solution and P the probability of a CO_2 effect on those plants.

			N supply rate			Prob.			
x	**y**	**CO_2**	**Low**	**Mid**	**High**	**CO_2**	**N**	**Hoagland**	***P***
C_P	N_P	Con	0.68 ± 0.04	0.77 ± 0.05	0.91 ± 0.05	***	***	0.93 ± 0.01	***
		Enr	0.64 ± 0.06	0.75 ± 0.08	0.83 ± 0.07			0.86 ± 0.02	
C_{SH}	C_R	Con	1.28 ± 0.04	1.12 ± 0.05	1.03 ± 0.05	ns	***	0.89 ± 0.04	ns
		Enr	1.33 ± 0.06	1.14 ± 0.08	1.01 ± 0.07			0.86 ± 0.05	
C_P	C_L	Con	0.88 ± 0.01	0.95 ± 0.02	0.98 ± 0.02	ns	ns	1.00 ± 0.01	ns
		Enr	0.87 ± 0.02	0.94 ± 0.03	0.98 ± 0.02			1.00 ± 0.02	
C_P	C_S	Con	0.97 ± 0.03	1.03 ± 0.04	1.03 ± 0.04	ns	ns	1.06 ± 0.02	ns
		Enr	0.94 ± 0.04	1.01 ± 0.05	1.05 ± 0.05			1.06 ± 0.03	
C_P	C_R	Con	1.17 ± 0.03	1.09 ± 0.03	1.02 ± 0.03	ns	***	0.91 ± 0.03	ns
		Enr	1.19 ± 0.04	1.10 ± 0.05	1.01 ± 0.05			0.88 ± 0.05	
C_P	N_L	Con	0.63 ± 0.05	0.76 ± 0.06	0.91 ± 0.06	ns	***	0.93 ± 0.02	**
		Enr	0.59 ± 0.07	0.73 ± 0.09	0.81 ± 0.08			0.85 ± 0.03	
C_P	N_S	Con	0.70 ± 0.05	0.78 ± 0.06	0.93 ± 0.06	***	***	0.99 ± 0.02	ns
		Enr	0.61 ± 0.07	0.79 ± 0.09	0.87 ± 0.08			0.94 ± 0.03	
C_P	N_R	Con	0.76 ± 0.05	0.81 ± 0.07	0.88 ± 0.06	ns	ns	0.89 ± 0.04	ns
		Enr	0.74 ± 0.08	0.78 ± 0.09	0.83 ± 0.09			0.80 ± 0.07	
N_{SH}	N_R	Con	1.17 ± 0.07	1.05 ± 0.08	0.96 ± 0.08	ns	***	0.94 ± 0.04	ns
		Enr	1.20 ± 0.11	1.05 ± 0.13	1.01 ± 0.12			0.94 ± 0.05	
N_P	N_L	Con	0.93 ± 0.02	0.99 ± 0.03	1.00 ± 0.03	ns	ns	0.99 ± 0.01	ns
		Enr	0.92 ± 0.04	0.97 ± 0.04	0.98 ± 0.04			0.98 ± 0.02	
N_P	N_S	Con	1.03 ± 0.05	1.00 ± 0.06	1.02 ± 0.05	ns	ns	1.06 ± 0.02	ns
		Enr	0.96 ± 0.07	1.05 ± 0.08	1.05 ± 0.08			1.09 ± 0.03	
N_P	N_R	Con	1.12 ± 0.05	1.04 ± 0.06	0.97 ± 0.06	ns	***	0.96 ± 0.05	ns
		Enr	1.14 ± 0.08	1.04 ± 0.09	1.01 ± 0.09			0.93 ± 0.08	

Table 5.5 Allometric relationships involving carbon or nitrogen allocation. Summary of values of the intercept (*b*) of the allometric relationship for the full model, where C and N are the total C and N contents of the plant ($_P$), shoot ($_{SH}$), leaf ($_L$), sheath ($_S$), or root ($_R$), and A is the leaf ($_L$) or root ($_R$) surface area. Mean is followed by the standard error. Probabilities (Prob.) given are those for model improvement when the term is included in the model by stepwise-multiple linear regression, $P<0.001$ and $P<0.05$ represented by *** and * respectively, ns represents non-significance, nd not determined and - shows values which may have changed due to differences in the allometric constant. There were no significant interactions between CO_2 and N level. Hoagland shows *b* for the pots supplied with Hoagland solution and *P* the probability of a CO_2 effect on those plants.

x	y	CO_2	N supply rate Low	Mid	High	Prob. CO_2	N	Hoagland	*P*
C_P	N_P	Con	-2.40 ± 0.07	-2.00 ± 0.11	-2.01 ± 0.10	-	-	-1.88 ± 0.03	-
		Enr	-2.48 ± 0.12	-2.12 ± 0.16	-1.96 ± 0.16			-1.75 ± 0.06	
C_{SH}	C_R	Con	-0.84 ± 0.06	-1.30 ± 0.09	-1.37 ± 0.09	ns	ns	-1.53 ± 0.10	ns
		Enr	-0.91 ± 0.09	-1.24 ± 0.14	-1.32 ± 0.13			-1.45 ± 0.12	
C_P	C_L	Con	-0.59 ± 0.03	-0.50 ± 0.04	-0.50 ± 0.04	ns	ns	-0.50 ± 0.03	ns
		Enr	-0.57 ± 0.04	-0.51 ± 0.06	-0.49 ± 0.05			-0.48 ± 0.05	
C_P	C_S	Con	-1.79 ± 0.05	-1.67 ± 0.07	-1.65 ± 0.07	ns	ns	-1.59 ± 0.05	ns
		Enr	-1.71 ± 0.08	-1.67 ± 0.12	-1.71 ± 0.11			-1.65 ± 0.09	
C_P	C_R	Con	-1.26 ± 0.05	-1.56 ± 0.07	-1.61 ± 0.07	ns	-	-1.72 ± 0.09	ns
		Enr	-1.31 ± 0.07	-1.52 ± 0.10	-1.56 ± 0.10			-1.64 ± 0.15	
C_P	N_L	Con	-2.92 ± 0.09	-2.46 ± 0.12	-2.47 ± 0.12	***	-	-2.30 ± 0.05	-
		Enr	-3.12 ± 0.13	-2.63 ± 0.19	-2.41 ± 0.18			-2.13 ± 0.09	
C_P	N_S	Con	-4.31 ± 0.08	-3.72 ± 0.12	-3.71 ± 0.12	-	-	-3.53 ± 0.06	ns
		Enr	-4.15 ± 0.13	-3.88 ± 0.18	-3.66 ± 0.18			-3.45 ± 0.10	
C_P	N_R	Con	-3.77 ± 0.09	-3.67 ± 0.13	-3.69 ± 0.13	ns	ns	-3.88 ± 0.12	ns
		Enr	-3.75 ± 0.14	-3.62 ± 0.20	-3.68 ± 0.20			-3.69 ± 0.22	
N_{SH}	N_R	Con	-0.62 ± 0.13	-1.34 ± 0.14	-1.55 ± 0.13	***	ns	-1.93 ± 0.05	ns
		Enr	-0.33 ± 0.19	-1.13 ± 0.20	-1.50± 0.20			-1.89 ± 0.08	
N_P	N_L	Con	-0.68 ± 0.04	-0.50 ± 0.04	-0.46 ± 0.04	ns	ns	-0.43 ± 0.02	ns
		Enr	-0.83 ± 0.05	-0.57 ± 0.06	-0.49 ± 0.06			-0.42 ± 0.03	
N_P	N_S	Con	-1.84 ± 0.07	-1.70 ± 0.07	-1.66 ± 0.07	ns	ns	-1.53 ± 0.03	ns
		Enr	-1.79 ± 0.10	-1.66 ± 0.11	-1.60 ± 0.11			-1.55 ± 0.05	
N_P	N_R	Con	-1.08 ± 0.08	-1.58 ± 0.08	-1.74 ± 0.08	***	-	-2.08 ± 0.07	ns
		Enr	-0.92 ± 0.11	-1.42 ± 0.12	-1.70 ± 0.12			-2.06 ± 0.12	

Table 5.6 Allometric relationships involving leaf of root surface area. Summary of values of the allometric constant (k) for the full model, where C and N are the total C and N contents of the plant ($_P$), leaf ($_L$), sheath ($_S$), or root ($_R$), and A is the leaf ($_L$) or root ($_R$) surface area. Mean is followed by the standard error. Probabilities (Prob.) are those for model improvement when the term is included in the model by stepwise-multiple linear regression, $P<0.001$ and $P<0.01$ represented by *** and ** respectively, ns represents non-significance and nd is not determined. There were no significant interactions between CO_2 and N level. Hoagland shows k for the pots supplied with Hoagland solution and P the probability of a CO_2 effect on those plants.

			N supply rate			Prob.			
x	**y**	**CO_2**	**Low**	**Mid**	**High**	**CO_2**	**N**	**Hoagland**	***P***
C_P	A_L	Con	0.68 ± 0.03	0.80 ± 0.04	0.86 ± 0.03	ns	***	0.72 ± 0.02	ns
		Enr	0.69 ± 0.04	0.81 ± 0.05	0.82 ± 0.05			0.74 ± 0.03	
C_P	A_R	Con	0.82 ± 0.04	0.83 ± 0.05	0.78 ± 0.05	ns	ns	nd	
		Enr	0.86 ± 0.06	0.87 ± 0.07	0.80 ± 0.07			nd	
A_L	A_R	Con	1.19 ± 0.07	1.03 ± 0.08	0.90 ± 0.08	ns	***	nd	
		Enr	1.24 ± 0.10	1.08 ± 0.11	0.89 ± 0.11			nd	
A_L	N_L	Con	0.94 ± 0.05	0.96 ± 0.06	1.05 ± 0.06	***	**	1.04 ± 0.01	*
		Enr	0.86 ± 0.07	0.90 ± 0.08	0.99 ± 0.08			0.99 ± 0.02	
A_L	C_L	Con	0.77 ± 0.03	0.84 ± 0.04	0.88 ± 0.04	ns	ns	0.88 ± 0.02	ns
		Enr	0.79 ± 0.05	0.85 ± 0.06	0.84 ± 0.06			0.85 ± 0.03	
A_R	C_R	Con	0.70 ± 0.03	0.76 ± 0.04	0.76 ± 0.04	ns	ns	-	
		Enr	0.72 ± 0.05	0.80 ± 0.06	0.79 ± 0.06			-	

Table 5.7 Allometric relationships involving leaf of root surface area. Summary of values of the intercept (*b*) of the allometric relationship for the full model, where C and N are the total C and N contents of the plant ($_P$), leaf ($_L$), sheath ($_S$), or root ($_R$), and A is the leaf ($_L$) or root ($_R$) surface area. Mean is followed by the standard error. Probabilities (Prob.) are those for model improvement when the term is included in the model by stepwise-multiple linear regression, P<0.001 and P<0.05 represented by *** and * respectively, ns represents non-significance, nd not determined and - shows values which may have changed due to differences in the allometric constant. There were no significant interactions between CO_2 and N level. Hoagland shows *b* for the pots supplied with Hoagland solution and *P* the probability of a CO_2 effect on those plants.

			N supply rate			Prob.			
x	**y**	**CO_2**	**Low**	**Mid**	**High**	**CO_2**	**N**	**Hoagland**	***P***
C_P	A_L	Con	-0.61 ± 0.05	-0.50 ± 0.07	-0.50 ± 0.07	***	-	0.27 ± 0.06	*
		Enr	-0.83 ± 0.08	-0.70 ± 0.11	-0.52 ± 0.11			0.05 ± 0.10	
C_P	A_R	Con	1.75 ± 0.07	1.47 ± 0.10	1.50 ± 0.10	ns	ns	nd	
		Enr	1.66 ± 0.11	1.32 ± 0.16	1.37 ± 0.15			nd	
A_L	A_R	Con	2.48 ± 0.05	2.00 ± 0.08	1.96 ± 0.08	***	ns	nd	
		Enr	2.71 ± 0.07	2.09 ± 0.13	1.87 ± 0.12			nd	
A_L	N_L	Con	-2.35 ± 0.04	-2.00 ± 0.06	-1.95 ± 0.06	-	-	-1.86 ± 0.03	-
		Enr	-2.40 ± 0.05	-2.00 ± 0.09	-1.90 ± 0.09			-1.63 ± 0.06	
A_L	C_L	Con	-0.16 ± 0.04	-0.08 ± 0.06	-0.07 ± 0.06	ns	ns	0.03 ± 0.05	ns
		Enr	-0.38 ± 0.06	-0.27 ± 0.09	-0.10 ± 0.09			-0.09 ± 0.08	
A_R	C_R	Con	2.63 ± 0.04	2.66 ± 0.06	2.73 ± 0.05	ns	ns	-	
		Enr	2.61 ± 0.06	2.53 ± 0.09	2.61 ± 0.08			-	

Table 5.8 Specific surface areas of leaf and root averaged over harvest, expressed on a carbon mass basis. $P<0.001$ represented by ***, not significant by ns, and not determined by nd.

	Nitrogen level			Probabilities			LSD $P<0.05$		
	Low	**Mid**	**High**	**CO_2**	**N**	**C*N**	**C*N**	**Hoagland**	***P***
Specific leaf area cm^{-2} leaf area g^{-1} C									
Con CO_2	0.73	0.73	0.77	***	***	ns	0.04	0.84	***
Enr CO_2	0.56	0.59	0.65					0.65	
Specific root surface area cm^{-2} root surface area g^{-1} C									
Con CO_2	3.99	3.96	4.37	***	***	ns	0.33	nd	
Enr CO_2	3.55	3.23	3.56					nd	

Table 5.9 Relative growth rates, derived for the period day 0 to 16, calculated (instantaneous) for the period between day 16 to 37 using HPCURVES (Hunt,R & Parsons, 1974), and derived for the period day 16 to 37. Derived RGR is the average RGR for day 0-16 based on 0.70 mg seeds with an assumed carbon concentration of 40%. There were no significant effects of CO_2 enrichment on measured RGR (*t*-test, n = 3). Derived RGR average for day 16-37 disregarding other harvests. Instantaneous RGR calculated as;

$$RGR = \frac{dC_P}{dt}$$

Average relative growth rate ($\overline{RGR}$) calculated as;

$$\overline{RGR} = \frac{\ln(C_{Pt2}) - \ln(C_{Pt1})}{t_2 - t_1}$$

		Derived $\overline{RGR}$ day 0-16	**Measured RGR day 16-37**	**Derived $\overline{RGR}$ day 16-37**
			g C g^{-1} C d^{-1}	
Low-N	Con	0.097	0.106 ± 0.009	0.111
	Enr	0.121	0.101 ± 0.008	0.102
	$R_{e/c}$	*1.25*	*0.95*	*0.92*
Mid-N	Con	0.110	0.147 ± 0.008	0.147
	Enr	0.132	0.144 ± 0.008	0.147
	$R_{e/c}$	*1.20*	*0.98*	*1.00*
High-N	Con	0.109	0.159 ± 0.005	0.160
	Enr	0.130	0.178 ± 0.007	0.181
	$R_{e/c}$	*1.19*	*1.12*	*1.13*
Hoagland	Con	0.119	0.180 ± 0.015	0.182
	Enr	0.142	0.193 ± 0.011	0.192
	$R_{e/c}$	*1.19*	*1.07*	*1.06*

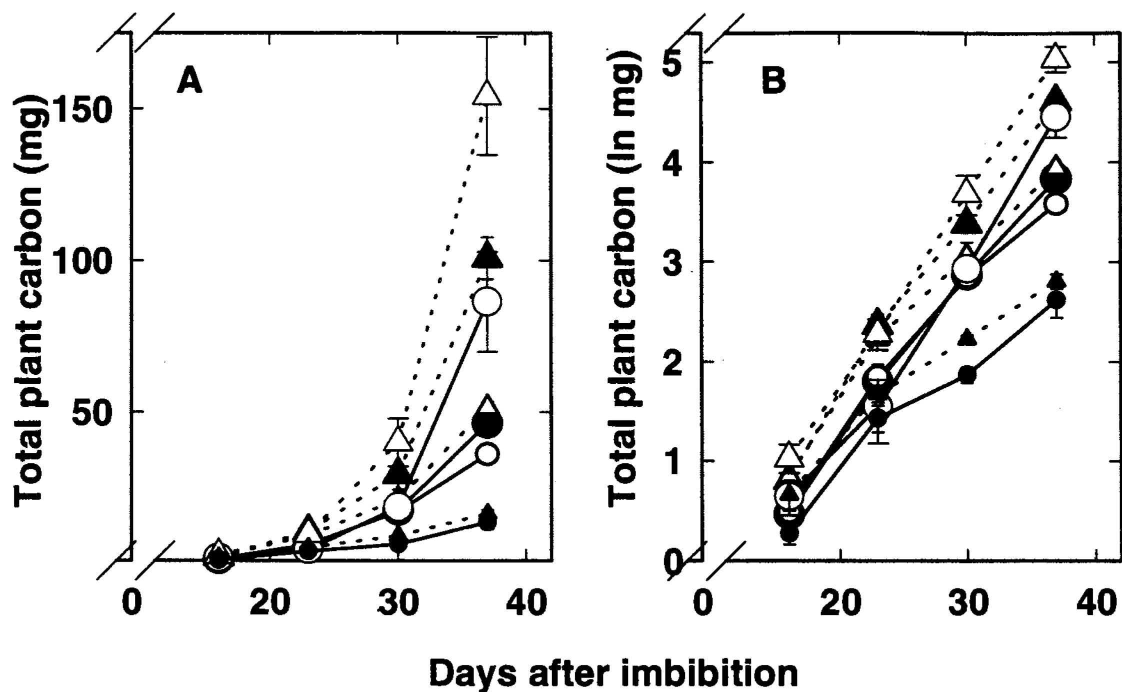

Figure 5.1 Accumulation of total plant carbon over the 37 days of the experiment. (A) Untransformed, (B) natural log transformed. (●) Control low-N, (○) mid-N, (⬤) high-N, (◯) Hoagland with solid line. (▲) Enriched low-N, (△) mid-N, (▲) high-N, (△) Hoagland with dotted line. Error bars are ± one standard error, which may be concealed by the symbol.

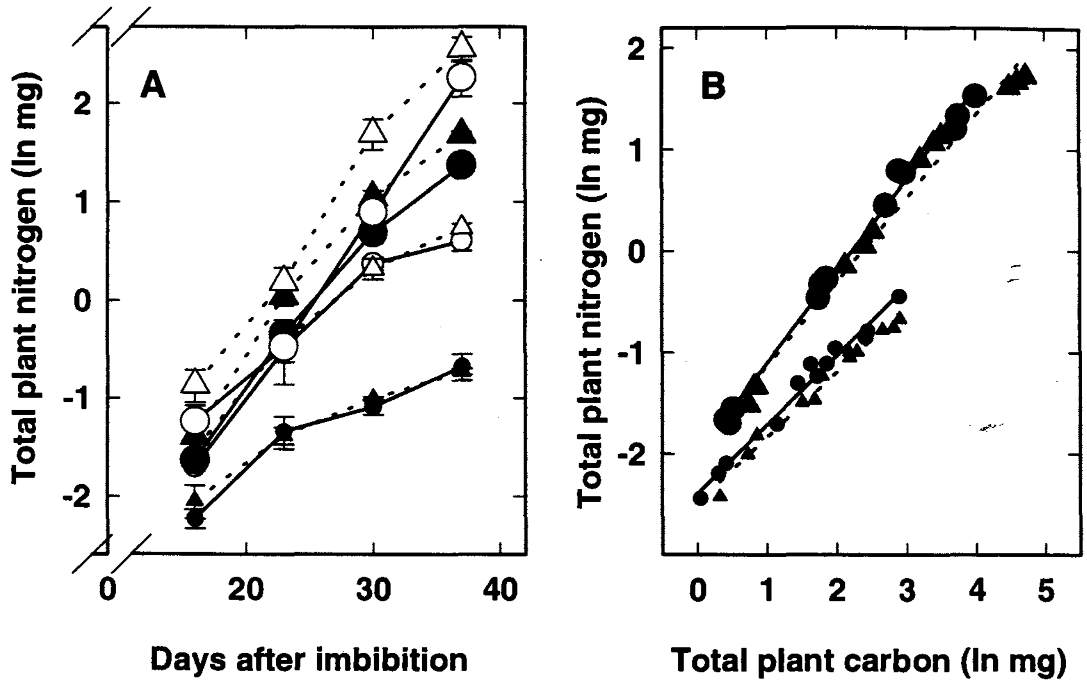

Figure 5.2 Total plant nitrogen dynamics. (A) Accumulation over the 37 days of the experiment, natural log transformed. (B) Allometric relationship between total plant nitrogen and total plant carbon. (●) Control low-N, (○) mid-N, (⬤) high-N, (◯) Hoagland with solid line. (▲) Enriched low-N, (△) mid-N, (▲) high-N, (△) Hoagland with dotted line. Error bars are ± one standard error on (A), which may be concealed by the symbol. In (B) lines are linear regressions for each CO_2 x N treatment, for clarity only low- and high-N treatments included.

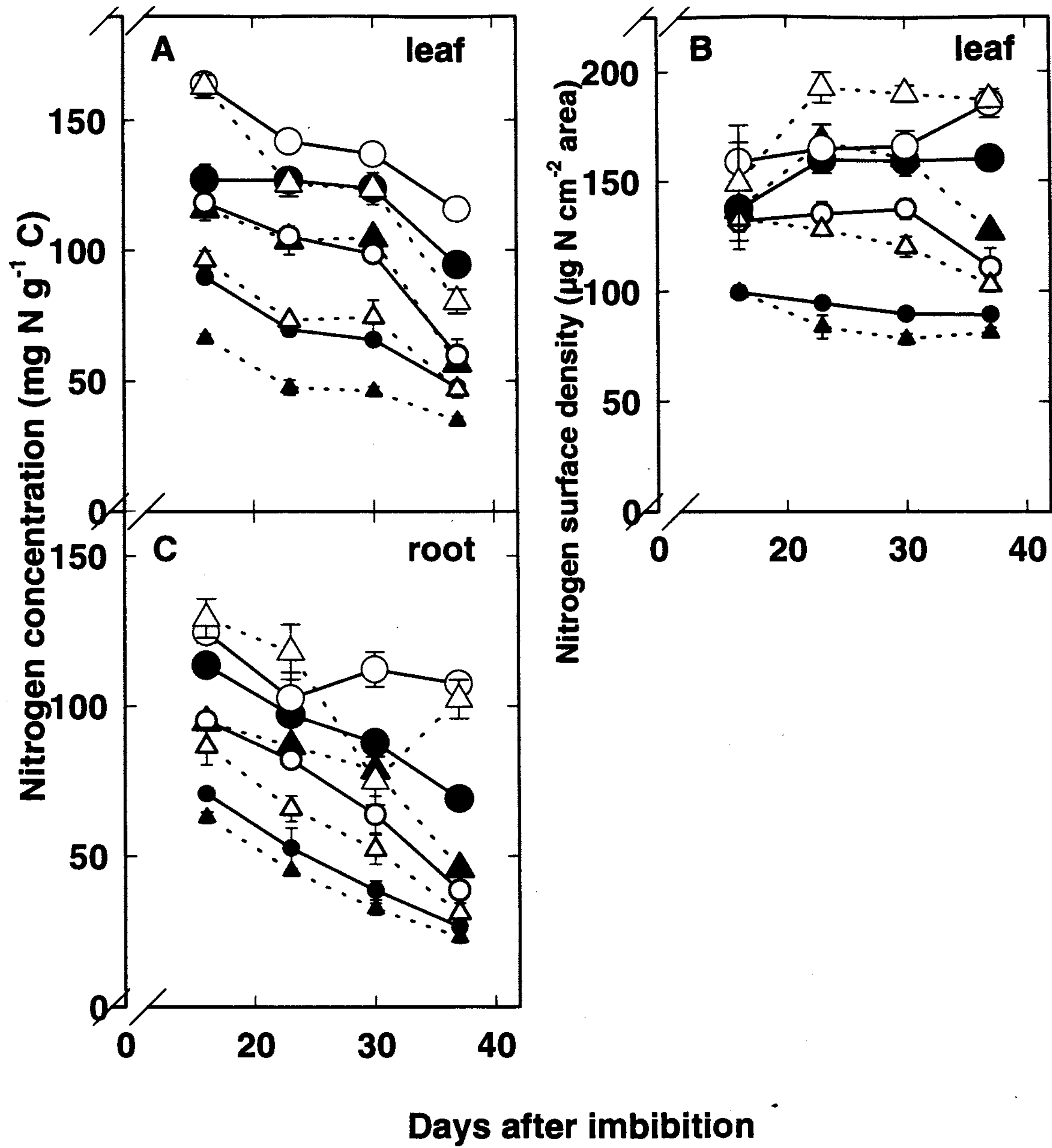

Figure 5.3 Nitrogen parameters. (A) Leaf nitrogen concentration, (B) leaf nitrogen surface density, (C) root nitrogen concentration. (●) Control low-N, (○) mid-N, (●) high-N, (○) Hoagland with solid line. (▲) Enriched low-N, (△) mid-N, (▲) high-N, (△) Hoagland with dotted line. Error bars are ± one standard error, which may be concealed by the symbol.

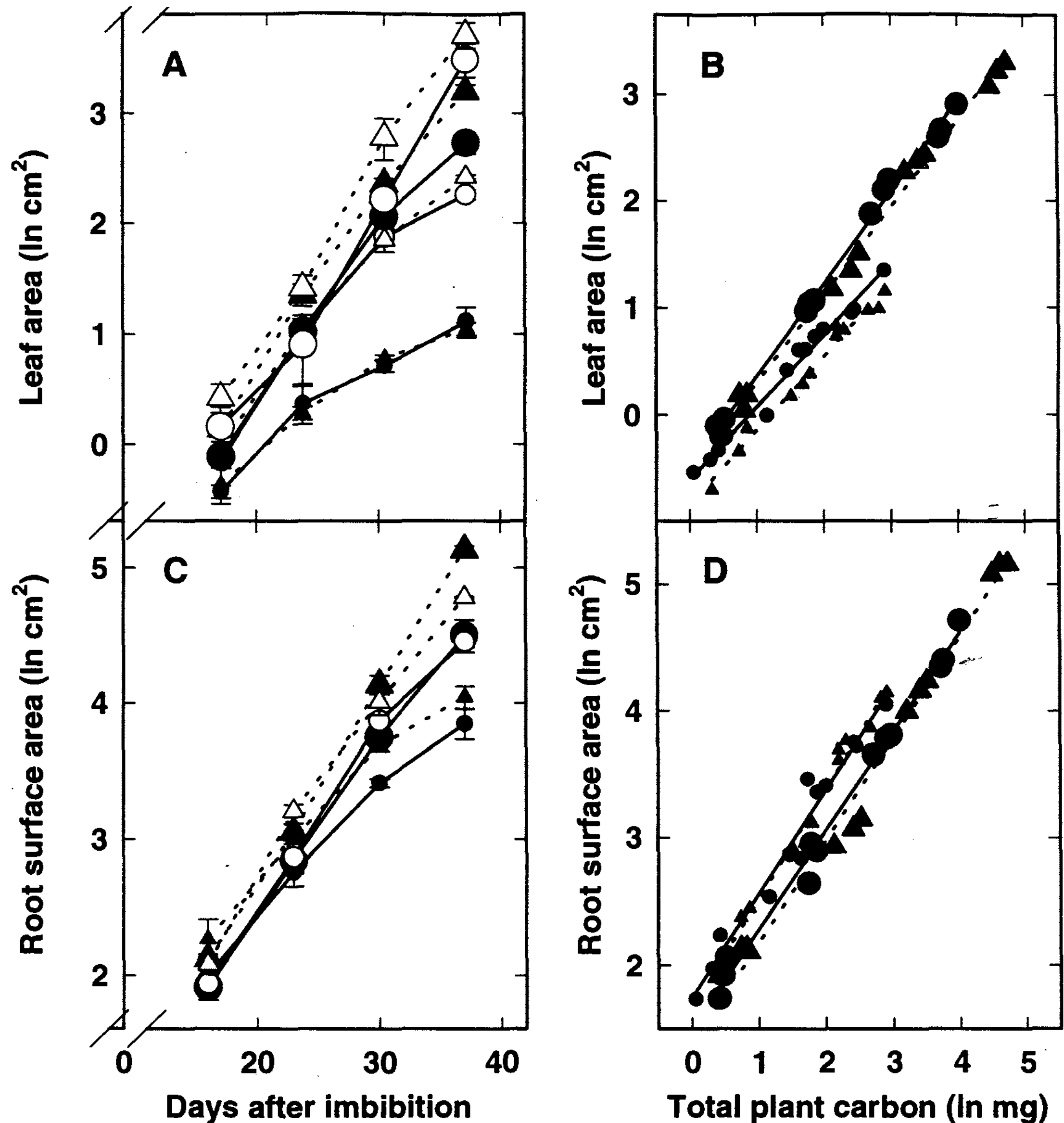

Figure 5.4 Surface area development and allometric relationships over the 37 days of the experiment. (A) Leaf area development, natural log transformed. (B) Allometric relationship between leaf area and total plant carbon. (C) Root surface area development, natural log transformed. (D) Allometric relationship between root surface area and total plant carbon. (●) Control low-N, (○) mid-N, (⬤) high-N, (◯) Hoagland with solid line. (▲) Enriched low-N, (△) mid-N, (▲) high-N, (△) Hoagland with dotted line. In (B) and (C) lines are linear regressions for each CO_2 x N treatment. Error bars are ± one standard error on (A) and (C), which may be concealed by the symbol. Hoagland experiment excluded from (B) for clarity. Root surface area not determined for Hoagland treatment.

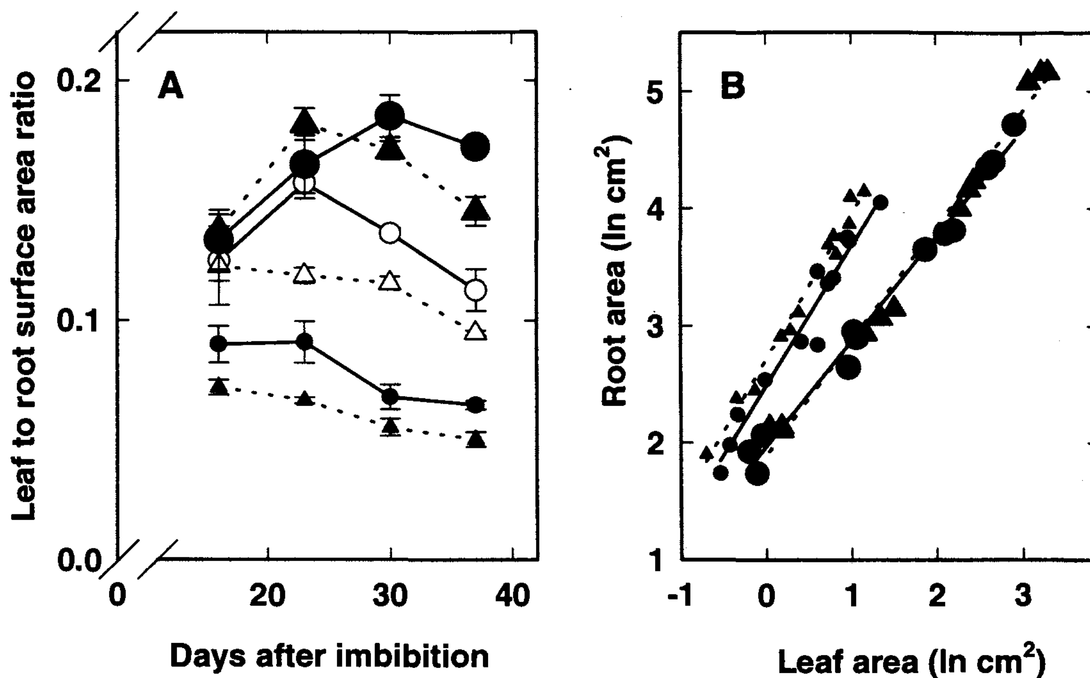

Figure 5.5 Leaf area to root surface area relationships. (A) Ratio of leaf area to root surface area. (B) Allometric relationship between root surface area and leaf area. (●) Control low-N, (○) mid-N, (⬤) high-N with solid line. (▲) Enriched low-N, (△) mid-N, (▲) high-N with dotted line. Error bars are ± one standard error, which may be concealed by the symbol. Root surface area not determined in Hoagland treatment. The allometric relationship of (B) may not be linear at high-N. The statistical model explained 98% of the variance, and it is evident that the conclusion of little effect at high N would not be altered if an alternate model could be justified.

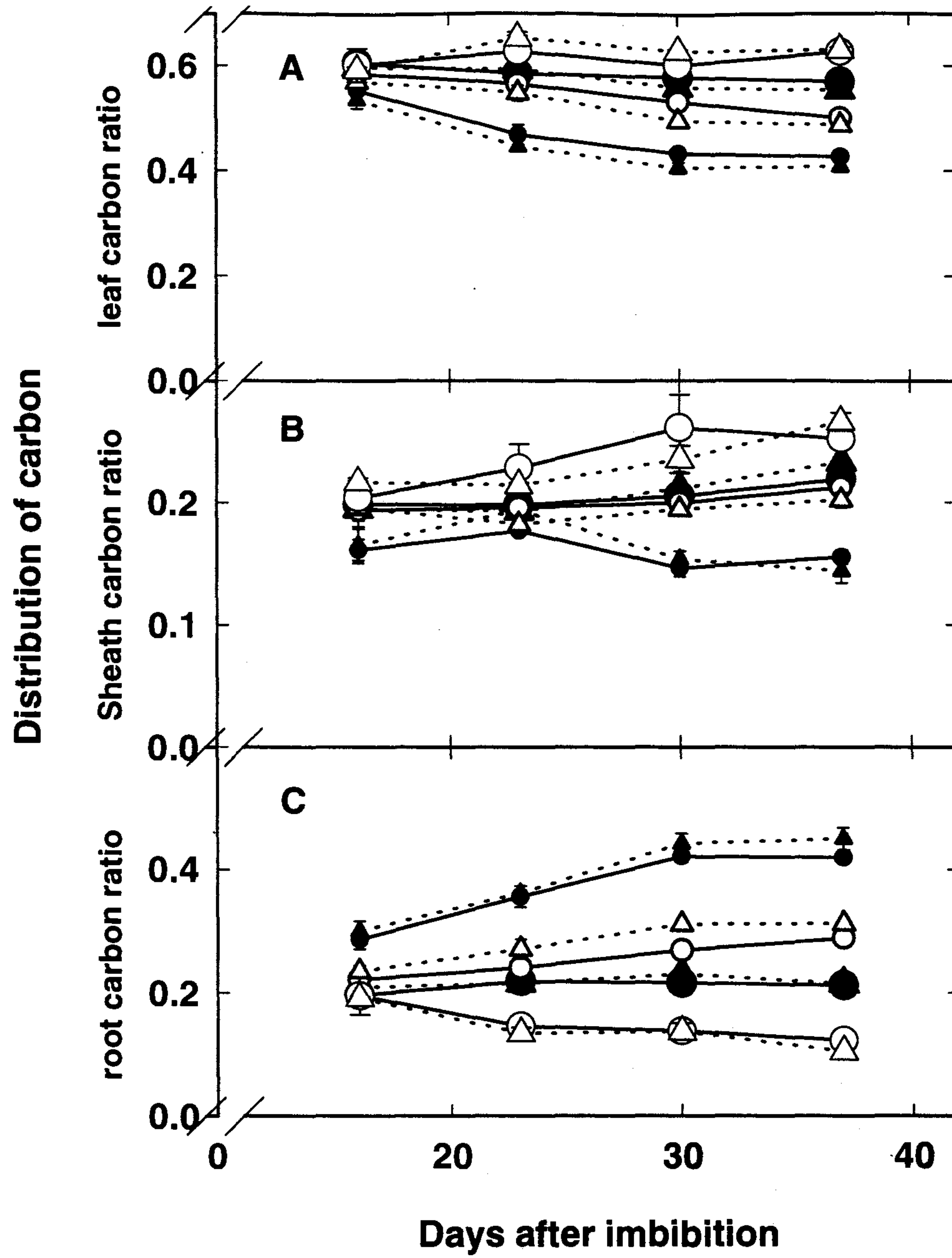

Figure 5.6 Distribution of carbon within the plant with relation to time (A) Leaf carbon ratio, (B) sheath carbon ratio, (C) root carbon ratio. (●) Control low-N, (○) mid-N, (●) high-N, (○) Hoagland with solid line. (▲) Enriched low-N, (△) mid-N, (▲) high-N, (△) Hoagland with dotted line. Error bars are ± one standard error, which may be smaller than the symbol.

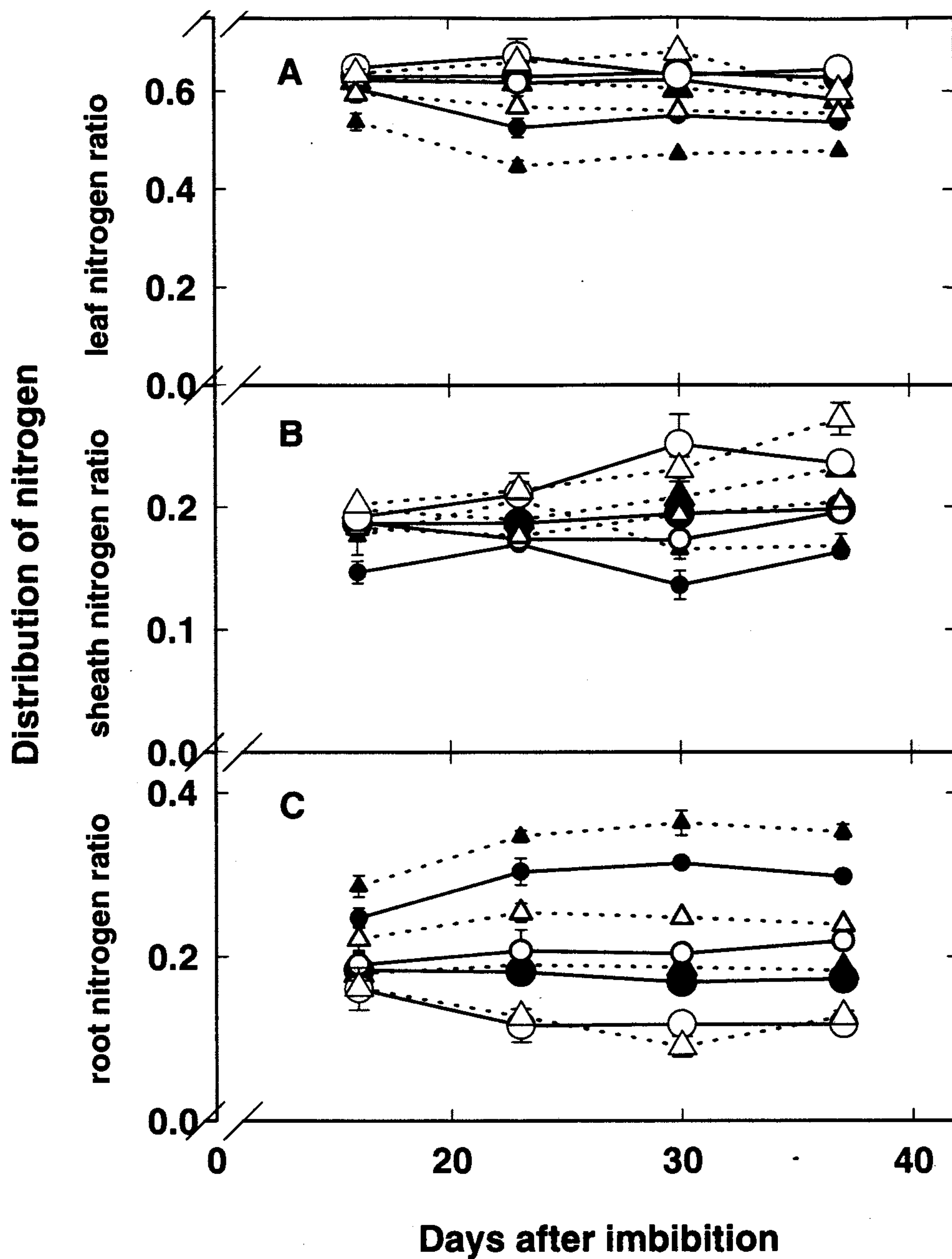

Figure 5.7 Distribution of nitrogen within the plant in relation to time (A) Leaf nitrogen ratio, (B) Sheath nitrogen ratio, (C) root nitrogen ratio. (●) Control low-N, (○) mid-N, (⬤) high-N, (◯) Hoagland with solid line. (▲) Enriched low-N, (△) mid-N, (▲) high-N, (△) Hoagland with dotted line. Error bars are ± one standard error, which may be smaller than the symbol.

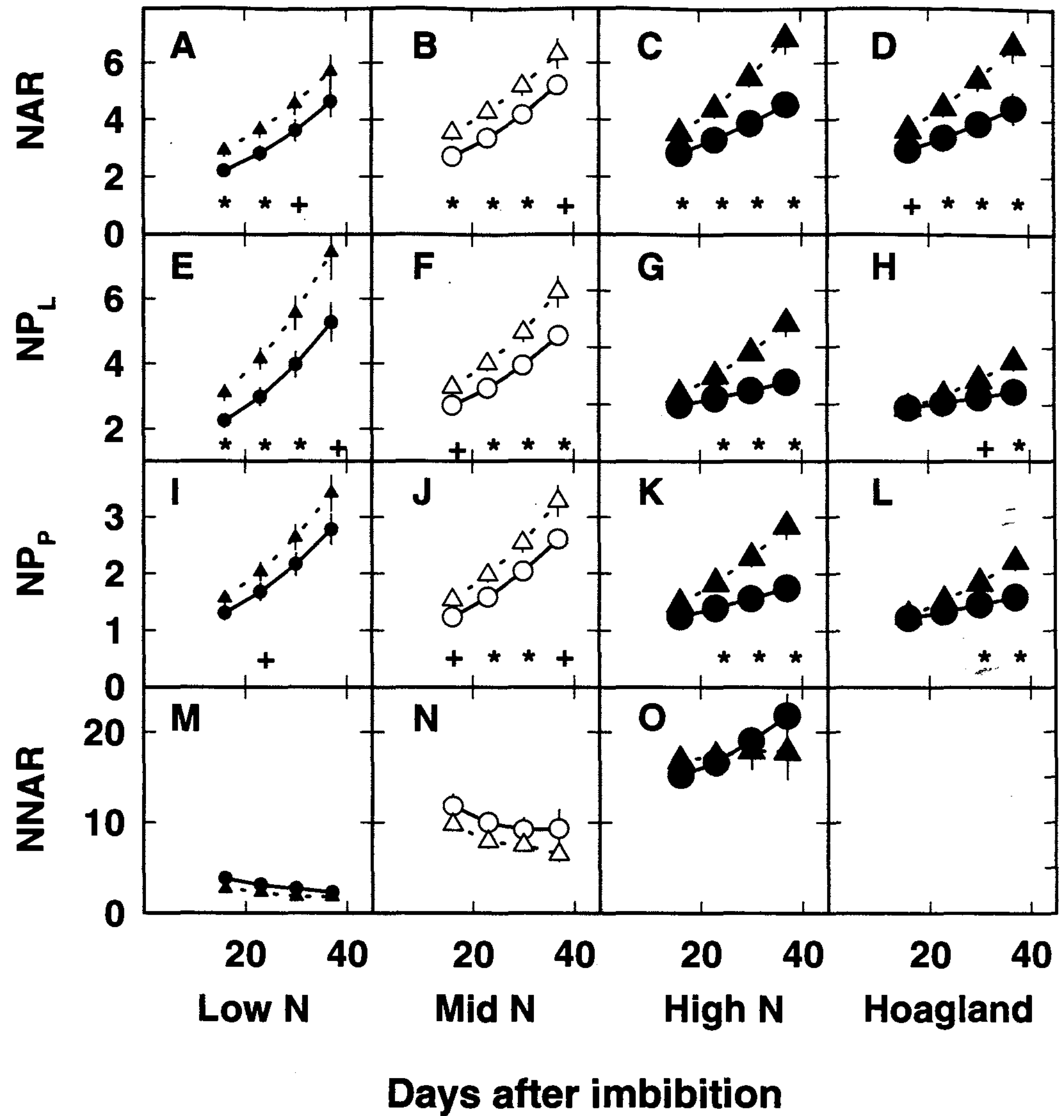

Figure 5.8 Functional growth analysis parameters calculated with HPCURVES (Hunt & Parsons, 1974) showing variation with time. Net assimilation rate (NAR; A, B, C, D; g C m^{-2} leaf-A d^{-1}), leaf nitrogen productivity (NP_L; E, F, G, H; g C g^{-1} leaf-N d^{-1}), plant nitrogen productivity (NP_P; I, J, K, L; g C g^{-1} plant-N d^{-1}), net nitrogen absorption rate (NNAR; M, N, O; mg N m^{-2} root-A d^{-1}) for the low-, mid-, high-N and Hoagland treatments. NNAR was not calculated for the Hoagland treatment. Control, circles with solid line, enriched, triangles with dotted line. Net nitrogen absorption rate not determined for Hoagland grown plants. + $P<0.1$, * $P<0.05$ by *t*-test. Error bars show one standard error of the parameter estimation and may be concealed by the symbol.

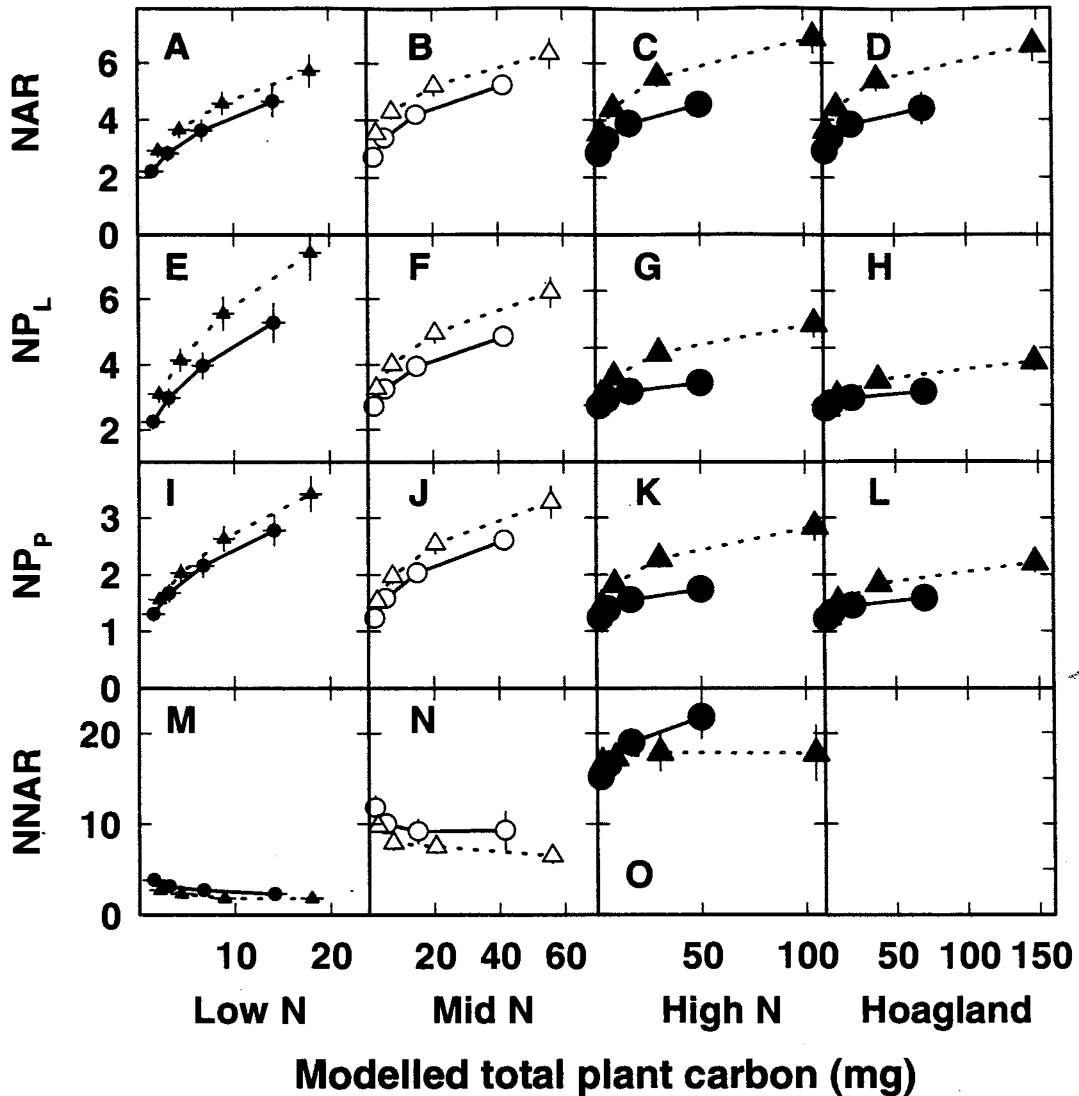

Figure 5.9 Functional growth analysis parameters calculated using HPCURVES (Hunt & Parsons, 1974) showing variation with total plant carbon. Net assimilation rate (NAR; A, B, C, D; g C m^{-2} leaf-A d^{-1}), leaf nitrogen productivity (NP_L; E, F, G, H; g C g^{-1} leaf-N d^{-1}), plant nitrogen productivity (NP_P; I, J, K, L; g C g^{-1} plant-N d^{-1}), net nitrogen absorption rate (NNAR; M, N, O; mg N m^{-2} root-A d^{-1}) for the low-, mid-, high-N and Hoagland treatments. NNAR was not calculated for the Hoagland treatment. Control, circles with solid line, enriched, triangles with dotted line. Net nitrogen absorption rate not determined for Hoagland grown plants. Each point has error bars showing one standard error for both parameter estimation and total plant carbon estimation, which may be concealed by the symbol.

Chapter 6. Carbon accumulation, distribution and water use of *D. richardsonii* swards in response to CO_2 and nitrogen supply over four years of growth

Introduction

Most plant species, when grown under favourable conditions as isolated plants show a positive growth response to CO_2 enrichment (eg. this study; Kimball, 1983). Growth at high CO_2 has been shown to increase the efficiency of use of resources, such as radiation (eg. Gifford & Morison, 1993), water (eg. Morison, 1993), and mineral nutrients such as nitrogen (eg. this study; Bowes, 1993) and phosphorous (eg. Barrett & Gifford, 1995a,b), resulting in higher levels of whole plant net carbon fixation. High atmospheric CO_2 concentrations have also been observed to reduce the effects of other limitations on plant growth, such as high temperature, salinity and atmospheric pollutants (Nicolas *et al.*, 1993; Idso & Idso, 1994).

The response of plant communities to high atmospheric CO_2 concentrations is less well known, as there are added complexities of plant/plant and plant/microbe competition for resources, and feedbacks on growth from nutrient cycling. The characterisation of community responses to CO_2 is critical to understanding the response of terrestrial ecosystems to climate change (Gifford *et al.*, 1996b), and in understanding the current imbalances in the global carbon budget (Schimel *et al.*, 1995). With these questions in mind, it is important to consider the longer term response of months to years, as opposed to days to weeks, as is commonly addressed in isolated plant studies (Gifford *et al.*, 1996a).

This and the next two chapters will address the CO_2 response of microcosms of the C_3 grass *Danthonia richardsonii* Cashmore, grown as swards, under severe nitrogen limitation, over a four year (1469 day) period of exposure to CO_2 enrichment from seed imbibition. Nitrogen was supplied to the swards throughout the experimental period. This can be conceptualised as nitrogen release from a very slow cycling pool of organic matter, of which the size and rate of turnover, and hence nitrogen release, is not affected by current plant growth at the temporal scale of this experiment. The lowest level of nitrogen application in this experiment is of the order of estimates of nitrogen deposition in parts of industrialised Europe (Gifford *et al.*, 1996c). These were monotypic swards, bypassing complexity of between species competition. Atmospheric CO_2 increase is the only environmental aspect of global change which is

examined. That is, the direct effects of elevated atmospheric CO_2 on microcosm function are under examination, not the indirect, follow-on effects of changes in temperature and rainfall.

This chapter addresses the hypothesis that *a)* elevated CO_2 will increase net carbon accumulation by the microcosms, resulting in higher levels of soil carbon, and *b)* elevated CO_2 will reduce microcosm water use, due to the "anti-transpirant" effect of CO_2, as observed in isolated plants (chapter 4). The following chapters will address the accumulation and distribution of nitrogen in the microcosm, and the effects of elevated CO_2 on decomposition and related soil parameters.

Materials and Methods

Microcosm experiment one

Soil

A yellow podzolic subsoil was collected from a pasture paddock at Ginninderra Experiment Station (CSIRO, Division of Plant Industry) which had no recorded fertiliser addition. The soil was passed through a 3 mm sieve to remove stones and large fragments of organic material, and dropped through a lateral air stream to remove root fragments. After this pre-treatment the physical composition of the soil was 39% coarse sand, 38% fine sand, 14% silt and 7% clay. Initial soil pH (1 soil:5 H_2O v/v) was 6.2 and electrical conductivity 0.01 mS cm^{-1}. Cation exchange capacity was 3.5 milliequivalents 100 g^{-1} (meq 100 g^{-1}; 1.0 N ammonium acetate) with exchangeable Ca, Mg, K, and Na of 1.90, 0.54, 0.19 and 0.03 meq 100 g^{-1} respectively, giving a base saturation of 81%[5]. Initial total carbon and total nitrogen concentrations were 0.16% and 0.023% (by mass) respectively. The soil was not sterilised.

The equivalent of 66 g m^{-2} of superphosphate (~5.9 g P m^{-2}, ~12.5 g Ca m^{-2}, ~7.3 g S m^{-2}), 63 g m^{-2} of rock phosphate (~8.5 g P m^{-2}, ~21 g Ca m^{-2}), 223 g m^{-2} of gypsum (~42 g Ca m^{-2}, ~36 g S m^{-2}) and 500 mL of a trace element mixture equivalent to 2.5 g Ca m^{-2}, 1.9 g S m^{-2}, 1.4 g Fe m^{-2}, 0.4 g Mg m^{-2}, 0.3 g Mn m^{-2}, 0.12 g Zn m^{-2}, 0.06 g Cu m^{-2}, 0.012 g B m^{-2}, and 0.006 g Mo m^{-2} were individually mixed with the soil for each microcosm using a cement mixer.

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[5] Preliminary soil physical, pH, EC and exchangeable cation analysis was carried out by Dr. Jim Beatty, CSIRO Division of Soils.

Microcosm design and sward establishment

Rectangular-section, slightly tapered plastic bins with top outer dimensions of 250 mm x 330 mm and a depth of 290 mm were used as microcosms. Holes were drilled in the bottom of both the microcosms and jar-lids which were attached to the microcosms to hold jars for leachate collection. Three layers of fabric (70% shadecloth, MARIX fine synthetic mesh, and nylon curtain fabric) were placed in the bottom of the microcosm before soil addition to minimise soil loss from the microcosm. To aid drainage 500 mL of coarse washed river sand was placed over the layers of fabric in each microcosm. Approximately 17.5 kg (dry weight) of moist soil was added, resulting in an average soil depth of 225 mm. Due to unforseen delays in construction of the automated watering system and the benches needed to support the microcosms in the glasshouses, the filled microcosms were stored outdoors under black plastic for 200 days prior to sowing. During this time they were kept moist by spraying weekly with demineralised water to avoid hard-setting problems. At sward establishment approximately 4.5 g m^{-2} of mineral nitrogen was present.

The experimental treatments were 2 CO_2 levels * 3 N levels * 8 harvests, as outlined below. The microcosms were closely packed on each of 6 benches to form swards, with 2 swards per bench ("top" or "bottom" of bench, Diagram 6-1). All microcosms forming a sward were of the same treatment, with two swards per treatment. Swards were further delineated into core and guard microcosms. Initially there were 20 microcosms per sward. Prior to bench packing 3 benches were assigned to each CO_2 treatment, and nitrogen treatments were assigned to each bench end, so that all 3 nitrogen treatments were present at each bench end in each glasshouse. In each glasshouse the 3 benches were placed side by side to form a continuous canopy. To remove any possible positioning effects the benches and their associated microcosms were rotated end for end and shifted one position to the east within each glasshouse monthly. In addition, to remove any glasshouse associated effects, benches and their associated microcosms were moved between glasshouses every three months. Glasshouse CO_2 concentrations were also switched at those times. Thus in any six month period a sward from each treatment was exposed to one month's growth in each of the possible bench locations. At each six monthly harvest the remaining microcosms were re-randomised within each sward and core - guard designation and packed to form a closed canopy. The last of the core microcosms were harvested at the 1104 day harvest. For the harvests after day 1104 the microcosm which had the greatest exposure to the edge of the sward in the preceding harvest interval was designated the guard microcosm for statistical purposes. No significant core-guard effects were noted in major

experimental parameters apart from total root nitrogen, where the core pots had 9% more root nitrogen than the guard pots, although a similar effect was not noted for root carbon ($P<0.05$).

Monospecific communities of mixed ecotypic *Danthonia richardsonii*[6] were established in their growth CO_2 concentration by seeding at 12 g of clean seed m^{-2}. Atmospheric CO_2 concentrations were nominally 360 and 720 $\mu L\, L^{-1}$ (24 hr enrichment), referred to as control and enriched, respectively (Table 6.1, Figure 6.1). For the first 7 days (day 0 was 29/5/91) the microcosms were watered daily with a fine spray of demineralised water. During this time emergence occurred and on day 8 watering with a semi-automated system which supplied known amounts solution commenced. Nitrogen, as analytical grade NH_4NO_3 was applied to the soil surface in the irrigation solution at every regular watering, to simulate the gradual release of mineral nitrogen via mineralisation. In a previous experiment *D. richardsonii* biomass production was not affected by nitrogen source (NH_4^+ or NO_3^-; Gifford & Lutze unpublished). The concentration of nitrogen in the nutrient solution was regularly modified to maintain a uniform rate of nitrogen supply which averaged 2.2, 6.7 or 19.8 g N m^{-2} yr^{-1}, hereafter low-, mid-, and high-N. Individual treatments are referred to as CLN, CMN, CHN for the control CO_2, low-, mid- and high-N treatments, and ELN, EMN, EHN for the corresponding enriched CO_2 treatments.

Water application

Irrigation solution was applied two or three times weekly, initially at 44% of potential evaporation. Initially, equal quantities of solution were supplied to all treatments. Every six weeks demineralised water was applied in abundance to return the microcosms to field capacity and to leach 6 L m^{-2}, averaged over all treatments. Nutrient solution was not added to any of the water used to induce leaching. During the first 6 months this resulted in significantly more leaching in the high CO_2 treatments than in the ambient treatments ($P<0.05$; not presented). To avoid water-logging, water application between day 191 and 380 ceased when the treatment-average volume of leachate reached 6 L m^{-2}. The leachate was collected, bulked within sward and analysed for its C and N content.

On day 492 monitoring of volumetric soil water content (θ_v) commenced using a TRASE 6050XI time domain reflectometry system (SOILMOISTURE Equipment Corp., Santa Barbara, CA). Buriable three-element wave guides were permanently pushed into each of two microcosms per sward. A wave guide was removed only when the microcosm was selected for

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[6] Seed supplied by Dr Richard Groves, CSIRO Division of Plant Industry.

harvest. It was then inserted in another microcosm of the same sward. Observations were made weekly, in the morning (starting at ~9 am EST) prior to the first watering of the week. A calibration was developed between instrumental θ_v and actual θ_v. Three of the microcosms having θ_v measurements taken were weighed at each measurement event over a 12 week drying cycle (defined below). Actual soil volume, soil mass and hence actual θ_v were calculated after harvest.

After monitoring θ_v over one 6 week watering cycle it was apparent that there were large differences in water use between treatments, supporting data on leachate volume (not presented) and spot estimates of θ_m (Table 6.2). This resulted in differing patterns of soil water depletion, with the enriched low-N treatment almost continually waterlogged and the high-N treatments almost continually droughted (Figure 6.2). It was decided that such large differentials in soil water content were not in line with experimental objectives, as differences between nitrogen treatments would be confounded with soil θ_v.

The watering strategy was changed on day 548 so that different amounts of water were supplied to each nitrogen treatment (above-ground plant nitrogen is often correlated with rainfall; Bremen & Krul, 1991), such that its enriched CO_2 treatment would dry down linearly from field capacity (~32% θ_v) to wilting point (defined as that moisture content at which the plants could not extract further moisture from the soil, ~10% θ_v) over a 12 week period (Figure 6.2). During this time the control CO_2 treatment from each nitrogen treatment was supplied with the same amount of irrigation solution as its corresponding enriched CO_2 treatment. Following this, the microcosms were re-wet with demineralised water until 6 L m^{-2} of leachate was collected for analysis from each treatment. The period from field capacity through wilting point to field capacity was defined as a *"drying cycle"*.

These changes resulted in less water being applied to the low- and mid-N treatments, and more to the high-N treatments (Table 6.3, Figure 6.2), and the nitrogen treatments within each CO_2 treatment having a similar θ_v (Figure 6.2, Figure 7.1). The average θ_v within a drying cycle was reduced at mid- and low-N, and in the CHN treatment, and increased in the EHN treatment (Table 6.4). Thus confounding of nitrogen response with θ_v was removed. However, there were still differences in θ_v between CO_2 treatments. These differences were considered internal to the system, and an integral part of the CO_2 response. Therefore this experiment is examining the integrated effects of CO_2 on carboxylation efficiency and transpiration efficiency.

On day 664 a weekly spraying of the canopy with 1.0, 1.5 or 2.0 mm (low-, mid- & high-N respectively) of demineralised water commenced. This spraying was instigated to aid decomposition of surface litter. As the watering system applied water under the surface litter the bulk of the surface litter was not exposed to water before this spraying started.

Environmental control and monitoring

The temperature and dew point targets (for environmental control see chapter 2) were set higher during the summer months than the winter months both to allow more accurate environmental control, and to accommodate other experiments in the glasshouses with plants such as maize and cotton which required high temperatures. The variations in daily average CO_2 concentration, temperature and total short-wave radiation passing through the glass are outlined in Figure 6.1. The alterations to temperature between summer and winter also allowed a more realistic relationship between radiation and temperature, although temperatures could not be lowered enough in winter to properly simulate field conditions. Temperature was controlled on a diurnal sine wave throughout the experiment, with maxima and minima 3 to 4°C above and below the daily average. Environmental conditions over the whole experimental period are summarised in Table 6.1.

Harvesting

Destructive harvests were undertaken at six-monthly intervals and were completed over 12 working days. Table 6.5 shows the harvest number, months after sowing and the date and day of experiment at the mid-point of harvest. Prior to each harvest those microcosms which were not destructively harvested were defoliated at 8 cm above soil level. Defoliated material was weighed, chopped into 5-20 mm lengths (approx.) and distributed over the same microcosm to form a surface litter layer. The surface litter layer would not have formed naturally in the absence of grazing or heavy rainfall.

Four microcosms from each treatment, one core and one guard from each sward, were destructively harvested. The whole microcosm was sub-sampled for total C and total N by dividing it into the following fractions (outlined in Diagram 6-2); green leaf from above the 8 cm defoliation height (green leaf lamina), green leaf and sheath below 8 cm (green leaf base), senesced leaf above 8 cm (senesced leaf lamina), senesced leaf and sheath below 8 cm (senesced leaf base), stem, surface litter, root and soil. Leaf area was determined on a Li-cor LI-3000 leaf area meter. All live plant fractions were freeze dried, other fractions were oven dried at 80°C.

Root and soil fractions were obtained by cutting the soil monolith into 7 layers, the depths of which are given in Diagram 6-2. Root was dry sieved from the sand layer, and the root and sand dried, weighed and ground for total C and N analysis. Each soil layer was sectioned into 32 cubes. A subsample of each layer ($^{16}/_{32}$ for 0-50 mm, $^{8}/_{32}$ for 50 mm to bottom, less other samples as outlined below) was collected on a predetermined grid pattern which varied from layer to layer to uniformly sample the microcosm (Diagram 6-3), and oven dried after its fresh weight was obtained for moisture content determination. The subsample was then finely ground for total C and N analysis. Another subsample of each layer ($^{16}/_{32}$ for 0-50 mm, $^{8}/_{32}$ for 50 mm to bottom, less other samples as outlined below) was collected, its fresh weight determined and root obtained by washing. Root samples were freeze dried. The remaining soil in the lower layers was oven dried to determine the total mass of each layer for total microcosm C and N calculations. Root mass and total below-ground C and N were determined assuming uniform distribution of root and C and N throughout the soil layer. Root-free total soil C and N contents were determined as the difference between the total C or N content in the soil plus root sample, after conversion to a total layer basis, and that in the washed root sample, after conversion to a total layer basis. At the 191 day harvest leaf lamina and leaf base were not separated, and a different method of soil harvest was employed. Root was obtained from the soil by dry sieving the entire layer and analysing the root and root-free soil obtained for total C and N.

Small soil samples were obtained from each of the soil layers (not sand) at each harvest for pH determination (1 soil:5 H_2O v/v). These sample were collected from the soil remaining after subsampling for the layers below 50 mm, and from the root subsample in the 0-50 mm layers. Collection of soil subsamples ($^{4}/_{32}$) commenced at the harvest on day 922 for microbial biomass determinations, on a predefined grid from the non-subsampled soil of the 50-100 mm layer, and equally from each subsample (root / total C & N) of the 0-50 mm layers (Diagram 6-3). Samples from the 50-100 mm layer were not collected at day 922. Commencing at the harvest on day 1285 these samples were also used for potentially mineralisable nitrogen assays. Root from these samples was assayed for arbuscular mycorrhizal infection at day 1285. At harvests on day 1285 and 1469 litter samples were collected for microbial biomass and potentially mineralisable nitrogen determinations.

Calculation of total plant-soil system carbon and nitrogen

Carbon and nitrogen concentrations in each fraction were determined as outlined in chapter 2. Total microcosm C and N was calculated as the sum of the total C and N content of each

fraction, the stem, green and senesced leaf lamina and base, surface litter, soil and root of each soil layer, root in the sand layer, and sand.

Calculation of total plant-soil system carbon and nitrogen increment from sowing

The total increment in microcosm C and N was calculated by subtraction of initial microcosm C and N from microcosm C and N at harvest. Initial soil C and N were obtained from initial soil C and N concentrations and root-free soil dry weight at harvest.

Statistical analysis

Data were analysed with GENSTAT 5.3 by ANOVA (Genstat 5 Committee, 1993) (probability levels *P*) as a split plot design with benches ends as blocks, N treatments as main plots, and exposure (core/guard) as the split plot. Exposure was also included as a treatment effect. Protected least significant difference tests (probability levels P_L) were performed where appropriate. Regression analysis, analysis of covariance and *t*-tests (P_t) were undertaken as necessary. Natural logarithm transformations were performed on data when needed to remove heteroscedasticity.

Microcosm experiment two

On May 24 1993, day 726 of microcosm experiment one, another set of microcosms were established. These were identical to those of the core experiment, and were watered with the second, or final watering strategy from establishment. These microcosms were packed onto the benches as guard microcosms for the core experiment, and thus were all exposed to the sward edge. On day 493 of this experiment, day 1219 of microcosm experiment one, these microcosms were harvested using the same methodology as used in the core experiment. A summary of environmental conditions is given in Appendix 4. These results are only discussed where pertinent. A summary of the results of this experiment is given in the appendices (Appendix 5, Appendix 6 & Appendix 7).

Results

Results pertain to microcosm experiment one only. Not all significant treatment effects and interactions will be discussed. Most 3 way interactions between CO_2, N level and harvest are not described in the text, and were generally indicative of the CO_2 effect developing over time. Significant differences between CO_2 treatments within N level and harvest are marked on the graphs when 3 way interactions were present. Complete tables of significance from ANOVA analysis for LAI and major total carbon fractions included in the appendices (Appendix 1).

Total system carbon increment

Growth at high CO_2 increased total system carbon gain when averaged over harvest and nitrogen supply rate (Figure 6.3; $P<0.001$) with an average enhancement ratio, $R_{e/c}$ of 1.25 . The CO_2 effect interacted with N supply ($P<0.05$). The CO_2 effect was significant at all rates of N supply ($P_L<0.001$), the $R_{e/c}$ being 1.15, 1.17 and 1.34 for the low-, mid- and high-N supply rates respectively when averaged over harvest. There was a significant interaction between harvest and N supply ($P<0.001$), with only a small effect of N level on carbon increment on day 191, owing to the large initial flush of mineralised N.

Multiple linear regression (Figure 6.3) showed that the absolute rate of net carbon accumulation was increased by CO_2 enrichment ($P<0.001$). The CO_2 effect on the absolute rate of net carbon accumulation increased as N supply increased ($P<0.001$; $R_{e/c}$=1.06, 1.11 & 1.32 for low-, mid- & high-N respectively), and the CO_2 effect on the rate of carbon accumulation was significant at all N levels ($P_t<0.01$).

Leaf area

Growth at high CO_2 *reduced* total leaf area index (LAI) at harvest when averaged over all other treatments with an $R_{e/c}$ of 0.91 (Figure 6.4; $P<0.05$). There was no significant interaction between CO_2 and N, although there was little effect of CO_2 at low N.

Carbon content of fractions

Live shoot carbon

The response to CO_2 of green leaf carbon per unit ground area at harvest varied with time ($P<0.001$; Figure 6.5). Initially growth at high CO_2 increased green leaf carbon per unit ground area at harvest when averaged over N supply rates ($P_L<0.01$), although this effect was not evident after the 554 day harvest.

Non-structural carbohydrates

Non-structural carbohydrate (NSC) concentrations (Figure 6.6) were determined on green leaf at day 191, 380 (control low-N not analysed due to small sample size), 922 and 1104. NSC concentration was increased by CO_2 enrichment ($P<0.01$). There was an interaction between harvest and CO_2 level on total green NSC concentration ($P<0.01$). The CO_2 effect was significant at all harvests ($P_L<0.05$), although the response at day 922 was greater than that at day 1104 ($R_{e/c}$=2.03 & 1.27 respectively, averaged over N levels), with an intermediate response at day 380 ($R_{e/c}$=1.72, av mid- & high-N only).

Structural green leaf carbon was determined on the samples which underwent analysis for NSC concentration. There was an interaction between N, harvest and CO_2 on structural green leaf carbon (P<0.05), with the only significant CO_2 effect an increase at high-N at day 191 (P_L<0.05).

Senesced leaf carbon

Growth at high CO_2 increased standing senesced leaf carbon per unit ground area at harvest when averaged over all treatments with an $R_{e/c}$ of 1.31 (Figure 6.7; P<0.001). Over time, accumulation of total senesced leaf carbon has slowed when averaged over all other treatments, and there was no statistical increase in total senesced carbon after day 1104 (P_L<0.05). The response of the different N levels to harvest differed (P<0.001), with greater stabilisation of total senesced carbon at low- and high-N than at mid-N. Total senesced leaf carbon continued to increase in the enriched high-N treatment, although there were no significant interactions of CO_2 level with harvest or N supply rate.

Leaf turnover

The ratio of senesced leaf lamina carbon to green leaf lamina carbon at harvest, an index of leaf turnover, is shown in Figure 6.8. Growth at high CO_2 increased this ratio ($R_{e/c}$=1.21; P<0.001). As N supply rate was increased the ratio decreased (P<0.05), which was largely accounted for by high values at low-N at days 380 and 554 (P_L<0.001).

Surface litter carbon

Surface litter carbon recovered at harvest was increased by growth at high CO_2 (Figure 6.9; P<0.01). The CO_2 effect had no interaction with harvest or N supply rate. There was a significant interaction between harvest and N level (P<0.001). Accumulation of carbon in this pool ceased after day 922 at low-N, and day 1104 at mid-N, while at high N carbon accumulation continued throughout the experiment.

Root carbon

The CO_2 effect on root carbon was dependant on the rate of N supply when averaged over harvest (Figure 6.10; P<0.001). At low- and mid-N, root carbon was decreased by CO_2 enrichment ($R_{e/c}$=0.87 and 0.84 respectively; P_L<0.01) while at high-N, root carbon was increased by growth at high CO_2 ($R_{e/c}$=1.30; P_L<0.001).

Root non-structural carbohydrate concentrations were not affected by CO_2 or N treatment at the 922 and 1104 day harvests, although NSC concentrations were higher at day 922 than day 1104 (23.7 & 18.4 mg NSC-C g^{-1} structural C respectively; P<0.05).

Distribution of plant carbon - Apparent root carbon ratio

The effect of growth at high CO_2 on the apparent proportion of total live plant carbon present in root (apparent root carbon ratio, RCR_A; Figure 6.11) was dependant on N supply rate (P<0.01). At low-N growth at high CO_2 reduced RCR_A when averaged over harvest ($R_{e/c}$=0.95; P_L<0.001). At mid-N growth at high CO_2 also reduced RCR_A when averaged over harvest ($R_{e/c}$=0.94; P_L<0.001). At the high rate of N supply growth at high CO_2 increased RCR_A when averaged over harvest ($R_{e/c}$=1.05; P_L<0.01). As N supply rate increased RCR_A decreased (P<0.001), and RCR_A tended to increase with time (P<0.001). There was also an interaction between CO_2 level, N level and harvest (Figure 6.11; P<0.01).

Root-free soil carbon

The size of the soil carbon pool changed with time (P<0.001). After the accumulation of soil carbon in the first 191 days (P_L<0.05) soil carbon declined until day 753 (P_L<0.001), after which it increased to day 1104 (P_L<0.001). When averaged over all treatments growth at high CO_2 increased soil carbon content (Figure 6.12; P<0.001) with an $R_{e/c}$ of 1.10. There were interactions between CO_2 level and N level (P<0.001). When averaged over harvest soil nitrogen content was increased by growth at high CO_2 at all N levels (P_L<0.05) and the increase was greater as the rate of N supply increased ($R_{e/c}$ of 1.04, 1.09 and 1.17 for the low-, mid- and high-N supply rates respectively). When expressed as gain of carbon in the soil pool above the level present at sowing, the average $R_{e/c}$ was 1.15, 1.32 and 1.57 for the low-, mid- and high-N treatments respectively. There was an interaction between CO_2, N level and harvest (Figure 6.12; P<0.001).

Leachate carbon loss

Loss of carbon in leachate was determined between days 753 and 1469 and averaged 320 mg C m^{-2} yr^{-1}. Loss increased as N supply rate increased from 236 mg C m^{-2} yr^{-1} at low-N to 452 mg C m^{-2} yr^{-1} at high-N (P<0.001). There was an interaction between N supply rate and CO_2 level (P<0.01). At low- and mid-N leachate carbon loss was lower at high CO_2 ($R_{e/c}$=0.74, P_L<0.05 & $R_{e/c}$=0.81, P_L<0.10 respectively) and at high-N leachate carbon loss was higher at high CO_2 ($R_{e/c}$=1.28; P_L<0.01). Harvest effects were disregarded as the method used for carbon determination was changed from a wet chromic acid digestion to a combustion method at day 1104 (chapter 2). As the annual leachate carbon loss was less than 0.5% of the net yearly increment in total system carbon (Figure 6.3) these losses were considered insignificant.

Microcosm water use

The change in watering strategy on day 548 resulted in similar applications of water to the high-N treatments and lower applications to the low- and mid-N treatments when summed over harvest intervals (Table 6.3). Before this change, the mid- and low-N treatments did not dry to wilting point (eg. Figure 6.2). The change in soil water conditions had the largest impact on the enriched low-N treatment, which was no longer continuously close to waterlogged, and the enriched high-N treatment, which had an increase in average θ_v (Table 6.4).

Growth at high CO_2 reduced rates of water use for the initial 25 days after θ_v had been returned to field capacity with an $R_{e/c}$ of 0.75 (Figure 6.13; $P<0.001$). As N supply rate increased the rate of water use increased ($P<0.001$) while the CO_2 effect was statistically consistent across N treatments. Rates of water use were 28% higher ($P<0.001$) and the CO_2 effect was greater at the second event within each harvest period ($R_{e/c}$=0.78 & 0.73 for event 1 & 2 respectively; $P<0.05$), where the canopy was fully developed. The response to N supply was also greater at the second event ($P<0.001$).

Discussion

Total microcosm carbon gain was increased by CO_2 enrichment. This increase was achieved without increases in leaf area index (LAI) or total live leaf carbon. Senesced standing leaf carbon was increased, implying a greater turnover of leaf at high CO_2. More carbon was found in the surface litter layer at high CO_2. The response of total root carbon to CO_2 was dependant on the rate of N supply. Total soil carbon, the largest pool of carbon in grassland ecosystems (Anderson,JM, 1991) was higher at all N levels under CO_2 enrichment. Microcosm water use was decreased under CO_2 enrichment. This is potentially a very important factor in microcosm, and ecosystem response to CO_2 increase, as it may prolong the period of net carbon fixation between droughts, and improve conditions for soil microbial processes. These factors will now be discussed in more detail.

Total microcosm carbon gain above that present at sowing

Microcosms accumulated more carbon at all N levels when grown under CO_2 enrichment (Figure 6.3). Although the CO_2 effect on carbon accumulation was reduced under severe nitrogen limitation, attested to by the very low rates of net microcosm carbon gain, it was still significant. Thus the major hypothesis of this project, that growth at high atmospheric CO_2 would increase microcosm carbon gain under severe nitrogen limitation has been supported.

Response to changing atmospheric CO_2 concentrations is very well documented at the leaf level (eg. von Caemmerer & Farquhar, 1981). This response has been represented in many ways (Gifford *et al.,* 1996b). These include the use of forms of the Farquhar-von Caemmerer model of photosynthesis (von Caemmerer & Farquhar, 1981), or more simply (at least conceptually) at the whole plant level as a rectangular hyperbola (Gifford, 1993; Gifford *et al.,* 1996c). However, the response function of the soil-plant system is not known, as there are many processes working at different temporal scales (Gifford *et al.,* 1996a). If microcosm response to CO_2 concentration is assumed to be linear between 359 $\mu L\ L^{-1}$ and 718 $\mu L\ L^{-1}$, which is likely an underestimation of responsiveness at current atmospheric CO_2 concentrations, the minimum relative increase in carbon gain in this experiment at low-N of 15% gives an approximate increase in microcosm carbon gain of 0.15% for each percentage point increase in atmospheric CO_2 concentration. Although a single, large step change in atmospheric CO_2 concentration may elicit a different response to the relative continual increase in atmospheric CO_2 concentrations observed globally (Watson,RT *et al.,* 1990), this relative increase, if applicable to all terrestrial ecosystems would be enough to close the global carbon budget (Gifford *et al.,* 1996c). However, there are many factors which may alter this response under field conditions (Gifford *et al.,* 1996a), some of which will be discussed here and in the following chapters.

The rates of N supply in this experiment resulted in above-ground productivity similar to that observed in the field. Above-ground productivity of field grown *Danthonia* swards supplied with similar quantities of N as those in the current experiment were ~350 g C m^{-2} yr^{-1} (*D. linkii*; Robinson & Archer, 1988) and ~470 g C m^{-2} yr^{-1} (*D. racemosa* R. Br.; Robinson, 1976), compared to 140 to 453 g C m^{-2} for the first year after sowing of ambient CO_2 swards in this experiment.

Measurements of whole plant-soil system carbon pools under CO_2 enrichment are rare. Obtaining these estimates is difficult, as the heterogeneity of soil carbon concentrations is often greater than any expected treatment effect (Ross *et al.,* 1995). The selection of a soil of very low carbon and nitrogen concentration, and the care taken in its preparation eliminated the problem of heterogeneity. This can create problems with mineralisation of native soil organic nitrogen (Johnson,DW *et al.,* 1995). The disturbance of soil during its preparation did result in the mineralisation of a large quantity of nitrogen prior to sward establishment (4.5 g m^{-2}), which was approximately two years supply of nitrogen for the low-N treatment. This was despite there being virtually no partially decomposed root detritus in the soil, as indicated by the low initial C:N ratio of ~7. The large pool of mineral nitrogen contributed to the rapid gain in total system

carbon to the first harvest (Figure 6.3). Mineralisation of native organic nitrogen, in the absence of plants did not create a mineral nitrogen pool larger than this in a number of small pots filled with the soil and kept moist over a two year period (data not presented). This suggests that subsequent mineralisation of organic nitrogen which was present at sward establishment was minimal. Gaseous nitrogen loss from those pots was not determined, and mineralisation of soil organic nitrogen is often stimulated in the presence of plants (eg. Billès *et al.*, 1993), thus some contribution may have been made from native organic nitrogen to plant available nitrogen - attested to by declines in total soil nitrogen early in the experiment (Figure 7.14). However this was not large enough to compromise the N treatments (Figure 6.3).

Turves of *Lolium perenne* L. and *Trifolium repens* L. pasture grown under controlled environment conditions increased plant production at high CO_2. However no detectable change in soil carbon was noted, which the authors attributed to the large natural variation in soil carbon concentration (Ross *et al.*, 1995). Artificially constructed tropical plant communities exposed to high atmospheric CO_2 concentrations did not increase carbon accumulation relative to ambient concentrations (Körner & Arnone, 1992). This was largely due to losses of carbon from the soil, as the biomass carbon increment increased under CO_2 enrichment (+10%), although that increase was not statistically significant (Körner & Arnone, 1992). In a similar study where the community's growth was restricted by nutrient supply, there was a non-significant 4% increase in carbon acquisition in the total soil-plant system at high CO_2 (Arnone,JA & Körner, 1995).

Many authors have attempted to overcome the problems of measuring changes in carbon pool size of the soil-plant system by measuring net canopy carbon exchange. However, this is not usually monitored continuously, and thus does not fully integrate canopy carbon gain. *Lolium perenne* L. cv. Vigor swards increased net canopy carbon acquisition at high CO_2 over one season by a maximum of 77% (Nijs *et al.*, 1988). *Trifolium repens* L. cv Blanca swards grown on sterilised soil in chambers that doubled as gas exchange cuvettes approximately doubled net canopy carbon gain and above-ground biomass under CO_2 enrichment (Nijs *et al.*, 1989). Cotton canopies grown under FACE (Free-Air CO_2 Enrichment; Lewin *et al.*, 1994) had higher midday rates of photosynthesis (Hileman *et al.*, 1994), and field grown C_4 rangeland communities have also shown responses in canopy photosynthesis to CO_2 enrichment (Nie,D *et al.*, 1992).

Net carbon uptake by tundra microcosms was increased by growth at elevated atmospheric CO_2 concentrations (Billings *et al.*, 1983). In a further experiment, no significant CO_2 effects on

instantaneous rates of CO_2 uptake or biomass production of tundra microcosms were observed by Billings *et al.* (1984), although the authors did note that cumulated carbon uptake from carbon exchange measurements indicated a small increase in carbon gain at high CO_2 by nitrogen fertilised microcosms. The authors of these studies also show that changes in other environmental conditions, such as temperature (Billings *et al.*, 1982) or a lowering of the water table (Billings *et al.*, 1983; Peterson *et al.*, 1984) may moderate these responses. However, on those experiments it is not known how much soil (peat) respiration was stimulated by disturbance at core collection.

In experiments where chambers were placed over tussock tundra communities in the field, they exhibited higher rates of carbon acquisition under CO_2 enrichment over a growing season (Grulke *et al.*, 1990). The effect was reduced later in the season as some components of the plant community down-regulated photosynthetic rates in response to CO_2 enrichment (Grulke *et al.*, 1990). This down-regulation of tundra canopy CO_2 uptake was also noted by Oechel *et al.* (1992), although the CO_2 enriched canopies carbon balance was always more positive than that of the ambient CO_2 canopy. Net canopy carbon acquisition of an alpine grassland, dominated by *Carex curvula* was 40-80% higher under CO_2 enrichment than ambient concentrations in full sunlight (Diemer, 1994). Net canopy uptake of a weed infested *Medicago sativa* L. (lucerne) and *Dactylis glomerata* L. (orchard grass) sward was increased slightly under CO_2 enrichment, and the authors attributed the increase to increased growth of the weed species (Bunce, 1995). Experiments on annual grassland community response to CO_2 increase have also demonstrated increases of instantaneous net canopy carbon gain at high CO_2, both on relatively infertile and fertile sites, ranging from increases of 17 to 117% (Fredeen *et al.*, 1995).

The *D. richardsonii* microcosms in this experiment increased carbon acquisition under CO_2 enrichment at all levels of N supply, and this was maintained throughout the four years of the experiment. These data show that severely N limited systems can respond to CO_2 enrichment by increasing carbon acquisition and storage. The limited amount of data in the literature supports this conclusion.

Leaf area index and green leaf carbon

Growth at high CO_2 initially stimulated LAI at the highest rate of N supply, but not at mid- and low-N (Figure 6.4), a similar response to that observed in isolated plants (chapter 5). Over time this effect was reversed, with the higher rates of N supply tending to have a lower LAI at high CO_2, while there was little difference in the low-N treatment. This effect was also noted in

microcosm experiment 2 (Appendix 5). Accumulation of green leaf carbon showed similar trends to those of LAI (Figure 6.5).

No leaf area response to CO_2 was observed in the OTC-grown (open top chamber, field grown) woody shrub *Lindera benzoin* (Cipollini *et al.,* 1993). However, indirect measures suggested that above-ground biomass was increased by CO_2 enrichment (Cipollini *et al.,* 1993). Leaf area of OTC-grown *Liriodendron tulipifera* L. (yellow poplar) tended to be lower at high CO_2 in the last two years of a three year experiment, accompanied by increases in leaf photosynthetic rates (Norby *et al.,* 1992). LAI of an OTC-grown unfertilised tallgrass prairie community was increased after one year of growth at high CO_2, but not after two, with above-ground biomass showing the same trends (Owensby *et al.,* 1993b). Fertilised tallgrass prairie exhibited increases in community LAI and above-ground biomass over a two year period on similar site to the above cited study (Owensby *et al.,* 1994). No CO_2 effect was observed on leaf area in the artificial tropical plant communities of Körner & Arnone (1992).

Above-ground biomass of the dominant species of an OTC-grown alpine grassland, *Carex curvula* All. (sedge), *Leontodon helveticus* Merat. (forb) and *Trifolium alpinum* L. (legume) generally showed no response in above-ground biomass to three years of CO_2 enrichment, either with or without supplemental N (Schäppi & Körner, 1996). However, the most abundant grass, *Poa alpina*, which represented less than 2% of total community above-ground biomass, responded to CO_2 enrichment *without* supplemental N, *but not with* supplemental N (Schäppi & Körner, 1996). This conflicts with the gas exchange data of Diemer (1994), measured over the same swards. Schäppi & Körner (1996) attributed this discrepancy to a combination of an overestimation of seasonal gas exchange, a possible increase in undetectable carbon losses from the system at high CO_2, or an increase in soil carbon. However, changes in soil carbon were not reported.

Thus the increase in net carbon gain by the microcosm was obtained without an increase in LAI or of leaf carbon late in the experiment. This is indicative of no, or incomplete down-regulation of photosynthesis under CO_2 enrichment when expressed on either a leaf area or leaf carbon basis (chapter 1). Although net microcosm carbon gain is a function of canopy carbon gain and loss of carbon from the plant-soil system, it is not thought that growth at high CO_2 would have significantly reduced respiratory loss of carbon from the soil. In fact, increased respiratory carbon loss may have been expected (eg. Lekkerkerk et al., 1990; Körner & Arnone, 1992; Navas *et al.,* 1995) as microbial biomass carbon was greater at high CO_2 at the higher N levels (Figure 8.13). However, as respiratory loss of carbon from temperate grassland soils have been

measured at 70 to 450 g C m^{-2} yr^{-1} (Schlesinger, 1977), any small treatment effect on respiratory loss of carbon may have a large impact on conclusions on rates of gross carbon acquisition.

Increases in canopy carbon gain without corresponding increases in live plant biomass have been observed in some of the above cited studies (Schäppi & Körner, 1996 and Diemer, 1994; Billings *et al.,* 1984; Fredeen *et al.,* 1995). Photosynthesis has also been observed to up-regulate in OTC-grown *Scirpus olneyi* (sedge) exposed to CO_2 enrichment for a period of four years (Arp & Drake, 1991). Incomplete down-regulation of isolated plants of *D. richardsonii* was expressed as increases in net assimilation rate under CO_2 enrichment (chapter 5). Limited observations of leaf photosynthetic rate in the high-N treatments on day 938 showed an incomplete down-regulation of photosynthetic rate, expressed on a leaf area basis, under CO_2 enrichment (data not presented, n=2).

Thus in this experiment increases in net carbon accumulation by the microcosm were attained without increases in LAI or green leaf carbon. This has been observed in other experiments with grassland communities, as increases in net canopy carbon uptake without increases in live biomass.

Patterns of leaf area development

The system appeared to suffer perturbation in the period leading to the 753 day harvest, as shown by the dramatic reduction in LAI (Figure 6.4). The trends in green leaf carbon were similar, but not as dramatic (Figure 6.5). This decrease contributed to the lower than normal gain in microcosm carbon in the high-N and EMN treatments during that harvest interval (Figure 6.3). Grass communities, especially monocultures often exhibit near chaotic behaviour, due to the complex interactions between growth and the use of, and competition for resources, such as nitrogen, water, and light (Tilman & Wedin, 1991; Thornley *et al.,* 1995). Tilman & Wedin (1991) demonstrated with *Agrostis scabra* monocultures that near chaotic behaviour is more common in more fertile systems, and was linked to temporary immobilisation of nitrogen in litter, and to light interception by the litter inhibiting seedling establishment. The model of Thornley *et al.* (1995) produced chaotic oscillations of biomass in a simulated grass-legume sward, also associated with temporary immobilisation of nitrogen in the plant litter.

Was nitrogen immobilisation the driving force behind the LAI collapse in this experiment? The watering strategy also changed in this harvest interval. However, it is difficult to attribute the LAI reduction solely to the change in soil water dynamics, as all treatments exhibited similar changes in LAI, while experiencing different changes in θ_v and total water application (Table

6.3, Table 6.4 & Figure 6.2). It is also important to note that LAI in the low- and mid-N treatments appeared to follow a decreasing trend from the 191 day harvest to the 753 day harvest (Figure 6.4). Thus the reduction in LAI may have resulted from a reduction in nitrogen availability following rapid nitrogen uptake and growth to the first harvest, and nitrogen loss from the plants, as the senesced leaf (Figure 6.7), surface litter (Figure 6.9), and potentially the senesced root carbon pool increased in size.

The reduction in LAI may have resulted from a combination of a change in the pattern of water availability and a change in nitrogen availability. However, after day 753 LAI recovered to levels greater than that observed at the first harvest (Figure 6.4). It is important to note that the change in the watering strategy did not greatly affect either carbon gain by the microcosms, or the CO_2 response of total microcosm carbon gain. This is shown by the statistical linearity of carbon accumulation over time, and the similarity of the relative response of microcosm carbon accumulation to CO_2 enrichment between this experiment and microcosm experiment two (Appendix 5).

Green leaf non-structural carbohydrate concentration

Non-structural carbohydrate concentration of green leaf increased in response to CO_2 enrichment (Figure 6.6). This effect was noted in isolated plant experiments (see chapter 4 & 5) and is one of the most common responses to CO_2 enrichment, at least at the isolated plant level (eg. Wong, 1990). Observations on field grown plants generally show increases in NSC concentration in response to CO_2 enrichment (eg. Hendrix *et al.*, 1994; Jacob *et al.*, 1995). Large increases in leaf non-structural carbohydrate content were also observed in herbaceous plants and trees growing in naturally CO_2 enriched areas in Italy, relative to those growing close by under ambient conditions (Körner & Miglietta, 1994).

Oscillations of green leaf carbon

Green leaf carbon at high-N showed oscillations over harvests (Figure 6.5), which may be associated with changes in incident shortwave radiation in the weeks prior to harvest (*cf.* Figure 6.1). These changes were not solely explained by changes in non-structural carbohydrate content, as the differences were present when expressed on a structural carbon basis on days 922 and 1104 (not presented). This indicates that sward productivity may have been co-limited by radiation, at least over the winter months at the high rate of N supply.

Senesced leaf and leaf turnover

Growth at high CO_2 increased total standing senesced leaf carbon (Figure 6.7). Accumulation of carbon in the senesced leaf pool slowed over time, and, when averaged over CO_2 treatments the size of this pool at low- and high-N had statistically stabilised by the end of the experiment. However, at high-N the size of this pool still tended to increase at high CO_2. Thus a larger pool of senesced leaf carbon was maintained under CO_2 enrichment, even though green leaf carbon (Figure 6.5) was not increased by CO_2 enrichment. This is expressed in Figure 6.8 as the ratio of senesced leaf lamina to green leaf lamina. The ratio was initially large at low-N, probably owing to the initial flush of growth resulting from the mineralisation of native soil N, followed by leaf death as this source of nitrogen was exhausted prior to the first harvest. The ratio was increased by CO_2 enrichment, which suggests that the rate of leaf turnover, that is, the rate of leaf production and death, was higher under CO_2 enrichment. A similar response was seen in Mediterranean grassland microcosms, with plant litter the only phytomass pool to increase at high CO_2 (Navas *et al.,* 1995). However, leaf turnover was not changed by CO_2 in pasture turves dominated by *Lolium perenne* L. and *Trifolium repens* L. (Clark,H *et al.,* 1995). The presence of a legume and its associated nitrogen input may have influenced that result.

Rates of leaf initiation have been observed to increase slightly in response to CO_2 enrichment (Ackerly *et al.,* 1992). However, the effect of CO_2 enrichment on senescence is not consistent. It has variously been reported to delay senescence (Carter & Peterson, 1983; Latimore, 1984; Curtis *et al.,* 1989a; Arp *et al.,* 1993), have no effect (Gunderson *et al.,* 1993; Jackson *et al.,* 1994; Clark,H *et al.,* 1995; Pearson & Brooks, 1995), or advance senescence (Chang, 1975; Carter & Peterson, 1983; Mousseau & Enoch, 1989; Kimball *et al.,* 1995; Nie,GY *et al.,* 1995). A recent study of tropical forests on a global scale has shown increasing rates of forest turnover, which is correlated with productivity, since the 1950's (Phillips & Gentry, 1994). Although this can not be conclusively linked to atmospheric CO_2 increase over this period, it is the most likely cause, either directly by enhancing productivity, or by stimulating the growth of parasitic vines (Phillips & Gentry, 1994).

In this study, one of the responses to CO_2 enrichment has been to increase the turnover of leaf, resulting in more senesced leaf at high CO_2 *without* a corresponding increase in standing green leaf biomass. Thus the only above-ground carbon pools to contain more carbon at high CO_2 were the senesced leaf and surface litter pools (Figure 6.7 & Figure 6.9).

Root carbon and root carbon ratio

The CO_2 effect on root carbon was strongly dependant on the rate of nitrogen supply (Figure 6.10). The root pool contains both live and dead root. It very difficult to obtain estimates of the proportion of live and dead root in the total root mass (Dormaar, 1992). No attempt was made to partition live from dead root in this study. However, it is still instructive to examine apparent root carbon ratio (RCR_A), defined as the ratio of root carbon to total "live" plant carbon (root C + green leaf C), as shown in Figure 6.11. The proportion of "live" plant carbon below-ground is high, although grasslands often have RCR_A's in the order of 0.80-0.95 (Dormaar, 1992).

At high-N, growth at high CO_2 increased both absolute and relative distribution (RCR_A) of carbon to root, while at low- and mid-N, absolute and relative distribution of carbon to root at high CO_2 was reduced. These conclusions are based on the assumption that there is an equal proportion of live to dead root for both CO_2 treatments. When determined, there were no differences between treatments in non-structural carbohydrate content, so these masses accurately reflect structural mass. In chapter 8 root decomposability is discussed. Owing to small differences in decomposability and large differences in θ_v between treatments the validity of this assumption is difficult to assess. If root decomposition were slower at high CO_2, these responses would be amplified at low- and mid-N, but reduced at high-N.

Little or no direct effect of CO_2 on carbon allocation has been observed in isolated plants of *D. richardsonii* (chapter 5). However the large CO_2 induced changes in θ_v (Figure 6.2, chapter 8) could be expected to reduce carbon allocation to root at high CO_2 (Davidson, 1978; Turner & Begg, 1978). Other observations of root growth in herbaceous communities at high CO_2 include *Scirpus olneyi*, where total root biomass (live + dead) was increased when growing in monoculture (Curtis *et al.*, 1990), while total above-ground biomass was also increased (Arp *et al.*, 1993). Total root biomass under fertilised and unfertilised tallgrass prairie communities was observed to increase in response to CO_2 enrichment (Owensby *et al.*, 1993b, 1994). Total root biomass was increased by CO_2 in an alpine grassland in one of the two years in which it was determined (Schäppi & Körner, 1996), and total root biomass was increased in pasture turves exposed to CO_2 enrichment for 217 days (Newton *et al.*, 1994).

As soil conditions were so different between CO_2 treatments in this study it may be more instructive to examine changes in soil carbon. Soil carbon is the end repository for residual, senesced plant material that is not lost from the microcosm via microbial respiration or leaching.

Soil carbon

Soil carbon (Figure 6.12) was increased under CO_2 enrichment. Soil carbon is often the largest pool of carbon in grasslands (Anderson,JM, 1991), due in part to the large relative allocation of carbon to below ground structures (Dormaar, 1992). An increase in carbon storage in this pool suggests that a longer term response of microcosm carbon accumulation to CO_2 increase is likely. Few other studies that have reported changes in soil carbon in response to increased atmospheric CO_2 under herbaceous systems (Johnson,D *et al.,* 1994; Ross *et al.,* 1995), which has been largely attributed to the high spatial variability of soil carbon in natural systems (Ross *et al.,* 1995). Trends towards increased soil carbon have been observed under a cotton system exposed to CO_2 enrichment for three years (Wood *et al.,* 1994), and significant increases in soil carbon were found at some soil depths under a fertilised tallgrass prairie system exposed to CO_2 enrichment for three years (Rice *et al.,* 1994).

An interesting comparison to make here is of the "enriched" and "control" site in the study of a natural CO_2 vent in Italy by Körner and Miglietta (1994). The site near the vent, which has experienced natural CO_2 enrichment (~500 - 1000 $\mu L\ L^{-1}$) for a long period (at least since the turn of the century), has a soil organic matter content (hence C content) 3.5 times greater than that of the control site. Both sites are on similar soil types, and appear to have similar slopes and aspects. It would be interesting to have a more through analysis of this change, including documentation of possible management effects which may have influenced this change to determine if it could be attributed to CO_2.

Increases in soil carbon may result from either increased input of carbon into the soil, or reduced losses from microbial respiration. It is expected that respiratory loss of carbon from the soil would be higher under CO_2 enrichment, as discussed above. Thus the increase in soil carbon is largely thought to be the result of greater inputs of carbon from the plant. This may take the form of particulate input from the decaying surface litter layer, decomposition products and particulate matter from decomposing roots, or direct input from the roots by exudation. The increase in soil carbon appears to be related to the change in watering strategy (page 6-104) and the commencement of the weekly spraying of the canopy with water. This may have influenced soil carbon accumulation in two ways. Carbon input from the surface litter layer may have been increased owing to better conditions for litter decomposition (chapter 8), and the longer periods of low θ_v may have promoted root death and slowed soil respiration rates. The second factor

may have favoured carbon accumulation in the control CO_2 treatment, as it experienced water stress for a longer periods (Figure 6.2 & Figure 7.1).

Root mass at harvest was not increased at high CO_2 in the lower two N levels while soil carbon was. Thus the increase in soil carbon may have been either from increases in root turnover at high CO_2, or from increases in carbon deposition by the roots into the rhizosphere. As leaf turnover appears to be increased under CO_2 enrichment, root turnover may also be enhanced. Although there are no direct data from this experiment to support this, the increases in θ_v under CO_2 enrichment (Figure 6.2 & Figure 7.1) with the small changes in root decomposability (page 8-203, Figure 8.8) do not rule this out. Fine root turnover of OTC-grown *Liriodendron tulipifera* L. (yellow poplar) (Norby *et al.*, 1992) may have been increased at high CO_2, as live root responded to CO_2 where dead root did not. Alternately, root longevity may have been increased under CO_2 enrichment in that experiment. Fine root turnover of *Populus* x *euramericana* cv. Eugenei was increased both by growth under high CO_2 and by higher levels of nitrogen availability (Pregitzer *et al.*, 1995). This is an area of research that needs more attention, especially for herbaceous plants.

The other major source of carbon input to the soil is deposition by the root as exudate, mucilage and sloughed cortical cells (Stanton, 1988), collectively referred to as exudate (Rovira, 1969). This can account for large proportions of photosynthetically fixed carbon, with the sum of carbon lost from root by respiration and exudation ranging between 4-40% of carbon fixation (Whipps, 1984; Biondini *et al.*, 1988; van Veen *et al.*, 1991; Johansson, 1992) or 18-25% of dry matter accumulation (Barber & Martin, 1976) for Gramineae. Growth at under CO_2 enrichment has been observed to increase deposition of carbon into the rhizosphere of Gramineae (Whipps, 1985; Lekkerkerk *et al.*, 1990; Billès *et al.*, 1993; Rattray *et al.*, 1995) and tree species (Rouhier *et al.*, 1994). This increase in deposition was simply a response to increased plant size in *Zea mays* L. cv. LG 11 (maize), a C_4 (Whipps, 1985). However, rhizosphere microbial populations often increase carbon deposition (Merbach & Ruppel, 1992), and rhizosphere carbon deposition varies greatly with the environment (van Veen *et al.*, 1991), so changes in deposition other than those related to plant size are likely under CO_2 enrichment. Increases in specific exudation rates of citrate have been observed in response to growth at high CO_2 in excised root of *D. richardsonii* (DJ. Barrett, unpublished data, but see Gifford *et al.*, 1996c). Thus, deposition of carbon into the rhizosphere by the plant roots may well be a source for the extra soil carbon at high CO_2 in this experiment.

Soil carbon accumulated in the first 191 days of the experiment, and then declined until day 753, which was the first harvest after the change in watering strategy and commencement of the weekly spraying with demineralised water. This also corresponds with the LAI collapse in the high-N treatments. The recovery in LAI corresponds to the increase in soil carbon following day 753, which may indicate mineralisation of senesced root material and an associated release of plant available nitrogen allowing the recovery of LAI.

In summary, growth at high CO_2 resulted in higher levels of soil carbon at all N supply rates. This occurred without corresponding increases in root carbon at harvest at the two lower rates of N supply. Thus the increase may be due to a combination of increased root turnover and increased rhizosphere deposition of carbon at high CO_2. Although no evidence is available for the first mechanism, increases in microbial carbon, and decreases in an index of microbial C:N ratio at high CO_2 (Table 8.11) suggest that exudation may be a source of the extra soil carbon at high CO_2.

Microcosm water use

Rates of water use on a ground area basis, immediately following the return of the microcosms to field capacity were reduced by CO_2 enrichment (Figure 6.13). At this stage in the drying cycle water use should not have been largely influenced by treatment differences in θ_v (Figure 6.2 & Figure 7.1). Thus, it was this change in rates of water use which created the differences in θ_v, an internal system variable. This water use data is available only after the change in watering strategy (day 548). However, it can be assumed that this effect was present early in the experiment, at least in the low- and mid-N treatments, as shown by spot measurements of θ_m (Table 6.5). This reduction in water use and corresponding increase in average θ_v may have important implications for microcosm, and potentially ecosystem function, perhaps as important as those of CO_2 directly on carboxylation rate (Field *et al.,* 1995). Repercussions may be via effects on decomposition rate (chapter 8), and maintenance of stomatal opening, and hence photosynthesis rates for longer periods in drying soils. This effect would be important in both C_3 and C_4 communities (Nie,D *et al.,* 1992; Knapp *et al.,* 1993; Samarakoon & Gifford, 1995), and may have contributed to the CO_2 responsiveness of carbon gain in this experiment. Growth responses to CO_2 enrichment are often observed to be greater under water stress, such as in *Arachis hypogaea* L. (groundnut) swards, were the CO_2 response in dry matter production increased from 16% under well watered conditions to 112% under severe water stress (Clifford *et al.,* 1993).

Reductions in water use on a whole plant basis have been observed in isolated plants of *D. richardsonii,* other C_3 grasses in this study (chapter 4) and in other Gramineae (eg. Samarakoon & Gifford, 1995), the mechanisms of which were discussed in chapter 2. Stomatal conductance was not determined routinely. A limited dataset exhibited a reduction in conductance at high CO_2 in high-N grown microcosms (n=2, not presented). The reduction in microcosm water use in this study may not solely be attributable to stomatal closure under CO_2 enrichment, as LAI was reduced at high CO_2 (Figure 6.4), and surface litter load was increased (Figure 6.9), both potentially reducing evapotranspiration under CO_2 enrichment. The CO_2 effect on water use was not influenced by N level, even though the CO_2 effect on LAI tended to be less at low-N, suggesting that LAI differences did not contribute greatly to the reductions in water use, at least at low-N. Reductions in water use were evident under CO_2 enrichment early in the experiment when there was little difference in LAI between treatments (Table 6.2 & Table 6.4). Thus, although it is impossible, owing to lack of data, to attribute all of the reduction in water use to stomatal closure it is thought that it was a major contributing factor.

Few other microcosm or field studies have shown direct reductions in canopy water use of herbaceous plants under elevated CO_2 which are more than "spot" or instantaneous measurements. Over a growing season OTC-grown *Avena fatua* monocultures had a lower water use on a ground area basis under CO_2 enrichment (Fredeen & Field, 1995). Wheat, grown in a FACE system exhibited a reduction of evapotranspiration of 8% over a growing season (Kimball *et al.,* 1995), while FACE grown cotton showed no difference in seasonal evapotranspiration (Hunsaker *et al.,* 1994), potentially due to LAI compensation.

Instantaneous measurements have often shown canopy conductance to decrease under CO_2 enrichment, such as in *Medicago sativa* L. or *Dactylis glomerata* L. swards, where canopy conductance was reduced at high CO_2, and there was no increase in above-ground biomass at high CO_2 (Bunce, 1995). Canopy conductance of an OTC-grown alpine grassland has been observed to decline under CO_2 enrichment (Diemer, 1994). An OTC-grown *Andropogon gerardii* Vitman. community (big bluestem, C_4) exhibited reductions in transpiration rate (leaf area) and increased leaf temperature at high CO_2 (Kirkham *et al.,* 1991), while canopy evapotranspiration was reduced under CO_2 enrichment in a similar community (Nie,D *et al.,* 1992). Canopy water use is not always reduced under CO_2 enrichment if there are compensatory increases in LAI. Swards of *Trifolium repens* L. cv. Blanca exhibited stomatal closure at elevated CO_2, although increases in LAI resulted in similar rates of canopy water use (Nijs *et al.,* 1989), a similar effect to that observed in FACE cotton (Hileman *et al.,* 1994).

There is not a 1:1 proportionality between stomatal conductance and canopy evapotranspiration in the field. The relationship is stronger (ie. closer to 1) for aerodynamically rough canopies such as forests than for aerodynamically smooth canopies such as grasslands, owing to interactions with the canopy boundary layer involving leaf temperature and air humidity feedbacks (Field *et al.,* 1995). The relationship is stronger with lower stomatal conductance, that is, in dry environments (Kelliher *et al.,* 1993; Field *et al.,* 1995). Field *et al.* (1995) speculated that for a well watered crop, the reduction in canopy evapotranspiration following a reduction in stomatal conductance may be only 25% of that of the decrease in stomatal conductance. However, following their argument, this reduction would be less (ie. a more direct transfer of stomatal to canopy conductance) for a grassland that is water limited, as this microcosm was, and as most grasslands are (Ripley, 1992). The reduction in the stomatal effect when transferred to the canopy may also be lower in a grassland than in a crop, as a native grassland could be expected to exhibit a greater degree of aerodynamic roughness due to the presence of more than one species, and the tufted or tussocky nature of native grass growth. Field *et al.* (1995) concluded that a significant proportion of a reduction in stomatal conductance (neglecting feedbacks on LAI) would be transferred to field where surface conductance (canopy + soil) to water vapour is in the range of 0.1 - 1 mol m^{-2} s^{-1}. In review, Kelliher *et al.* (1993) concluded that grassland evapotranspiration became sensitive to changes in surface conductance at soil moisture contents below a range of 26 to 32% (θ_v). These soil moisture contents are quite close to saturation for many soils, especially those with a low clay content (Russell, 1973; Brady, 1984). As grasslands are characterised by periodic drought (Ripley, 1992), they may only have soil water contents above this level for very short periods. In another modelling exercise, de Bruin and Jacobs (1993) predicted for a grassland that a 50% decrease in stomatal conductance without any changes LAI would result in a reduction of regional canopy evapotranspiration of about 11%. The model employed in that study was a "big leaf" model, coupled to a planetary boundary layer model, potentially incorporating all atmospheric feedbacks on rates of evapotranspiration. Thus, it is apparent that the reductions of canopy water use observed in this experiment may well be transferred to the field, albeit with a reduced magnitude, as canopy evapotranspiration may not be affected by high atmospheric CO_2 concentrations under very high soil moisture contents.

Summary of carbon accumulation and distribution after 1469 days

After 1469 days of growth, microcosms grown under CO_2 enrichment accumulated more carbon in the senesced leaf, surface litter, root and soil pools, but not in the green leaf pool (Table 6.6).

As a proportion of total microcosm carbon, less carbon was present in the green leaf and root pools under CO_2 enrichment, while a greater proportion was present in the soil pool (Table 6.6).

Conclusions

Growth at high CO_2 increased microcosm carbon accumulation at all levels of nitrogen supply. The increase was of the right order to account for the "missing", or "terrestrial" carbon sink in global carbon models, if the results are transferable to all ecosystems in the field. The increase in carbon accumulation was achieved without increases in LAI or lasting increases in live above-ground carbon under CO_2 enrichment. Less root carbon accumulated under CO_2 enrichment at the two lower levels of N supply. This effect, of increased system carbon accumulation, measured as net canopy carbon uptake without corresponding increases in live plant biomass has been observed by other authors. Thus measures of changes in plant biomass under CO_2 enrichment are *not* effective indicators of changes in system carbon storage.

Factors contributing to the increase in carbon accumulation were an increase in leaf turnover, resulting in a greater accumulation of senesced leaf and leaf litter under CO_2 enrichment. Soil carbon was also increased, both in absolute and relative terms, possibly the result of increased root turnover, and/or increases in carbon deposition into the rhizosphere.

Microcosm water use was lower under CO_2 enrichment. This may have contributed to the CO_2 response, by reducing water stress during the drying cycles. This reduction in water use, and consequent increases in soil moisture content may have profound implications for microcosm function. This will be discussed further in subsequent chapters.

Table 6.1 Environmental conditions, as monitored over the whole experimental period of 1469 days. Data in parenthesis show one standard deviation, based on daily average data.

	Control	**Enriched**
Atmospheric CO_2 (daytime av; $\mu L\ L^{-1}$)	359 (15)	718 (30)
Dew point (°C)	12.1	12.1
Av air temperature (°C)	21.3	21.3
Temperature sum (air, degree days base 0°C)	31300	31291
Av below microcosm temperature (°C)	21.5	21.5
Total evaporation (mm)	19029	18914
Total short wave radiation (MJ m^{-2})	15457	

Table 6.2 Mass water content of the soil (θ_m, %) determined from microcosm weight and average soil DW at harvest on various days after imbibition prior to watering strategy change. Means followed by standard error, n was 2 before day 64 and 4 after. nd represents not determined. †Measurement on day 278 was immediately prior to drainage inducement, and on ‡279 was immediately after cessation of drainage following saturation, so θ_m was essentially field capacity.

	Low-N		Mid-N		High-N	
Day	**Control**	**Enriched**	**Control**	**Enriched**	**Control**	**Enriched**
29	20.5 ± 1.1	21.0 ± 0.0	19.3 ± 0.2	21.4 ± 0.9	18.1 ± 0.0	19.6 ± 1.0
34	15.6 ± 1.0	16.2 ± 1.0	14.9 ± 0.2	16.0 ± 0.9	12.9 ± 0.3	15.1 ± 1.2
37	15.0 ± 0.2	19.7 ± 2.3	16.5 ± 2.0	19.2 ± 0.3	18.5 ± 0.3	17.5 ± 1.0
44	12.7 ± 0.2	17.6 ± 2.3	14.2 ± 3.2	16.3 ± 0.7	15.8 ± 1.2	15.2 ± 1.0
64	16.7 ± 0.8	20.0 ± 1.3	14.3 ± 5.6	19.4 ± 0.5	17.3 ± 0.0	19.8 ± 0.3
237	12.2 ± 2.1	21.2 ± 0.1	13.8 ± 2.9	21.1 ± 0.4	8.8 ± 0.6	8.6 ± 0.3
241	nd	18.2 ± 0.2	24.0 ± 1.1	25.7 ± 0.5	24.7 ± 0.1	23.7 ± 0.7
†278	16.4 ± 0.8	18.9 ± 1.5	12.2 ± 0.5	18.4 ± 1.1	7.6 ± 0.3	9.1 ± 0.6
‡279	23.0 ± 0.3	23.4 ± 0.7	22.8 ± 0.9	24.3 ± 1.1	23.5 ± 0.7	24.6 ± 0.8
317	14.5 ± 1.1	19.8 ± 1.2	11.6 ± 0.5	15.3 ± 0.7	7.5 ± 0.3	8.5 ± 0.5
352	17.3 ± 1.4	21.9 ± 0.8	14.7 ± 1.5	17.3 ± 0.5	9.5 ± 0.6	9.0 ± 0.4

Table 6.3 Effective water application ([water applied] - [water leached], mm) in inter-harvest periods. Differences between CO_2 treatments result from differing minimum θ_v prior to saturation and leaching.

		Low-N		**Mid-N**		**High-N**	
	Day of harvest	**Control**	**Enriched**	**Control**	**Enriched**	**Control**	**Enriched**
initial watering strategy	191	353	342	365	358	386	375
	380	369	280	405	333	469	459
	554	270	196	344	287	408	392
final watering strategy	753	181	180	283	274	541	540
	922	146	135	241	238	460	466
	1104	200	197	245	237	405	399
	1285	180	163	243	236	360	365
	1469	230	222	313	297	355	351

Table 6.4 Average soil volumetric water content during the pre-harvest drying cycle immediately before (initial water strategy) and after (final watering strategy) the change in watering strategy. (mean ± one standard error, n = 4).

	Initial Watering Strategy Day 479 - 530	**Final Watering Strategy Day 637 - 727**
Control Low N	26.7 ± 0.5	13.1 ± 0.3
Enriched Low N	30.8 ± 0.5	20.0 ± 0.2
Control Mid N	21.6 ± 0.5	13.1 ± 0.5
Enriched Mid N	28.6 ± 1.2	20.4 ± 0.7
Control High N	13.7 ± 0.1	11.3 ± 0.7
Enriched High N	14.2 ± 0.7	19.2 ± 0.8

Table 6.5 Harvest numbers and dates. Dates and days are for the mid-point of harvest.

Harvest number	Date	Days after imbibition	Months after imbibition
1	6/12/91	191	6
2	12/6/92	380	12
3	3/12/92	554	18
4	20/6/93	753	24
5	6/12/93	922	30
6	6/6/94	1104	36
7	4/12/94	1285	42
8	6/6/95	1469	48

Table 6.6 Absolute and relative distribution of carbon within the microcosm at the 1469 day harvest. Absolute values for soil and total microcosm are gain above carbon present at sward establishment. Relative pool size is the fraction of the total microcosm carbon increment from sward establishment, expressed as a percentage. Control and enriched CO_2 are given as C and E respectively. Absolute pool sizes where the CO_2 effect is significant are indicated by (*). Probabilities are for the proportional distribution at day 1469. P<0.001 represented by ***, P<0.01 by **, P<0.05 by *, P<0.1 by +, and not significant by ns.

		Absolute pool size g m^{-2}			Relative pool size			P		
	CO_2	LN	MN	HN	LN	MN	HN	CO_2	N	C*N
green leaf	C	26	61	107	5.5	6.6	6.2	*	ns	ns
	E	27	55	97	4.7	5.2	4.3			
senesced leaf	C	155	271	343	32.9	29.3	20.0	ns	**	ns
	E	203*	344*	546*	35.7	32.4	24.5			
surface litter	C	46	145	561	9.7	15.4	32.4	ns	*	ns
	E	55*	192*	676*	10.1	18.0	30.3			
root	C	131	265	421	27.9	28.2	24.5	**	ns	ns
	E	127*	208*	473*	22.4	19.5	21.1			
soil	C	112	185	260	23.7	19.8	15.0	+ (5.6)	*	ns
	E	147*	252*	404*	25.8	23.8	18.1			
Microcosm total	C	471	934	1726	100	100	100			
- sward estab.	E	570*	1063*	2234*	100	100	100			

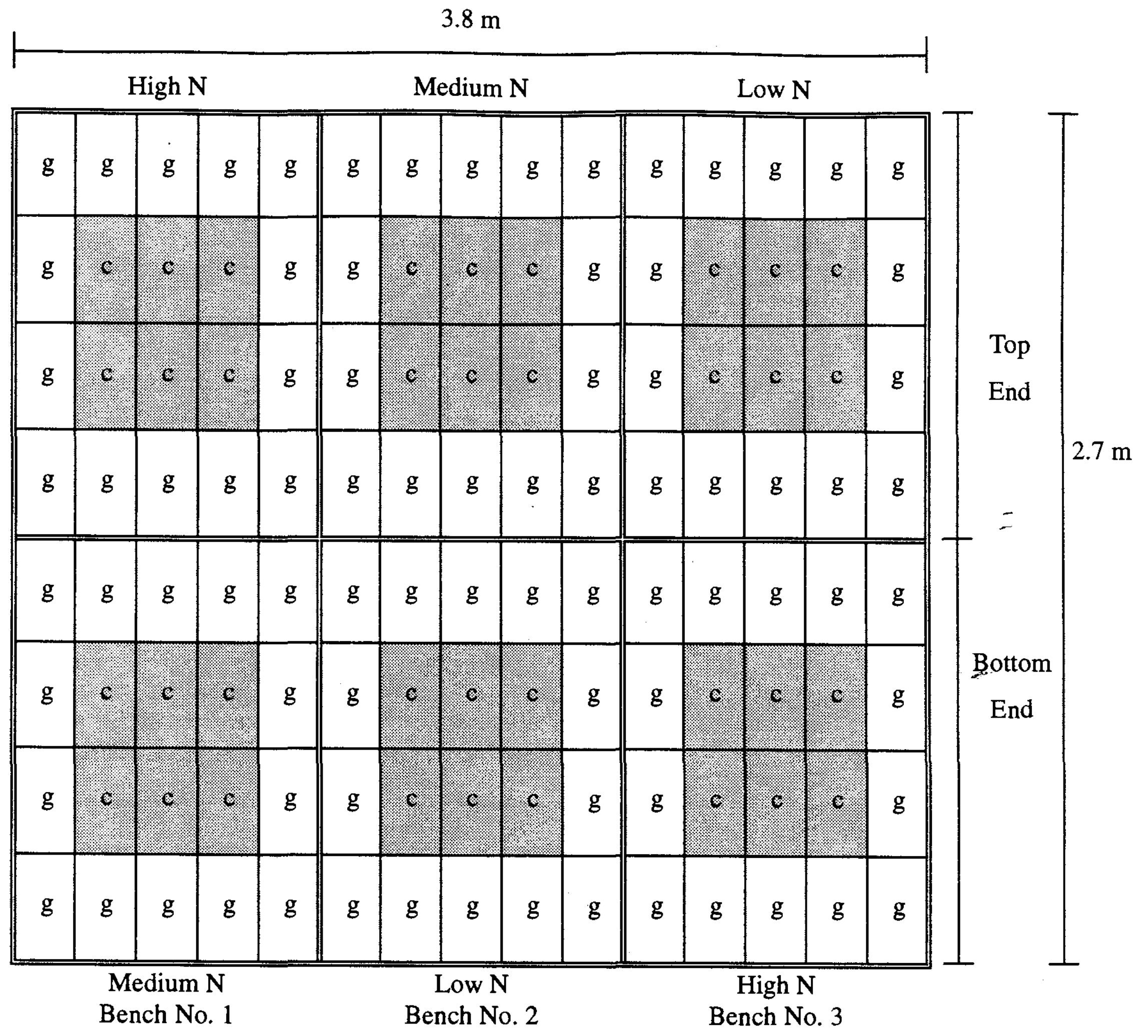


Diagram 6-1. Initial bench and microcosm layout within a glasshouse (CO_2 treatment). Guard microcosms are shown as "g" and core microcosms as "c".

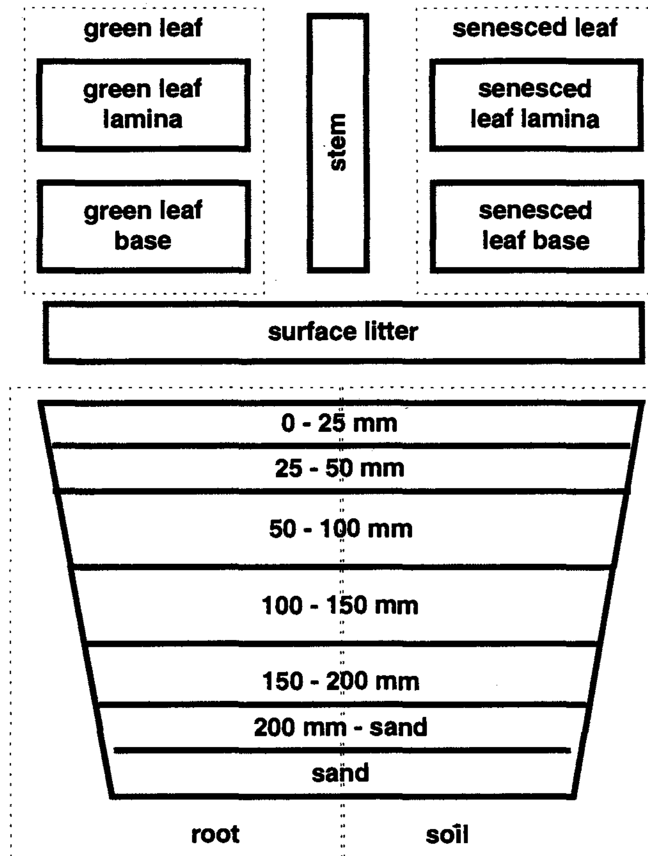
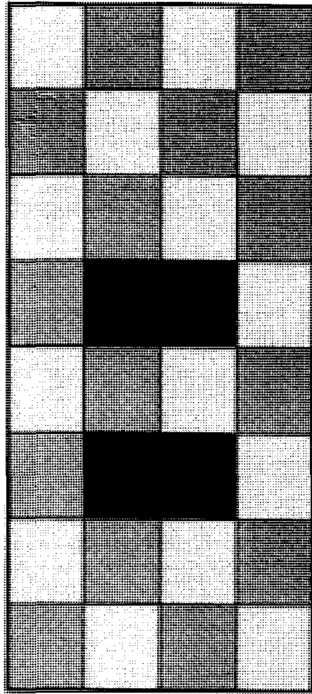
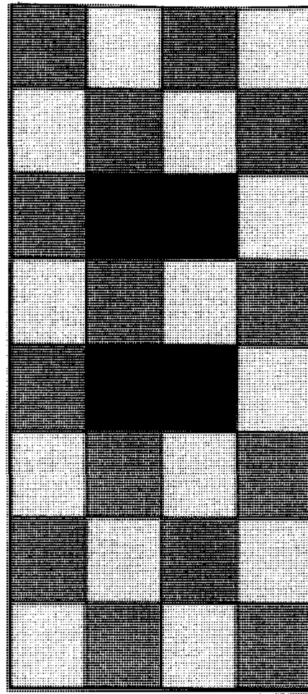
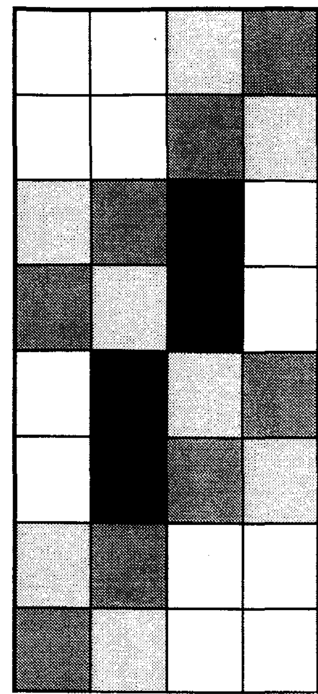
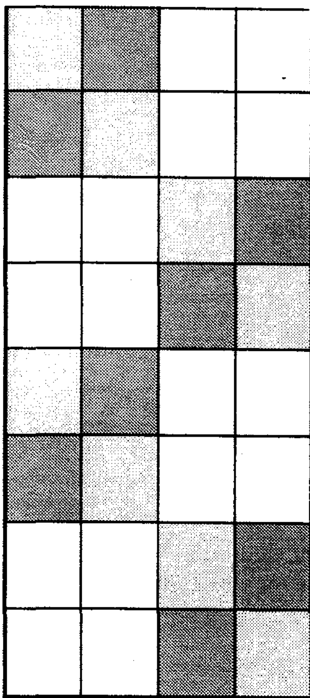
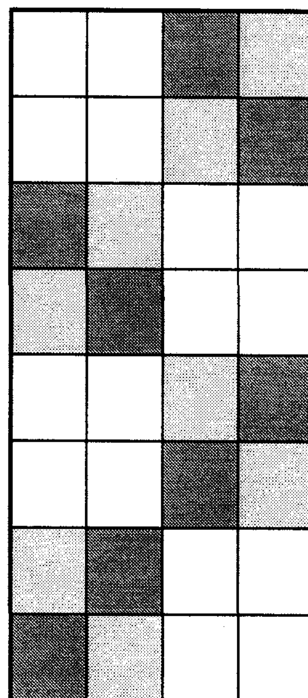
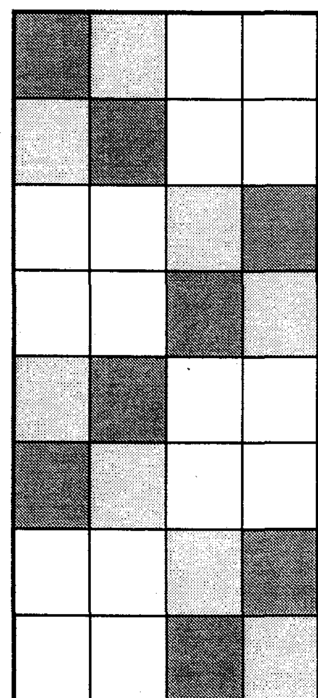


Diagram 6-2 Division of microcosm into fractions at harvest. Depths are given from soil surface (not to scale).

0-25 mm 25-50 mm 50-100 mm

100-150 mm 150-200 mm 200 mm sand

Diagram 6-3 Subsampling of soil layers at harvest. Light-spotted cubes were collected for total C and N analysis, mid-spotted cubes were collected for root weight estimation. This coding was reversed between each pot. Dark-spotted cubes were used for determination of microbial and extractable nitrogen parameters. Cubes without hatching were oven dried for dry mass determination.

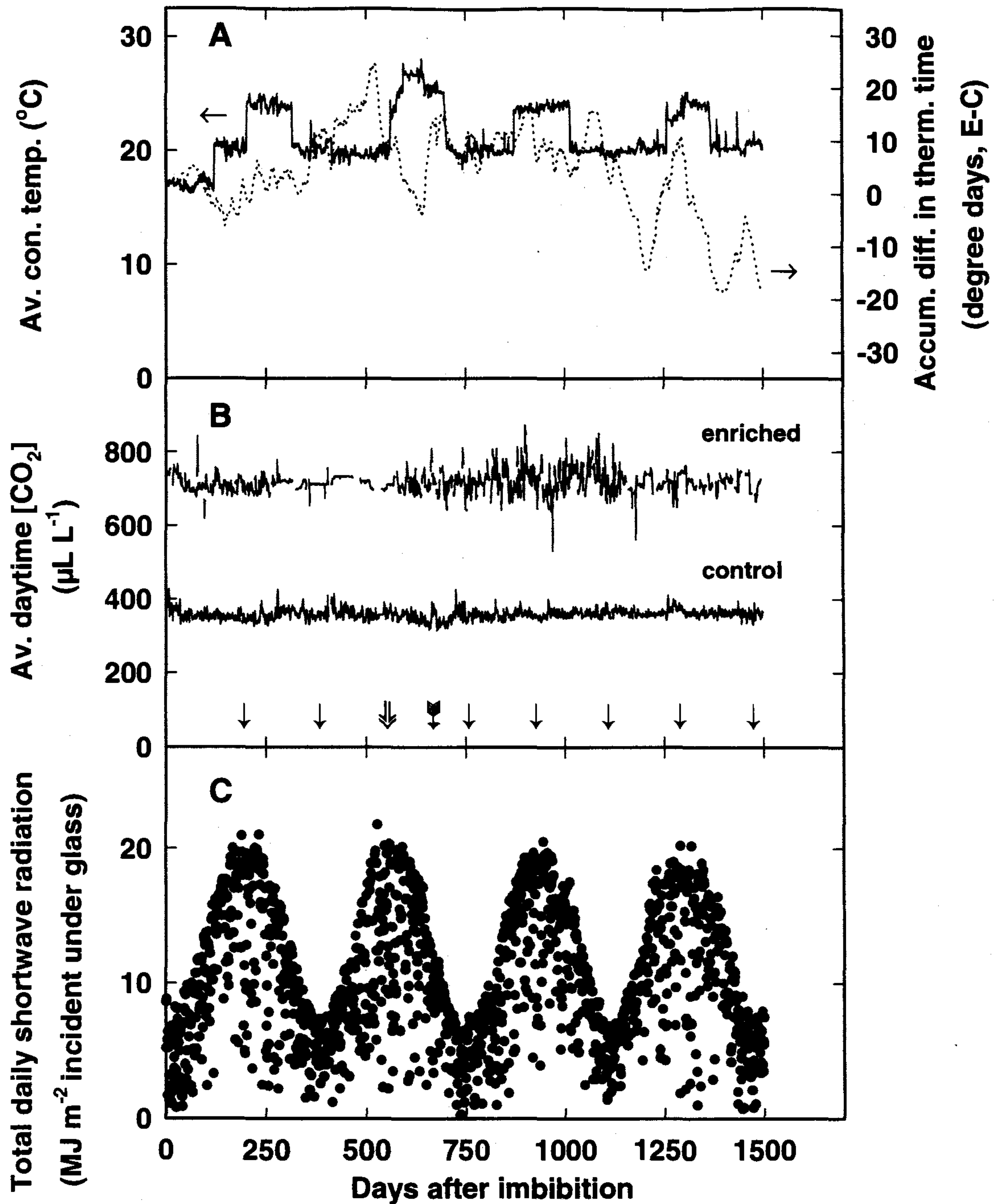


Figure 6.1 (A) Daily average temperature in the control glasshouse (left axis) and the differential in accumulated thermal time (base 0°C, right axis) between the enriched and control glasshouse. (B) Average daytime CO_2 concentration. (C) Total daily incident radiation under the glass and over the canopy, averaged over CO_2 treatments. Arrows in (A) show appropriate axis, in (B) show mid point of major harvests (→, ⇛), watering strategy change (⇛), and commencement of weekly spraying of canopy with water (➻).

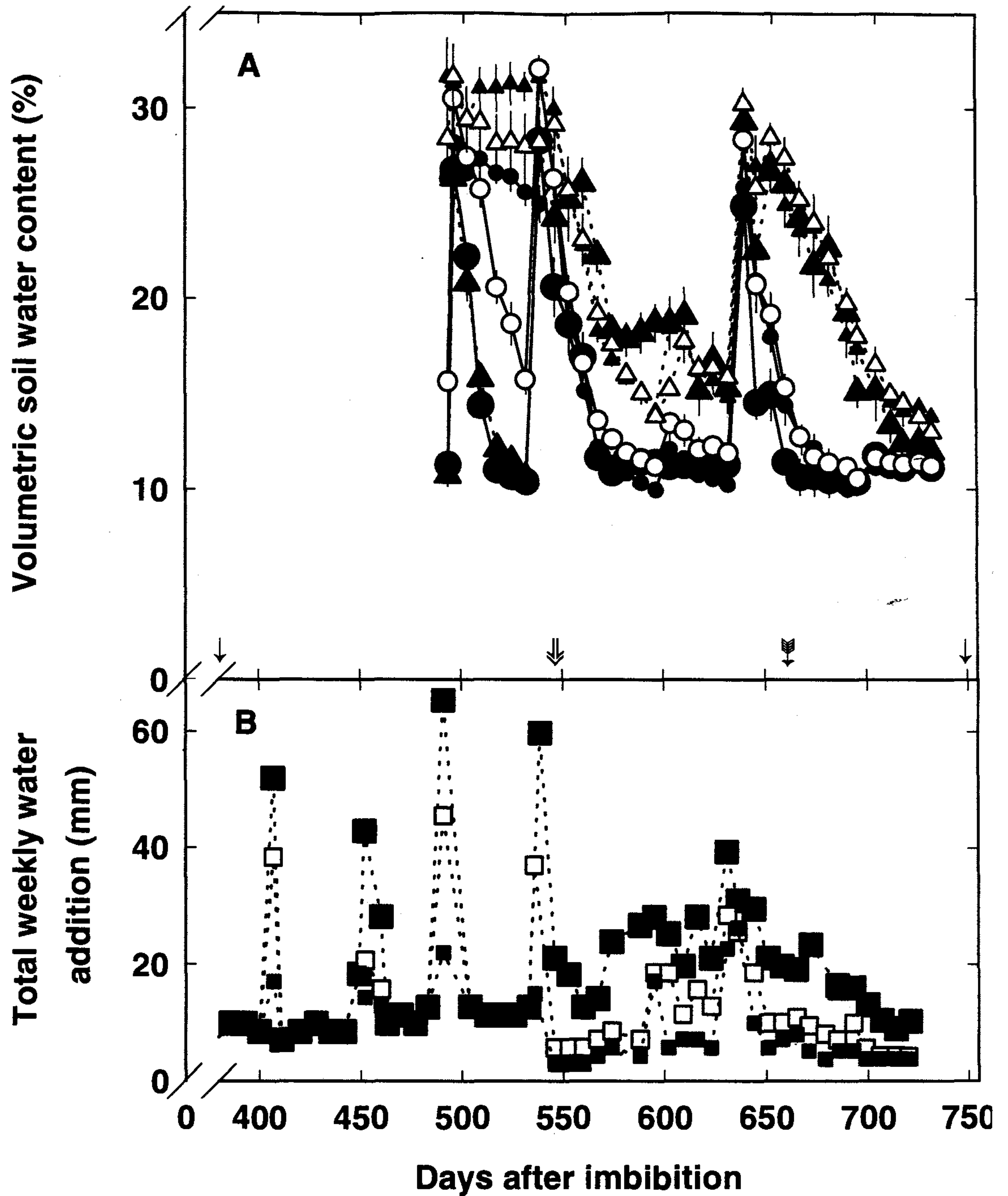


Figure 6.2 Patterns of soil volumetric water content, θ_v (A) before and after the change in watering strategy (⇛). Control low-N (●), mid-N (○), high-N (⬤) with solid line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted line. Error bars are ± one standard error. Arrows (⇛, →) denote harvests and (⤞) commencement of weekly spraying of canopy with water. Monitoring of θ_v did not commence until day 492. Difficulties in controlling the rate of soil moisture decline were experienced in the first cycle after watering strategy change. B) Total weekly water additions. Peaks show water applications for drainage inducement, low-N (■), mid-N (□), and high-N(■).

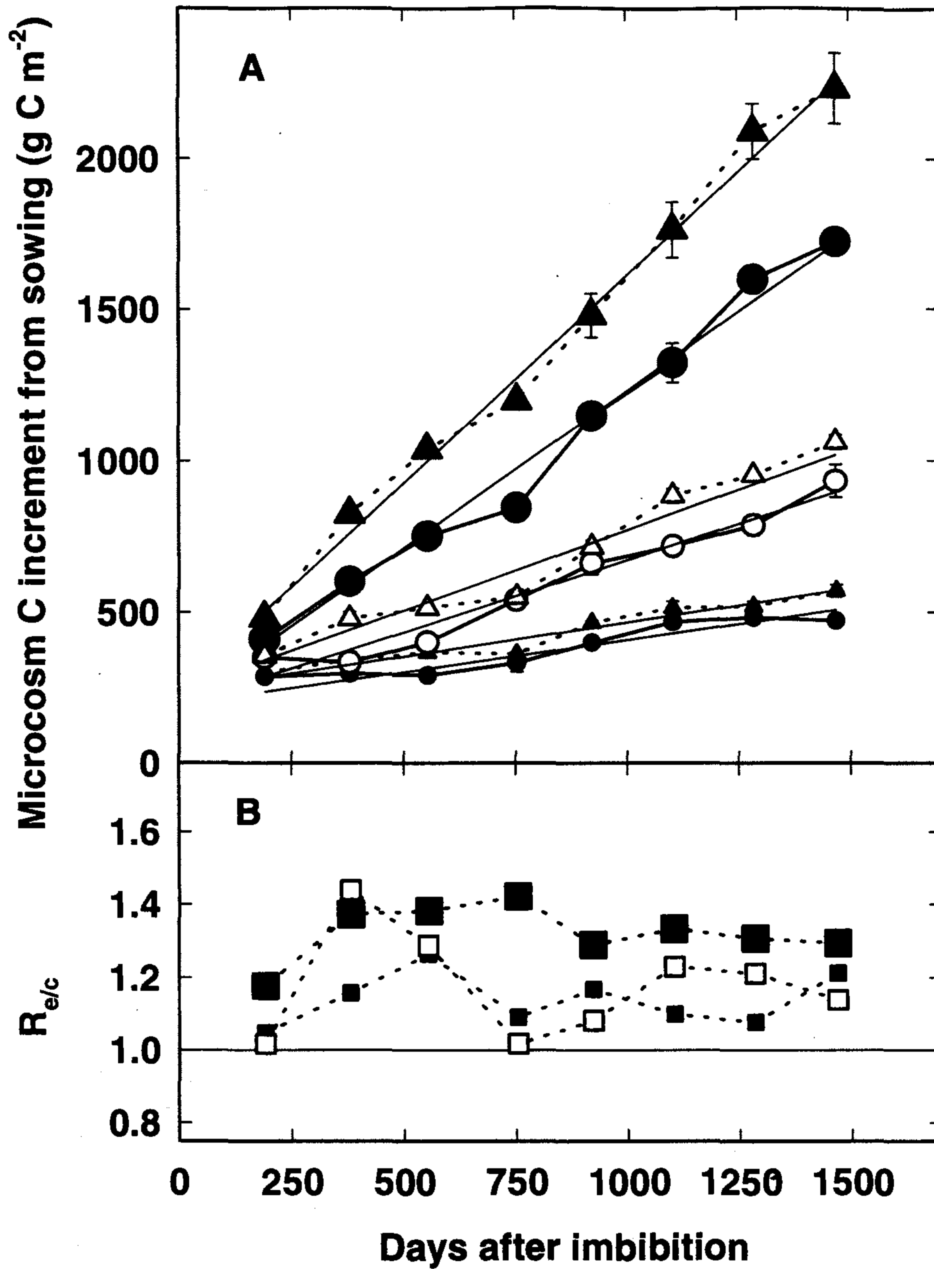


Figure 6.3 Total microcosm carbon increment above that present at sowing. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤) with solid, thick joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. Fine lines show best fit linear regressions for each treatment. Whole experiment average rates of carbon accumulation were 0.213, 0.226, 0.478, 0.531, 1.04 & 1.37 g C m^{-2} d^{-1} for the CLN, ELN, CMN, EMN, CHN & EHN treatments respectively. The linear regression explained 98% of the variance. (B) CO_2 response ratio, low-N (■), mid-N (□), and high-N(■).

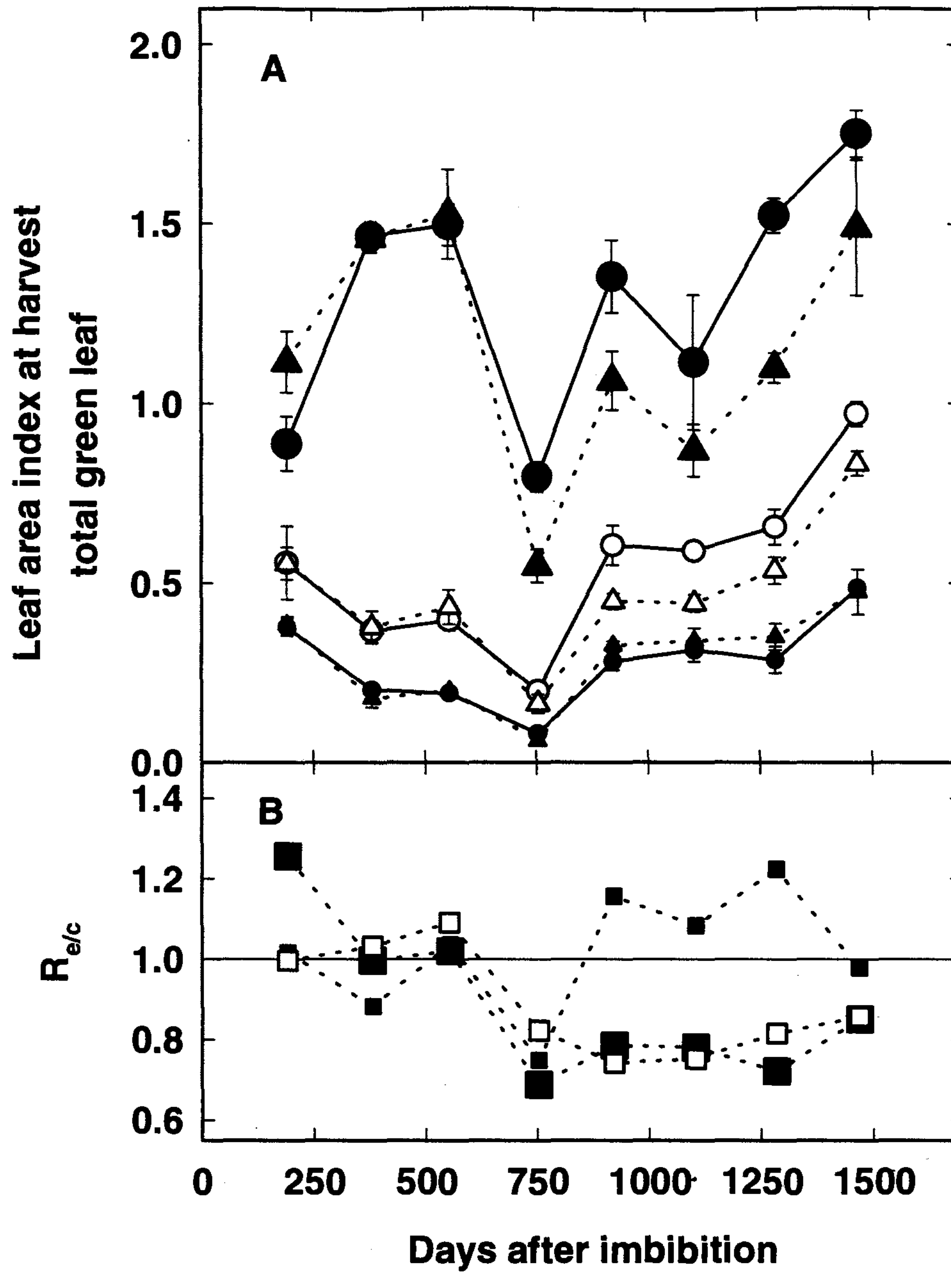


Figure 6.4 Leaf area index at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤) with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio, low-N (■), mid-N (□), and high-N(■).

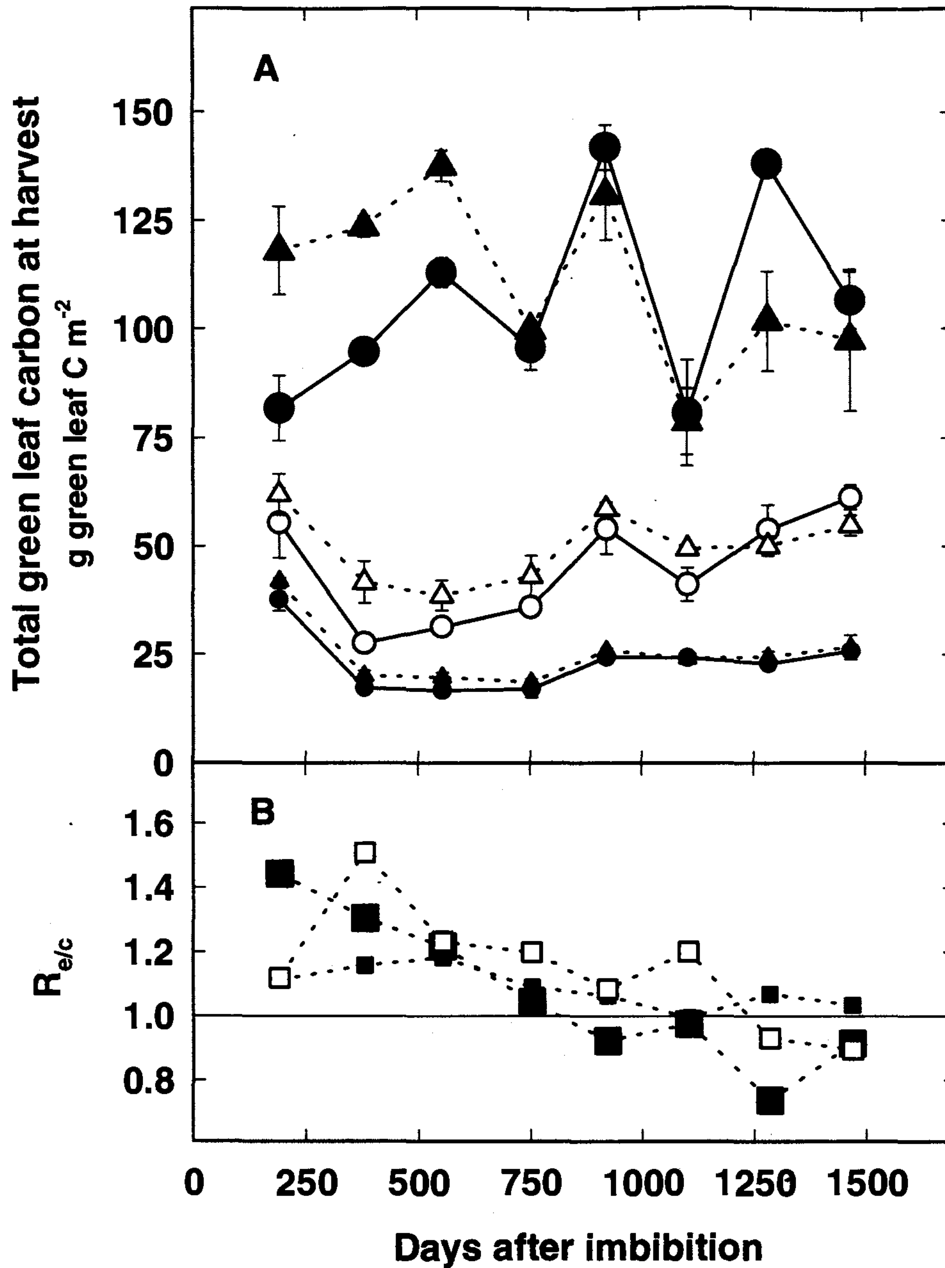


Figure 6.5 Total green leaf carbon per unit ground area at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤) with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio, low-N (■), mid-N (□), and high-N(■).

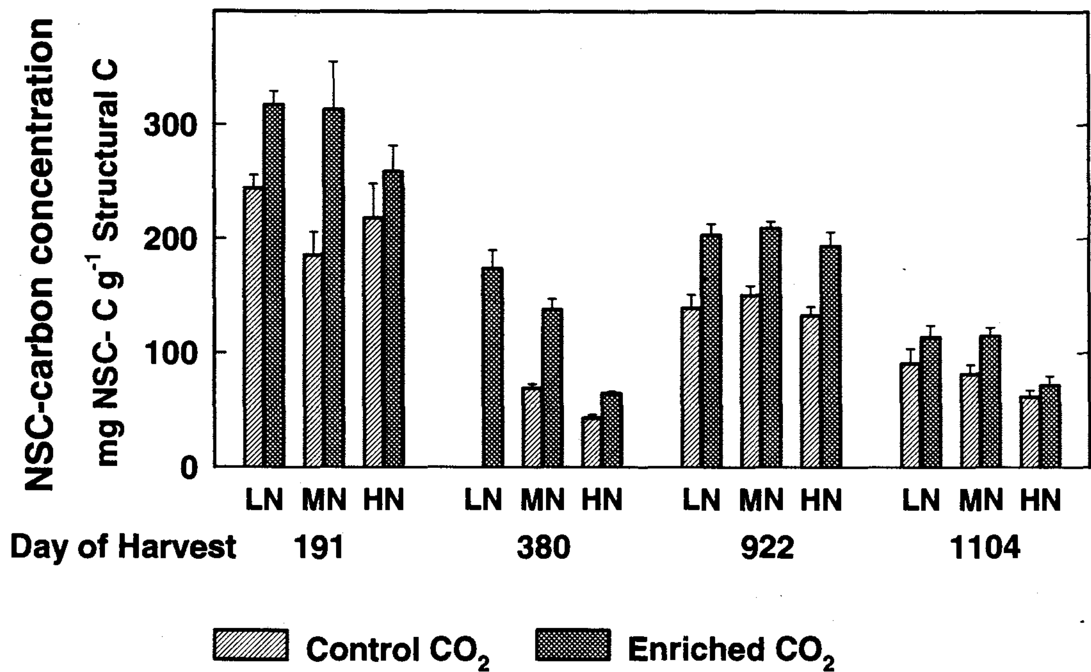


Figure 6.6 Non-structural carbohydrate (NSC) concentration of total green leaf. Low-N represented by LN, mid-N by MN, high-N by HN. NSC concentration was not determined at the 380 day harvest due to insufficient sample availability. Error bars show one standard error of the treatment mean.

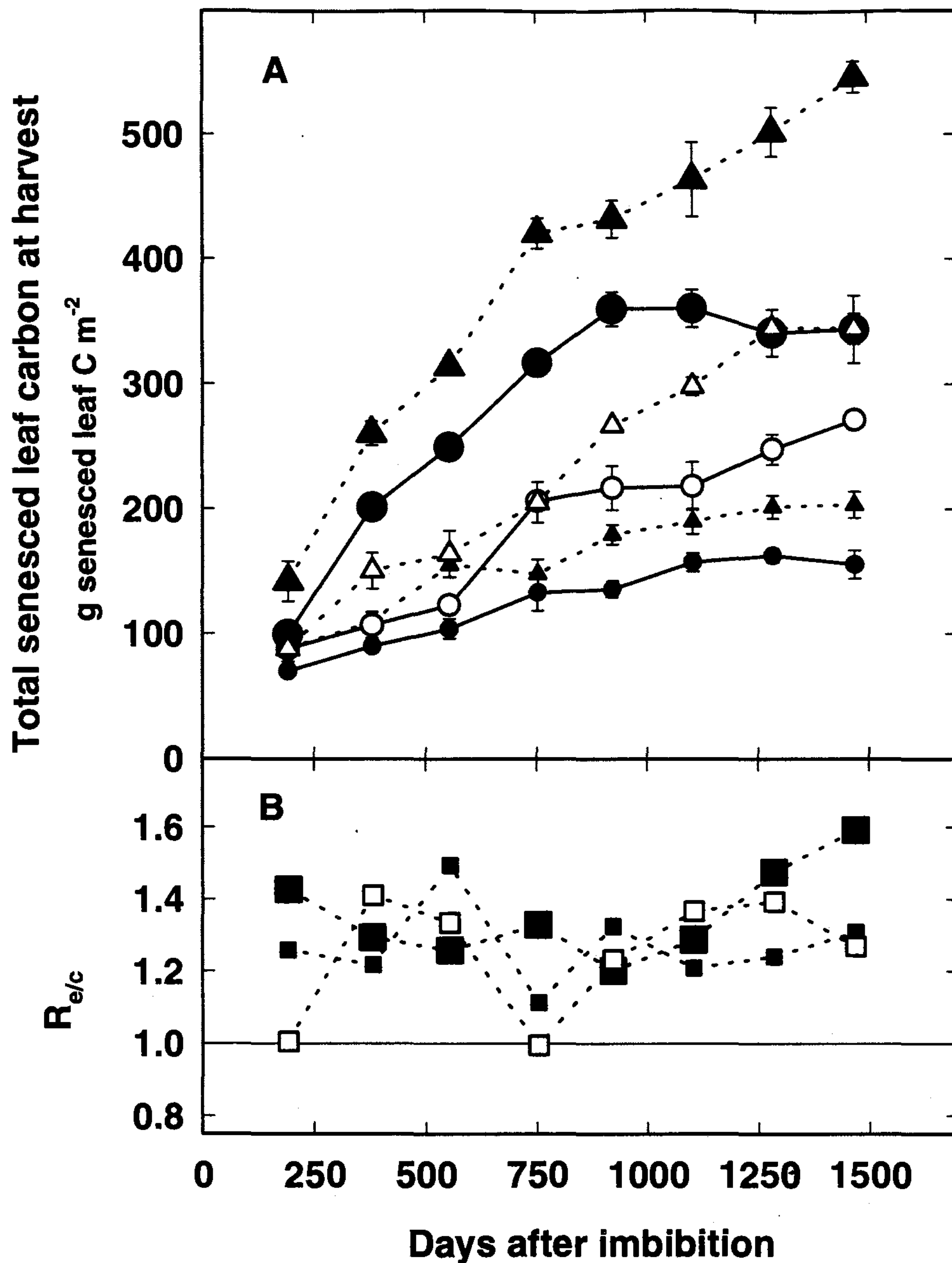


Figure 6.7 Total naturally senesced leaf carbon at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤) with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio, low-N (■), mid-N (□), and high-N(■).

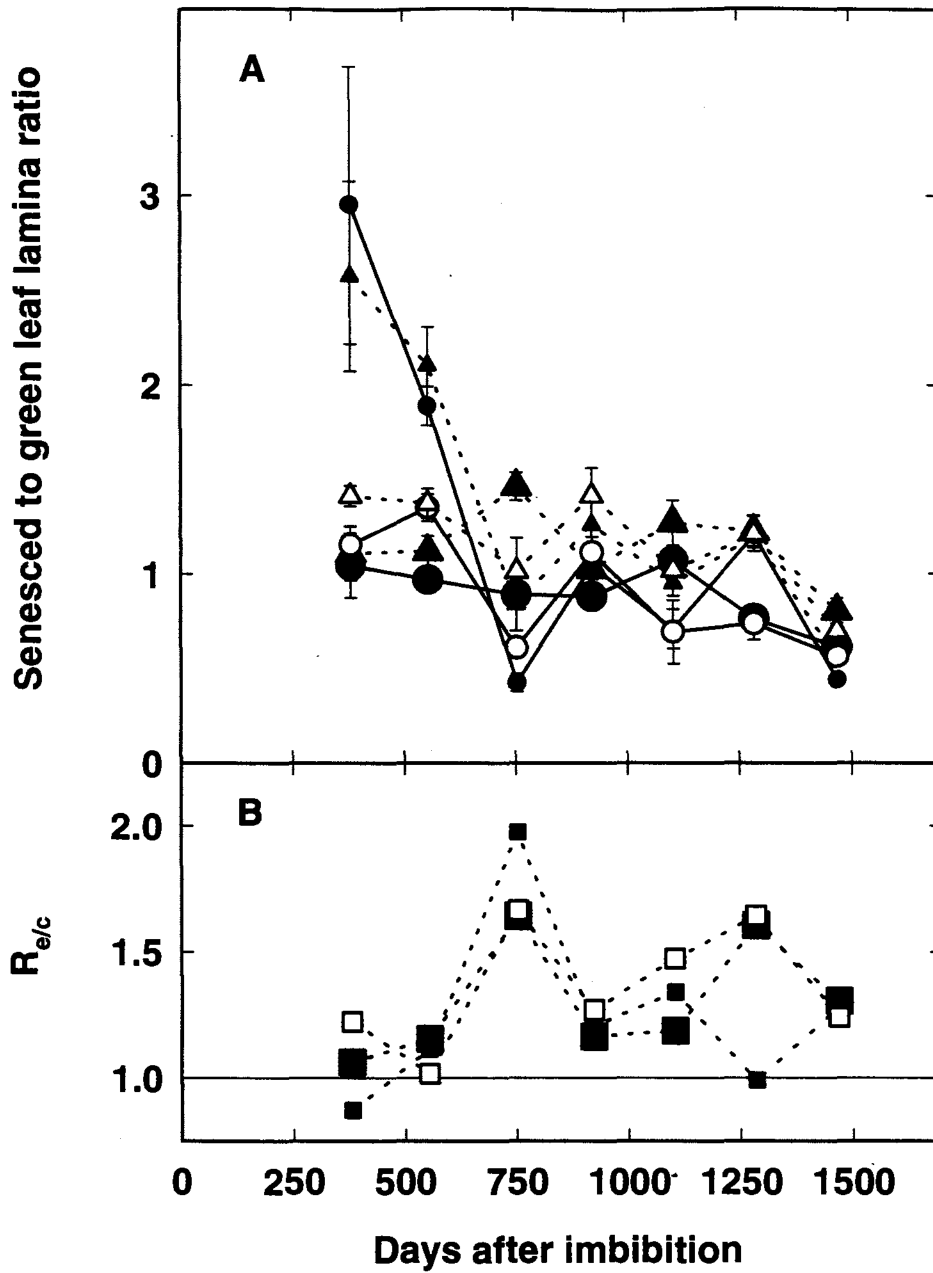


Figure 6.8 Ratio of senesced leaf lamina carbon to green leaf lamina carbon at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio, low-N (■), mid-N (□), and high-N(■).

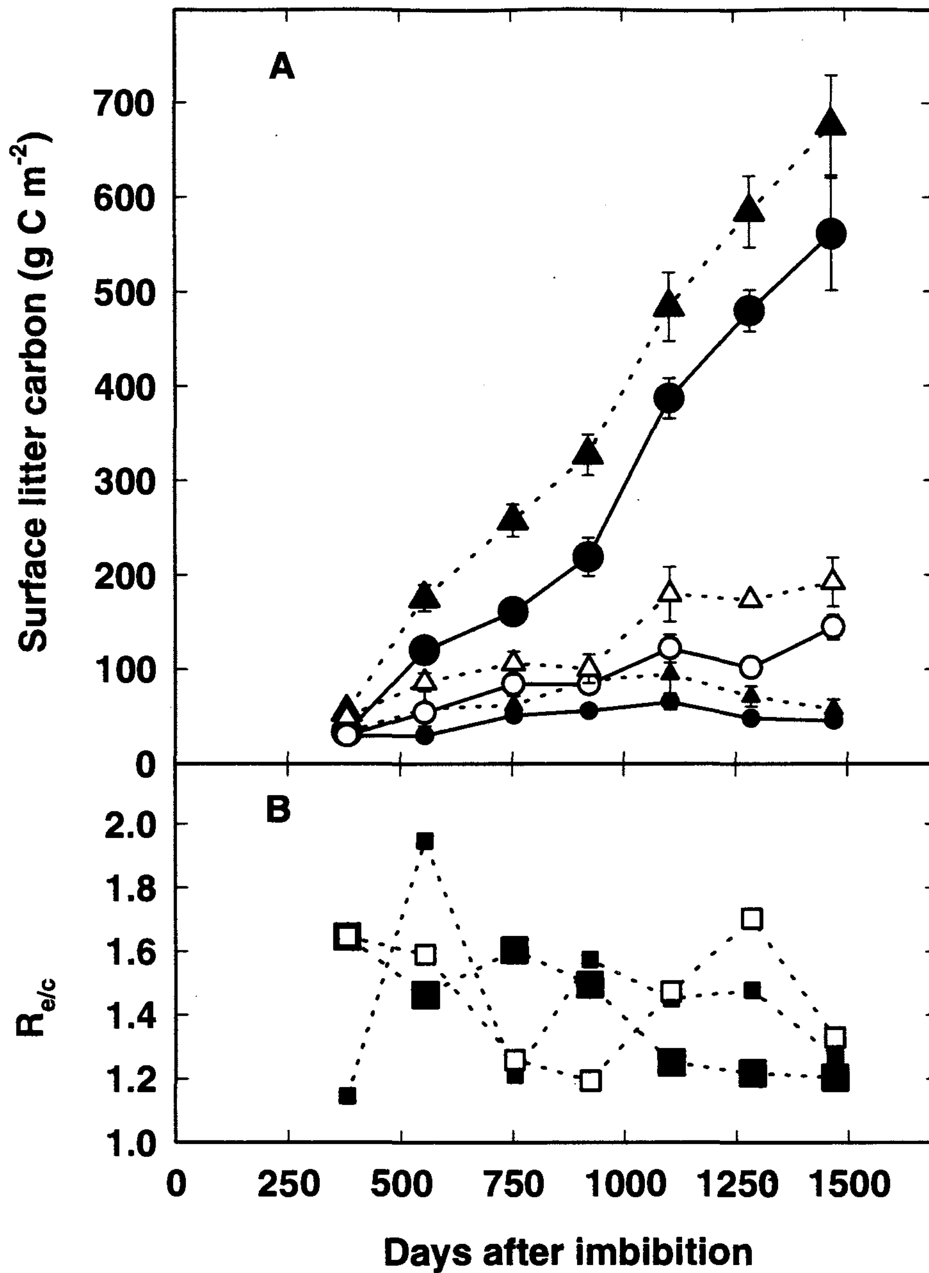


Figure 6.9 Total surface litter carbon at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤) with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio, low-N (■), mid-N (□), and high-N(■).

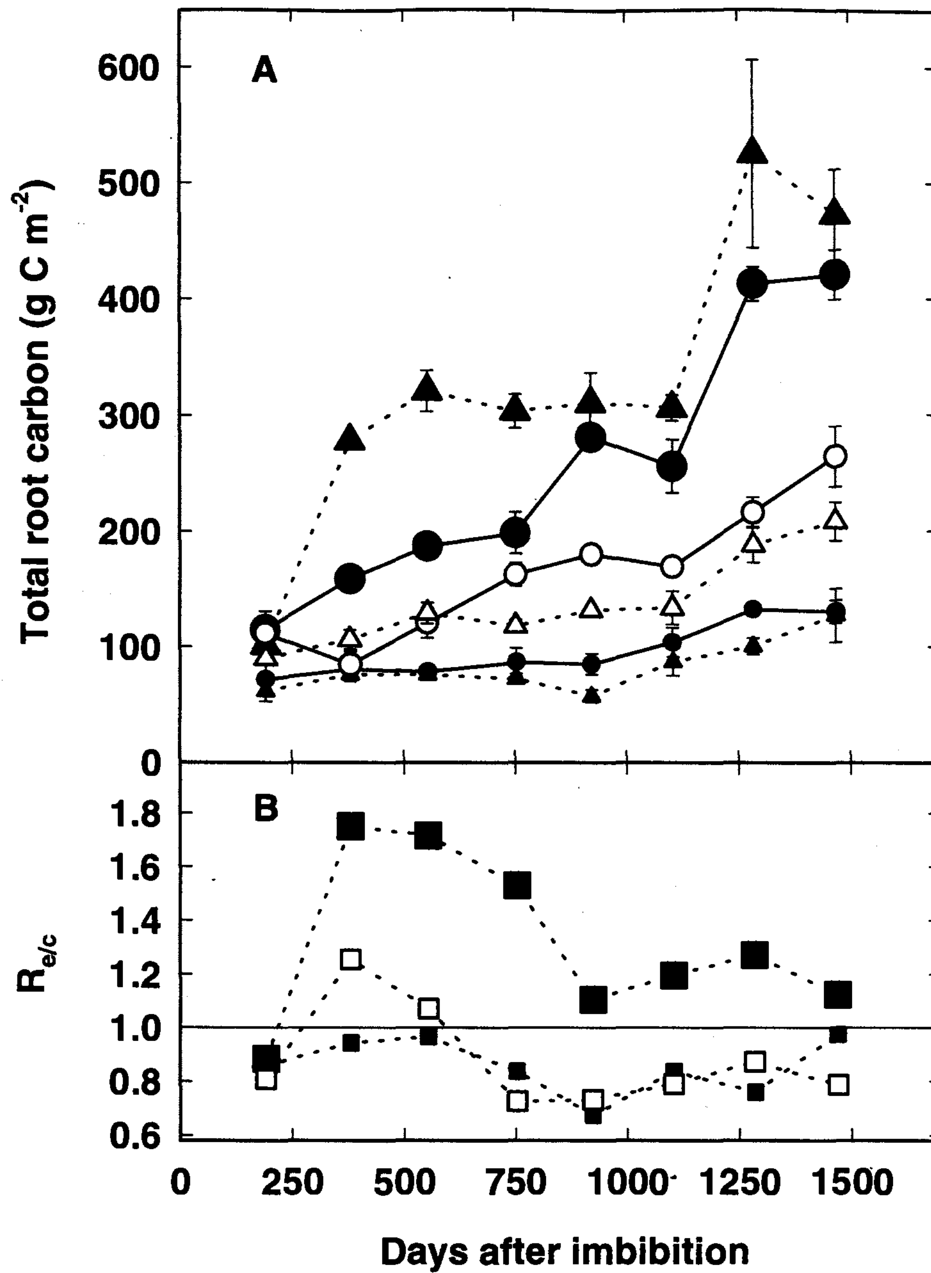


Figure 6.10 Total root carbon at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤) with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio, low-N (■), mid-N (□), and high-N(■).

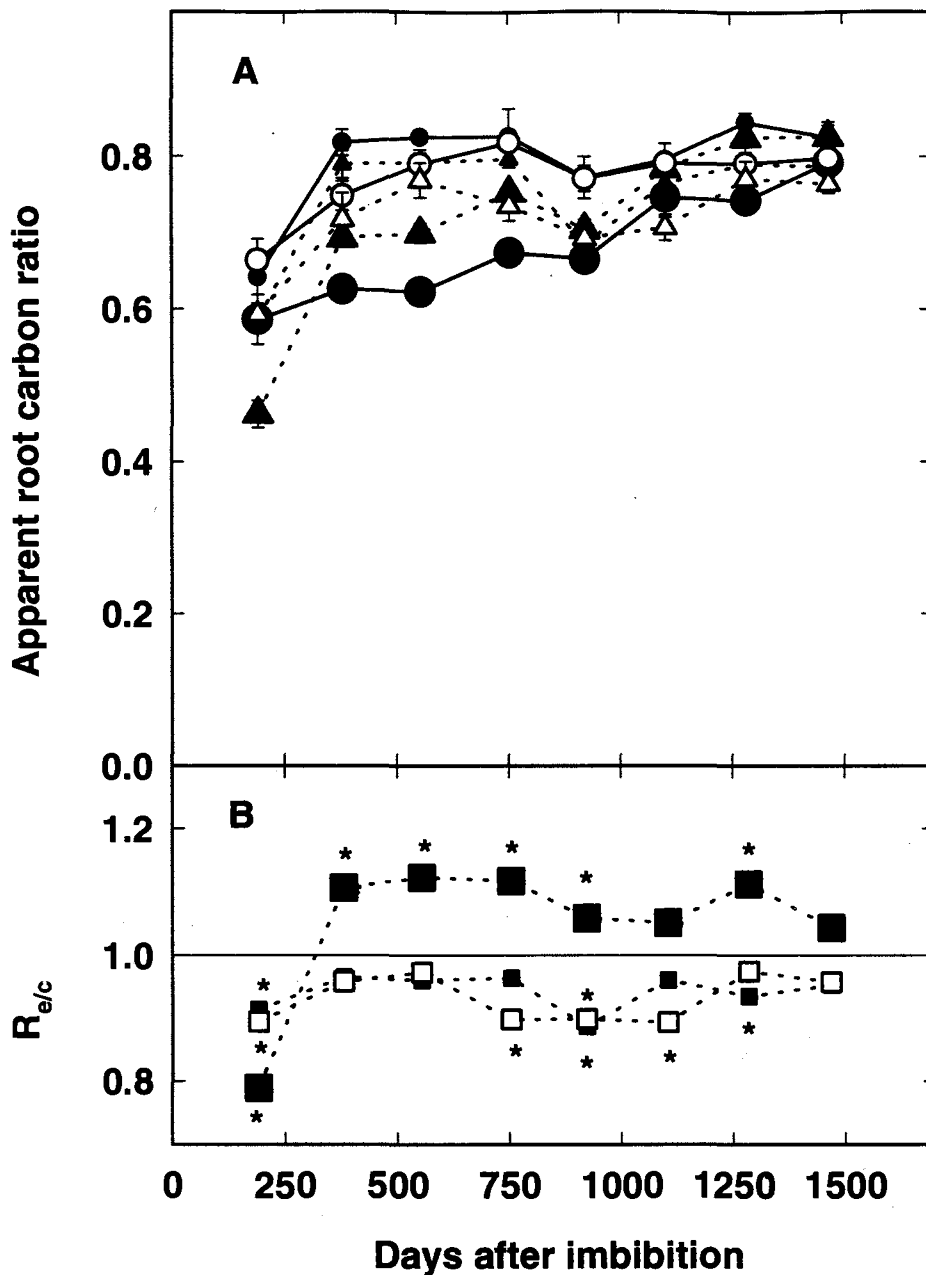


Figure 6.11 Apparent root carbon ratio (A) Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio low-N (■), mid-N (□), and high-N(■). The response of RCR_A to CO_2 was dependant both on harvest and N level (P<0.01). Harvest-N level combinations which exhibited a significant CO_2 effect within the N level (P_L<0.05) are marked by (*).

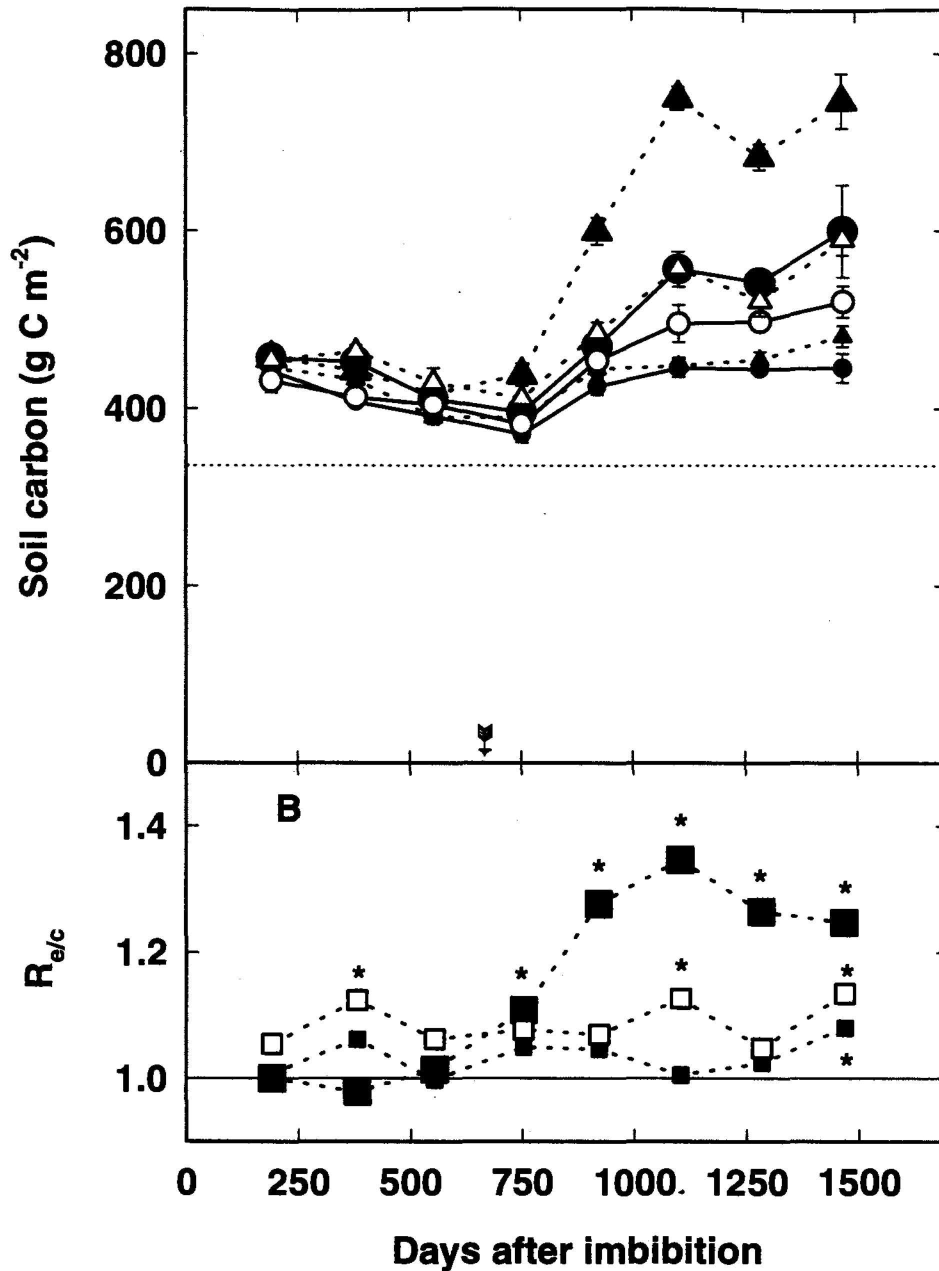


Figure 6.12 Carbon in root-free soil at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. Dotted line at 335 g C m^{-2} in shows initial soil carbon content. (B) CO_2 response ratio low-N (■), mid-N (□), and high-N(■). The response of soil carbon to CO_2 was dependant both on harvest and N level (P<0.001). Harvest-N level combinations which exhibited a significant CO_2 effect within the N level (P_L<0.05) are marked by (*).

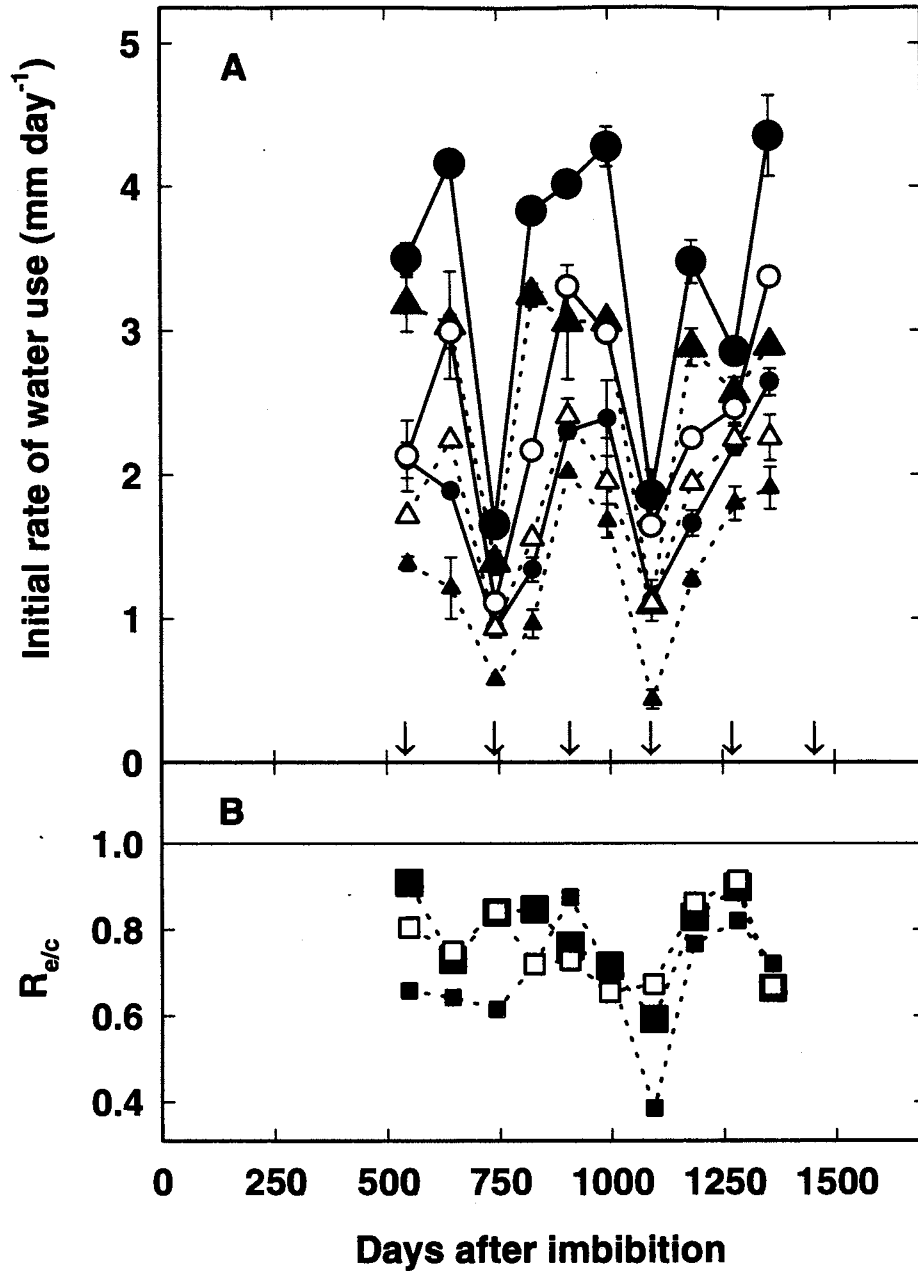


Figure 6.13 Rate of water use over the initial 25 days after re-wetting. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. Arrows show days of defoliation. (B) CO_2 response ratio low-N (■), mid-N (□), and high-N(■).

Chapter 7. Nitrogen accumulation and distribution in *D. richardsonii* swards in response to CO_2 and nitrogen supply over four years of growth

Introduction

Nitrogen is integral to plant and ecosystem function, the carbon and nitrogen cycles being tightly linked through production and decomposition (Parton *et al.,* 1988). Nitrogen limitation often places restrictions on grassland productivity, as well as that of many other natural and agricultural ecosystems (Vitousek & Howarth, 1991). We have seen in chapter 6 that growth at high CO_2 has enabled the simple, *Danthonia richardsonii* microcosm under study to increase its carbon accumulation at levels of nitrogen availability that greatly restrict productivity. In previous chapters the effect of CO_2 on nitrogen use in the single plant has been highlighted. In isolated plants of *D. richardsonii,* growth at high CO_2 increased leaf and plant nitrogen productivity, shifted nitrogen allocation towards tissue involved with nitrogen acquisition, and when nitrogen supply was in relative abundance the lower leaf nitrogen concentrations enabled a greater development of leaf area. These three effects combined to produce increases in total plant carbon at all levels of nitrogen supply tested.

When plants are grown as a community over a time-frame greater than several tens of days they face added stresses. Not only is there competition between plants for light and nutrient, but there is also competition between plants and microbes for nutrient. However, relationships between plants and soil microflora can be mutually beneficial. In the time-frame of weeks to years, there are questions of nitrogen availability as nitrogen becomes immobilised in senesced plant pools. It has been hypothesised that the CO_2 response will be reduced in the longer term owing to increased nutrient immobilisation in both the senesced plant or litter (Melillo *et al.,* 1990) and microbial pools (Díaz *et al.,* 1993). This is discussed further in the next chapter. However, as was shown in the previous chapter, even if this is occurring in this system it has not negated the CO_2 response in total microcosm carbon.

In the longer term (years to decades), if ecosystems are to maintain a response in total system carbon acquisition above that enabled by the increase in C:N ratios, or changes in the distribution of carbon to pools with a higher C:N ratio (Shaver *et al.,* 1992) there must be an increase in total system nitrogen. This may be attained via a decrease in gaseous or leachate

loss of nitrogen from the system, or via an increased gain of nitrogen by the system from biological nitrogen fixation, or from sorption of gaseous forms of nitrogen.

In this chapter, the effect of CO_2 enrichment on nitrogen gain and nitrogen loss from the microcosm is examined using mass balance and ^{15}N techniques. The effect of CO_2 on leaf nitrogen productivity for microcosm carbon gain (NP_M) is determined, defined as

$$NP_M = \frac{1}{N_L} \cdot \frac{dC_M}{dt}$$

where C_M total microcosm carbon content and N_L is total leaf nitrogen. The distribution of nitrogen between the high C:N ratio plant pools and the low C:N ratio soil pool is also examined. In the following chapter some aspects of nitrogen cycling within the microcosm will be explored, further addressing the long term response.

Materials and methods

Experimental protocol is given chapter 6 (page 6-102). Additional methodologies pertaining to the ^{15}N enrichment and ^{15}N natural abundance experiments are outlined below.

^{15}N *Methodology*

^{15}N enrichment experiment

On six occasions between day 901 and 1066, 28.7 mg m^{-2} of ^{15}N as 92.5 atom % ^{15}N enriched $^{15}NH_4{}^{15}NO_3$ (Sigma Chemical Co., nominal 98 atom % ^{15}N) was applied to eight microcosms from each N * CO_2 treatment. On each occasion this was the equivalent of approximately 4.7, 1.5 and 0.5 days of N supply for the low-, mid- and high-N treatment respectively. The $^{15}NH_4{}^{15}NO_3$ was added in solution, directly into the irrigation tubes in each individual microcosm and immediately flushed into the microcosm with each treatment's standard irrigation solution. The total quantity of nitrogen added was little different from a normal application at high-N, however at low-N it was approximately three times the size of a normal single-occasion N application. These applications were spaced evenly over two complete drying cycles (Figure 7.1, for details on "drying cycles" see page 6-104). Applications of ^{15}N commenced before defoliation on day 922, so ^{15}N entered the surface litter pool both via microbial uptake of ^{15}N on application, and from the return of ^{15}N enriched plant material. All microcosms harvested at day 1104 and 1285 had been ^{15}N enriched. Atom % ^{15}N and total nitrogen was determined on a Europa ANCA-NT 20 - 20 Stable Isotope Analyser. Samples were analysed in duplicate and the atom % ^{15}N of the first replicate sample disregarded to avoid problems of ^{15}N carryover between samples within the analyser (Boddey, 1987). Fractional

recovery of applied ^{15}N was calculated as:

$$\text{fractional recovery} = \frac{\text{total } ^{15}N \text{ recovered} - \text{background } ^{15}N}{\text{total } ^{15}N \text{ applied}}$$

Background ^{15}N was calculated individually for each treatment as the treatment-average atom % ^{15}N naturally present in the microcosm at the 1469 day harvest. This may slightly underestimate nitrogen loss, as some ^{15}N must have been added in normal fertilisation between ^{15}N enrichment and the 1469 day harvest. Microcosm natural abundance $\delta^{15}N$ was estimated at the 1104 and 1285 day harvest assuming a linear decline from the $\delta^{15}N$ at sowing to that at day 1469. Proportional loss of added ^{15}N was changed by less than 0.2 percentage points by the use of this correction. Thus any error in this estimation was assumed not to be significant.

Natural abundance ^{15}N

At the 1469 day harvest of microcosm experiment one $\delta^{15}N$ was calculated for each soil and plant fraction as presented in Boddey (1987),

$$\delta^{15}N\,(‰) = \frac{(\text{atom}\%\,^{15}N \text{ in sample} - 0.3663)}{0.3663} \cdot 1000$$

where 0.3663 is the atom % ^{15}N of atmospheric N_2 (Mariotti, 1983).

When the experiment commenced it was not known that a rapid and accurate means of determining atom % ^{15}N would be available and the $\delta^{15}N$ of the analytical grade NH_4NO_3 used in normal irrigation solutions was not determined. For estimations of potential microcosm $\delta^{15}N$ neglecting nitrogen loss or gain from the microcosm, $\delta^{15}N$ was determined for analytical grade NH_4NO_3 from suppliers that were used in the experiment. The $\delta^{15}N$ of these batches of NH_4NO_3 ranged from -2.3 to 1.0‰, compared to -3.0 to 0.85‰ for NH_4NO_3 from various sources as reported in Shearer *et al.* (1974) and Högberg (1990). Thus it is expected that the $\delta^{15}N$ of NH_4NO_3 applied throughout the experiment would be close to the measured range of -2.3 to 1‰.

The microcosms used in these calculations were growing over the period of ^{15}N enrichment for nitrogen loss estimation. There is the possibility that there was ^{15}N contamination of these microcosms via foliar exchange of $^{15}NH_3$ (Janzen & Gilbertson, 1994), especially from the high-N swards to the low-N swards (Lemon & van Houtte, 1980; Högberg, 1991). Some $\delta^{15}N$ data was obtained for samples collected prior to ^{15}N enrichment. Comparison of these with the appropriate samples from the 1469 day harvest did not reveal any systematic differences that could be attributed to ^{15}N contamination (not presented).

Leaf nitrogen productivity for microcosm carbon gain

Leaf nitrogen productivity for microcosm carbon gain (NP_M) was calculated with the computer program HPCURVES (Hunt,R & Parsons, 1974). Green leaf nitrogen on a ground area basis at harvest was used in NP_M calculation. This might underestimate NP_M as green leaf nitrogen was reduced at defoliation. Linear fits on microcosm C content and cubic fits on green leaf nitrogen content were applied. Differences between CO_2 treatments at each harvest were tested by *t*-test.

Results

Only pertinent significant main effects or interactions are presented. Complete tables of *F* probabilities for total nitrogen contents and concentrations of the major fractions are included in the appendices (Appendix 2 & Appendix 3).

Nitrogen balance - nitrogen gain and loss

The increment of microcosm total nitrogen content above that present at sowing, and its relationship to the quantity of nitrogen added is shown in Figure 7.2. This increment is net of all losses and gains of nitrogen by the microcosm. Averaged over all harvests there was a significant effect of N supply rate on the difference between the nitrogen increment from sowing and applied nitrogen ($P<0.001$). At low-N the increment was significantly greater than the quantity of nitrogen applied ($P_L<0.001$). At mid-N the quantity recovered from the system was not different from that applied, while at high-N less nitrogen was recovered than was applied ($P_L<0.001$). An interaction between CO_2, N level and harvest was present (Figure 7.2; $P<0.01$). At high-N there was no difference between recovered and applied N in the high CO_2 treatment. Significantly less nitrogen was recovered than applied over the last 2.5 years of the experiment in the control CO_2 treatment, although more N was recovered at the 380 day harvest. At mid-N the high CO_2 grown swards had accumulated less nitrogen than the controls on day 753 and 922 ($P_L<0.01$), and on those days less nitrogen was recovered from these swards than was applied ($P_L<0.01$). The reasons for the variable results early in the experiment are not known.

Leachate-nitrogen loss

Leachate-nitrogen loss data was not available for the initial 191 days of growth due to analytical problems associated with the low leachate-nitrogen concentrations. In the period following the 191 day harvest leachate-nitrogen loss differed between N levels ($P<0.001$), becoming greater as N supply rate was increased (31, 37 & 94 mg N m^{-2} yr^{-1} respectively; $P_L<0.001$). This accounted for, on average 1.40%, 0.55% and 0.47% of applied nitrogen, for the

low-, mid- and high-N treatments respectively. Growth at high CO_2 slightly, but significantly reduced leachate-nitrogen loss when averaged over all treatments ($R_{e/c}$=0.97; *P*<0.01).

Nitrogen input in demineralised water

The concentration of mineral nitrogen in the demineralised water used in the nutrient solution was monitored late in the experimental period. Total mineral nitrogen concentration (NH_4^+ + NO_3^-) averaged 31 ± 11 (SD) μg L^{-1} (n = 9). Assuming this concentration applied over the experimental period the total nitrogen application in demineralised water would have been approx 76, 92 and 132 mg m^{-2}, or 0.9, 0.3 and 0.2 % of applied N in the low-, mid- and high-N treatments respectively. These values may be over estimates of nitrogen addition as the solution was acidified (pH 3.1) with HCl to inhibit microbial growth during sample concentration (chapter 2, page 2-25). Thus NH_3 may have been absorbed from the atmosphere as the equilibrium for the reaction $NH_4^+ + H^+ \leftrightarrow NH_3$ is moved to the left under low pH conditions (Freney *et al.,* 1983). Glass distilled water yielded 32 ± 7 (SD) μg L^{-1} (n = 4) of mineral nitrogen in the same procedure, and when the distilled water was not acidified total mineral nitrogen was reduced by approximately 30%

^{15}N: enrichment experiment

There were no significant CO_2 or harvest effects on the fraction of applied ^{15}N lost from the microcosms (Figure 7.3). As the rate of N supply increased, the fractional ^{15}N loss decreased (*P*<0.001). The high-N treatment was the only treatment in which the daily nitrogen application on the days of ^{15}N addition was within the normal single-occasion nitrogen application range (see discussion). When the analysis was restricted to high-N, CO_2 enrichment reduced nitrogen loss (*P*<0.05).

Leachate-^{15}N loss

Total Leachate-^{15}N loss was 48 ± 9.2 μg ^{15}N m^{-2} over the experimental period, averaged over CO_2 and N levels. This accounted for less than 0.03 ± 0.01% (SE) of applied ^{15}N. There were no significant treatment effects on total leachate-^{15}N loss.

^{15}N: natural abundance measurements

Total microcosm $\delta^{15}N$ tended to be reduced by CO_2 enrichment in non-^{15}N enriched microcosms at day 1469 when averaged over N level, although the reduction was not significant (Figure 7.4). There was an interaction between CO_2 and N level (*P*<0.10) with $\delta^{15}N$ significantly lower in the EHN treatment than in the CHN treatment (P_L<0.05). As N level increased, $\delta^{15}N$ was reduced (*P*<0.05). Initial $\delta^{15}N$ of the soil at sowing was 4.6 ± 0.1‰. Total

system $\delta^{15}N$ was significantly lower than it was at sowing in the EMN, CHN & EHN treatments at harvest (P_t<0.05).

No experimental treatment affected $\delta^{15}N$ of leachate-nitrogen, which was determined in the intervals preceding the 1285 (non-^{15}N enriched microcosms) and 1469 day harvests. Average $\delta^{15}N$ was 7.5 ± 0.4‰ (SE).

Total green leaf $\delta^{15}N$ was lower at high CO_2 (Figure 7.4; *P*<0.001), as was total senesced leaf $\delta^{15}N$ (not presented; *P*<0.01) and root $\delta^{15}N$ (not presented; *P*<0.01), while there were no significant CO_2 effects on root-free soil $\delta^{15}N$, (Figure 7.4), although the trends were the same as those for total system $\delta^{15}N$ (Figure 7.4). Total green leaf $\delta^{15}N$ increased as N supply rate increased (*P*<0.001).

Total system nitrogen concentration

The nitrogen concentration of the total plant-soil system was lower at high CO_2 (Figure 7.5; *P*<0.001). The CO_2 effect interacted with N supply (*P*<0.01). The CO_2 effect was significant at all N levels (P_L<0.01), although the response increased from an $R_{e/c}$ of 0.93 at low-N to 0.88 and 0.82 at mid- and high-N respectively.

Nitrogen content of fractions

Green leaf nitrogen

Green leaf nitrogen concentration per unit green leaf C (leaf nitrogen concentration) was reduced on average at high CO_2 (Figure 7.6; *P*<0.001), with an $R_{e/c}$ of 0.69. The CO_2 response interacted with N supply rate (*P*<0.05), with the mid-N treatment exhibiting the greatest CO_2 response ($R_{e/c}$=0.66) and lowest CO_2 response was at low-N ($R_{e/c}$=0.72). Variation in leaf nitrogen concentration between harvests differed with N supply. At high-N each consecutive harvest had a different leaf nitrogen concentration (P_L<0.05), with the summer, high radiation harvests having a lower concentration than the winter, low radiation harvests. When corrected for non-structural carbohydrate content, total (structural) green leaf nitrogen concentration was reduced by CO_2 (*P*<0.001) at the harvests at which it was determined (Figure 7.7).

Growth at high CO_2 significantly reduced total green leaf nitrogen per unit ground area at harvest when averaged over other treatments (Figure 7.8; *P*<0.001), with an $R_{e/c}$ of 0.72. The CO_2 effect interacted with harvest and nitrogen supply as the CO_2 effect developed over time (Figure 7.8; *P*<0.01).

Leaf nitrogen productivity for microcosm carbon gain

Growth under CO_2 enrichment increased NP_M at all harvests at low- and high-N (P_t<0.05; Figure 7.9). At mid-N, NP_M was increased by CO_2 enrichment at all harvests (P_t<0.05) except those at day 380 and 554. However, the trend was always for an increase at high CO_2.

Senesced leaf nitrogen

Total senesced leaf nitrogen per unit ground area (Figure 7.10) was decreased by growth at high CO_2 when averaged over all treatments (*P*<0.01). There was a weak interaction between CO_2 and N supply rate (*P*<0.07), with reductions at high CO_2 at mid and high-N (P_L<0.05), but not at low-N when averaged over harvest.

Surface litter nitrogen

When averaged over all treatments total surface litter nitrogen per unit ground area was decreased by growth at high CO_2 (Figure 7.11; *P*<0.01). However, there was a significant interaction between CO_2, harvest and N supply (*P*<0.01) such that the only significant CO_2 effects were in the high-N treatment at and after day 1104 (P_L<0.05). At low-N, litter nitrogen peaked at day 1104 and declined slightly after that harvest, while at mid- and high-N litter nitrogen continued to rise over the experimental period.

Root nitrogen

Root nitrogen concentration (per unit root carbon Figure 7.12) was reduced by CO_2 enrichment when averaged over all other treatments (*P*<0.001). The reduction was significant at all N supply rates, (P_L<0.01), however the CO_2 effect increased with increasing N supply, from an $R_{e/c}$ of 0.89 at low-N to 0.76 at high-N (*P*<0.001). Root nitrogen concentration on a structural C basis was calculated at day 922 and 1104 (Figure 7.7). There was an interaction between CO_2 and N level (*P*<0.05), where the only significant reductions in structural root nitrogen concentration at high CO_2 were at mid- and high-N (P_L<0.05).

Total root nitrogen per unit ground area was on average lower in swards grown under CO_2 enrichment (Figure 7.13; *P*<0.001). However, there was a significant interaction between CO_2 level and rate of N supply (*P*<0.01). At low- and mid-N the $R_{e/c}$ was 0.76 and 0.70 respectively (P_L<0.001) while at high-N there as no difference between the CO_2 treatments.

Soil nitrogen

Total root-free soil nitrogen was increased by growth at high CO_2 when averaged over all other treatments (Figure 7.14; *P*<0.01). There was an interaction between CO_2, N level and harvest

(Figure 7.14; $P<0.01$). The CO_2 effect developed over time, and was not significant by the final harvest at low-N.

Soil pH

Soil pH decreased as N supply rate increased when averaged over all treatments (Table 7.1; $P<0.05$), and varied with depth ($P<0.001$). The surface 25 mm had a lower pH than the rest of the pot ($P_L<0.05$).

Soil Bulk Density

Growth at high CO_2 increased bulk density when averaged over all other treatments (Table 7.2; $P<0.001$). Bulk density decreased as N supply increased ($P<0.001$). An interaction occurred between N supply and CO_2 level ($P<0.01$). The CO_2 effect declined as N supply increased and was significant at low- and mid-N ($R_{e/c}$=1.08 & 1.06 respectively; $P_L<0.001$), but not at high-N.

Discussion

Three important results pertaining to the long term response of ecosystems to CO_2 increase are discussed here. First is the ability of the microcosm to retain nitrogen, or to sequester nitrogen from the environment. In this experiment, all treatments have sequestered nitrogen from the environment, and this effect was greater at the lower rates of N supply. Growth under CO_2 enrichment reduced leachate-nitrogen loss, and reduced gaseous losses of nitrogen from the high nitrogen treatment.

The second important group of results is that of nitrogen distribution. Less, or at least no greater, amounts of N were found in senesced plant pools at high CO_2. This may have reduced the response of microcosm carbon gain to CO_2 enrichment in the short term, as these pools have low-nitrogen concentrations, which are very responsive to CO_2 enrichment (Figure 8.1, Figure 8.3). However, the concurrent increase in size of the soil nitrogen pool under CO_2 enrichment may increase the rate of nitrogen supply in the long term. This will be further discussed in chapter 8.

Thirdly, the large increase in leaf nitrogen productivity under CO_2 enrichment in isolated plants was expressed in the microcosms, as increased nitrogen productivity for net microcosm carbon gain.

Microcosm exchange of nitrogen with the environment

Nitrogen loss

Total leachate-nitrogen loss increased as N supply rate increased. This loss accounted for less than 1.5% of applied nitrogen, and the greatest proportional loss was at the low rate of N supply (page 7-152). Proportional loss of applied-^{15}N as leachate (page 7-153) was an order of magnitude less than the loss of total leachate-nitrogen . This suggests that nitrogen loss via leachate was mostly derived from "old" mineralised organic nitrogen, rather than newly added fertiliser nitrogen.

Total loss of applied-^{15}N between application and harvest ranged from 26% at low-N to 10% at high-N, averaged over CO_2 treatments. As leachate loss was minute, processes involved with this loss of nitrogen may have been either leaf loss from the swards or gaseous loss of nitrogen. Losses of nitrogen (and carbon) from the sward in leaf fall were very small, as the microcosms were packed to form a continuous canopy, which was almost totally restricted to the dimensions of the microcosm. Visually little material was lost from the microcosms. If this were a significant source of nitrogen loss, it could be expected that there be a greater loss in the guard microcosms than the core microcosms, as the guard microcosms were exposed to the edge of the sward. No such effect was noted. Thus this source of nitrogen loss is regarded as insignificant, and gaseous loss processes are assumed to be the most import source of nitrogen loss.

Processes of gaseous nitrogen loss

Gaseous loss of fertiliser nitrogen is common in the field, and often ranges from 10-30% (Peoples *et al.,* 1995). The magnitude of loss in this experiment is within the expected range of loss for this soil type by ammonia volatilisation and denitrification (Meisinger & Randall, 1991). There was no significant increase in loss of applied-^{15}N between harvests, suggesting that the majority of the nitrogen loss occurred before or during the initial assimilation of the fertiliser nitrogen in this experiment.

The two major processes of gaseous nitrogen loss from soil are ammonia volatilisation and denitrification (Peoples *et al.,* 1995). Discrimination against ^{15}N in loss processes may have resulted in underestimates of total nitrogen loss, however in this context the discriminations of ~3% against ^{15}N in denitrification (Handley & Raven, 1992) and ~3% in ammonia volatilisation (Farquhar *et al.,* 1983) are minor.

More serious may be the addition of greater than normal amounts of nitrogen to the low- & mid-N swards on ^{15}N application. Rates of gaseous loss often increase as the size of the soil NO_3^- (Fillery, 1983) and NH_4^+ pools increase (Freney *et al.,* 1983). This effect is observed in the field, with losses of applied nitrogen often increasing as the rate of addition increases, resulting from an imbalance between nitrogen supply and demand, and the accumulation of mineral nitrogen (Peoples *et al.,* 1995). Thus the loss at low- and mid-N may have overestimated loss of total applied nitrogen.

The likely contribution of ammonia volatilisation and biological denitrification to the nitrogen loss will briefly be discussed.

Ammonia volatilisation

Volatile loss from the soil

Losses of nitrogen from the soil via ammonium volatilisation are strongly dependant on soil solution pH. As solution pH increases above 8 the $NH_4^+ + H^+ \leftrightarrow NH_3$ equilibrium moves sharply to the right. At a pH of 6.5 the relative NH_3 concentration is less than 1% of NH_4^+ concentration (Freney *et al.,* 1983). Soil pH of all treatments was below 6.5 in the top 25 mm of soil (Table 7.1). Less than 2% volatilisation loss of applied NH_4NO_3 fertiliser may be expected from the soil surface in these conditions (Meisinger & Randall, 1991). In addition the surface litter layer and canopy may form a strong sink for volatilised NH_3 (Denmead *et al.,* 1976; Lemon & van Houtte, 1980), further reducing net volatilisation from the microcosm. Thus volatile losses of nitrogen from the soil surface probably make only a small net contribution to gaseous nitrogen loss.

Volatile loss from the canopy

Exchange of NH_3 between the canopy and the atmosphere can be a major source of nitrogen loss (eg. Harper et al., 1987). Ammonia is present in the intercellular space of leaves, where it is in equilibrium with ammonia in solution, and originates from processes such as photorespiration (Farquhar *et al.,* 1983) and proteolysis (Peoples & Dalling, 1988; Schjoerring *et al.,* 1993b). In this experiment, exchange of nitrogen with the canopy is thought to form a net gain, rather than a net loss of nitrogen from the microcosm, at least at the lower N levels, as discussed later in this chapter.

Biological denitrification.

In relation to this experiment, two main factors may control biological denitrification, soil moisture content (Fillery, 1983), and the availability of readily degradable organic matter as a microbial energy source (Burford & Bremner, 1975; Weier *et al.,* 1993).

As the elevated CO_2 swards experienced higher average soil moisture contents (θ_v; Figure 7.1), the lack of an increase in nitrogen loss in this treatment may be surprising. In fact, at high-N, CO_2 enrichment reduced net nitrogen loss (Figure 7.2), which is supported by microcosm natural abundance $\delta^{15}N$ data, as discussed later. Denitrification generally only occurs when water filled pore space (WFPS, the proportion of total pore space that is occupied with water) is above 60% (Linn & Doran, 1984; Weier *et al.,* 1993). Assuming a particle density of 2.65 g cm^{-2} (Brady, 1984; Linn & Doran, 1984) WFPS was calculated and is shown in Figure 7.1. As bulk density was increased by CO_2 enrichment at the two lower N levels, and decreased with increasing N supply (Table 7.2) the θ_v at 60% WFPS was decreased as N level decreased, and the decrease was greater at high CO_2. Thus the length of time that soil water levels potentially favoured denitrification was short at high-N, and similar between CO_2 levels. As N level was reduced, the length of time that WFPS was above 60% increased. This increase was greater at high CO_2, due both to increases in bulk density and decreases in water use. Thus, soil moisture contents under CO_2 enrichment were more favourable for denitrification than under ambient CO_2, and conditions were more favourable for denitrification at the lower N levels.

The presence of readily degradable organic matter is necessary as an energy source for the bacteria involved in denitrification (Burford & Bremner, 1975; Weier *et al.,* 1993). Thus growth at high CO_2 may have been expected to promote denitrification as soil carbon (chapter 7), and potentially mineralisable nitrogen (chapter 8) were increased at high CO_2. However, if this material was of a lower nitrogen concentration at high CO_2, nitrogen may have been immobilised and denitrification reduced (Craswell, 1978).

Thus the reduction in nitrogen loss as N supply rate increased may be the result of a reduction in the amount of time that soil moisture conditions were favourable for denitrification. Alternately, they may have been induced at the lower N levels by an imbalance between nitrogen supply and demand at ^{15}N application.

Estimated gross nitrogen balance

As the microcosms exhibited a positive or neutral net nitrogen balance in most treatments (Figure 7.2), and significant quantities of nitrogen were lost from the system (Figure 7.3), a significant net input of nitrogen, above that applied to the microcosms must have occurred. A simple mass balance model was used to estimate gross nitrogen input from total microcosm nitrogen at day 1469, and results are shown in Table 7.3. As there are some uncertainties pertaining to the estimates of nitrogen loss and nitrogen application, three sets of parameters were used.

Model A addresses the problem of higher than normal applications of nitrogen to the low- and mid-N treatment during ^{15}N application, by assuming gaseous loss over all treatments to be equivalent to the average loss at high-N. Total leachate loss of nitrogen per leaching event was assumed to be the same in the initial 191 days of the experiment as that after (page 7-152). Background nitrogen concentration in demineralised water was assumed to be 31 µg L^{-1} throughout the experimental period (page 7-153). Thus model A assumes minimal gaseous loss of nitrogen with no CO_2 effect on this loss, maximal nitrogen gain in demineralised water (glass distilled blank not accounted for) and hence minimal nitrogen gain from the "unknown" source.

Model B assumes that the gaseous loss estimates are correct, each treatment's individual loss, averaged over harvest being applied. Consistency of leachate loss was assumed between the first 191 days of the experiment and that after. Background nitrogen concentration in demineralised water was assumed to be 23.5 µg L^{-1}, that found in unacidified distilled water.

Model C also assumes that the gaseous loss estimates are correct, each treatment's individual loss, averaged over harvest being applied,. There was large mineral nitrogen pool at sowing, possibly leading to greater leachate loss during the first 191 days as the swards established. Thus leachate loss of nitrogen was assumed to be 50% greater in the first 191 days than in the rest of the experiment. Background nitrogen concentration in demineralised water was assumed to be 23.5 µg L^{-1}, that found in unacidified distilled water.

The results from these estimations (Table 7.3) show that there were significant gains of nitrogen to the system at low-N and in the CMN treatment in each model, and in the EMN treatment in model B and C. Model B and C showed trends for a significant increment in the high-N treatments. There is little difference between model B & C, showing insensitivity to assumptions on leachate nitrogen loss. Considering natural abundance $\delta^{15}N$ results (below), model B or C probably gives the best approximation of total microcosm nitrogen gain above

that added as fertiliser. There are many reports of nitrogen accumulation in soil-plant systems of this magnitude under non-leguminous plants, some of which are outlined in chapter 1 (eg. Parker, 1953, 1957; Mariakulandai & Thyagarajan, 1958; Moore,AW, 1963; Dart, 1986; Powlson *et al.*, 1986).

Sources of nitrogen gain

The potential sources of nitrogen gain in this experiment were dry deposition of nitrogen and associative or free-living N_2 fixation. An estimation of input of nitrogen from atmospheric ammonia sorption follows, with supportive $\delta^{15}N$ data. The potential role of dinitrogen fixation in the accumulation of the extra nitrogen is then discussed.

Deposition of nitrogen

Empirical estimates

Wet and dry deposition of nitrogen onto the land surface is globally an important source of nitrogen for vegetation, accounting for between approximately 0.1 and 2.0 g m^{-2} yr^{-1} (see Gifford *et al.*, 1996c). As the swards were not exposed to rainfall, wet deposition would not have occurred. The air entering the Phytotron is passed through electrostatic precipitators, so most particulate matter should be removed from the incoming air (Morse & Evans, 1962), leaving NH_3 absorption as the major nitrogen entity potentially involved in dry deposition in this experiment. Uptake of ammonia by concentrated sulphuric acid in petri dishes in the glasshouses averaged 0.5 g m^{-2} yr^{-1} over a 1 week period. The sulphuric acid should have formed an infinite sink for ammonia. Hence these values would be an upper limit on N gain from dry deposition of ammonia, assuming this rate of accumulation applied over the whole experimental period.

Atmospheric ammonia concentrations in the Canberra region range from about 0-5 µg NH_3-N m^{-3} (Denmead *et al.*, 1974, 1976 and personal communication O.T. Denmead). Assuming the relationships for *L. multiflorum* (chapter 1) approximate those for *D. richardsonii*, the empirical model of atmospheric NH_3-N uptake of Whitehead and Lockyer (1987) was applied to the swards of this experiment (Table 7.4). At high-N the model was assumed to be inappropriate as leaf nitrogen concentrations in the high-N treatment were high enough to expect a canopy compensation point higher than the atmospheric NH_3-N concentration (Sutton *et al.*, 1993b). This may have also been the case in the control mid-N swards. The model predicts atmospheric nitrogen input to be between 1.2 and 2.6 g m^{-2} over the experiment period, or 0.3 to 0.7 g m^{-2} yr^{-1}, within the range of estimated input of 0.2 to 4 g m^{-2} yr^{-1} to unfertilised vegetation in the British Isles (Sutton *et al.*, 1993a). This rate approximates the uptake of the sulphuric acid

trap. Hence, if the atmospheric ammonia concentration were similar throughout the experimental period as when ammonia was trapped in the sulphuric acid, the modelled data should form an upper estimate of nitrogen gain from ammonia deposition in the low-N and EMN swards.

Support from $\delta^{15}N$ measurements

The $\delta^{15}N$ of atmospheric ammonia originating from biological sources is of the range -4.6 to -15.2‰, while that from a coal-fired power station was -4.3 to -7.2‰ (Moore,H, 1977; Freyer, 1978; Heaton, 1987). Thus strongly negative natural abundance foliar $\delta^{15}N$ levels (-2 to -5‰) have been used to infer depositional input of atmospheric nitrogen in rainforest systems (Vitousek *et al.,* 1989; Stewart *et al.,* 1995). To my knowledge no data on $\delta^{15}N$ of atmospheric NH_3-N is currently available for the Australian continent. As the major source of atmospheric ammonia over Australia is biological (Galbally *et al.,* 1980; Denmead, 1990), the $\delta^{15}N$ of NH_3-N in the Canberra region could be expected to be strongly negative. The strongly negative green leaf $\delta^{15}N$ of the low-N and EMN treatments support the sorption of atmospheric NH_3 as a source of nitrogen in this experiment (Figure 7.4). The positive $\delta^{15}N$ in the CMN and high-N treatments suggests that the leaf nitrogen concentration in these treatments may have been too high for significant NH_3 absorption at the encountered atmospheric NH_3 concentrations. Ledgard *et. al.* (1984) observed the plant available nitrogen pool to have a $\delta^{15}N$ about 4‰ lower than the total soil pool. The $\delta^{15}N$ of green shoot at high-N and in the CMN treatment are similar to that which may be expected with a soil of this $\delta^{15}N$ (Figure 7.4).

Thus sorption of NH_3 may have been a source of nitrogen for the low-N treatments and the mid-N enriched CO_2 treatment. However, green leaf nitrogen concentrations and green leaf $\delta^{15}N$ values suggest that NH_3 sorption was not an important source of nitrogen at high-N, or in the mid-N control CO_2 treatment.

Potential inputs of nitrogen from biological fixation

If the above estimates of NH_3-N absorption are correct, approximately 8 g m^{-2} of nitrogen is still unaccounted for in the low-N treatments, and 6 g m^{-2} of nitrogen at mid-N. The most likely source of this nitrogen is biological dinitrogen fixation. Biological dinitrogen fixation would introduce nitrogen into the microcosm with a $\delta^{15}N$ close to that of atmospheric N_2 (Hoering & Ford, 1960), moving total system $\delta^{15}N$ towards zero. Sorption of NH_3 would shift total system $\delta^{15}N$ towards that of the source NH_3-N, which was assumed to be strongly negative (above). However, concurrent gaseous loss processes would increase microcosm $\delta^{15}N$, as loss processes

discriminate against ^{15}N (chapter 1, eg. Högberg, 1990; Högberg & Johannisson, 1993). Changes in total system $\delta^{15}N$ were examined for support of the nitrogen balance data.

Natural abundance of ^{15}N

After four years of growth the natural abundance $\delta^{15}N$ of the microcosm was lower than the starting $\delta^{15}N$ of the soil in the high-N and EMN treatment (Figure 7.4). This might be explained by the addition of fertiliser with a $\delta^{15}N$ less than that of the initial nitrogen pool. Assuming no additional nitrogen input to the microcosm, no nitrogen loss causing fractionation and enrichment of the microcosm, and $\delta^{15}N$ of the fertiliser ranging between -2.3 and 1.0‰, final microcosm $\delta^{15}N$ in the high-N treatment would have been between -2.0 and 1.2‰, and in the low-N microcosms between -0.2 and 2.1‰ (Figure 7.4). Countering the possible decline in total system $\delta^{15}N$ due to nitrogen input (fertiliser addition, nitrogen fixation and deposition), nitrogen loss processes, apart from those involving leachate loss would tend to increase total system $\delta^{15}N$, as ^{15}N is discriminated against (Högberg, 1990; Högberg & Johannisson, 1993). Assuming that the $\delta^{15}N$ of fertiliser applied over the experiment approximated the range measured, these data are supportive of large losses of nitrogen from the system, as observed in the ^{15}N enrichment experiment (Figure 7.3). This then implies other significant inputs of nitrogen into the system, supporting the estimates of Table 7.3.

Microcosm $\delta^{15}N$ tended to be reduced by growth at high CO_2, especially at high-N. This suggests lower losses of nitrogen at high CO_2, assuming $\delta^{15}N$ of the fertiliser is within the range quoted above, supporting data on total leachate-nitrogen loss (page 7-152), total nitrogen balance (Figure 7.2), and loss of applied ^{15}N at the high-N level (Figure 7.3).

In conclusion, the microcosms have gained nitrogen over that which has been applied in fertiliser during the course of the experiment. Some, but not all, of this nitrogen may have been from atmospheric NH_3, especially in the low-N and EMN swards. This was supported by natural abundance ^{15}N measurements. The balance of the nitrogen gain may have been via biological N_2 fixation. Nitrogen loss was lower in EHN swards than CHN swards. Lower total system $\delta^{15}N$ of the EHN treatment support lower nitrogen losses at high CO_2. No CO_2 effect on nitrogen gain was evident. However, this does not preclude a CO_2 response in the longer term, as expected differences are small, and are likely to be lost in measurement error.

Nitrogen concentrations in live tissue and nitrogen distribution

Carbon and nitrogen cycles are tightly linked (Parton *et al.*, 1988), and nitrogen is a component of most ecosystem carbon pools. Thus, another possible route of increased carbon storage in the plant-soil system is a change in distribution of nitrogen within the system, towards those pools with higher carbon to nitrogen ratios (Shaver *et al.*, 1992). This may, however reduce the availability of nitrogen for further plant growth. In the remainder of this chapter the effect of CO_2 on the concentration of nitrogen in live plant tissue, and the distribution of nitrogen within the microcosm will be examined. In the following chapter the effect of CO_2 on some aspects of nutrient cycling, and hence the availability of nitrogen for further carbon fixation will be examined.

Green leaf nitrogen: concentration, content and productivity

Green leaf nitrogen concentration (per unit green leaf carbon) was reduced under CO_2 enrichment at all harvests and at all N levels (Figure 7.6). The oscillations in green leaf nitrogen concentration in the CHN treatment appear to be negatively correlated with radiation at harvest (cf. Figure 6.1). The decrease in nitrogen concentration in response to CO_2 enrichment was also present on a structural carbon basis (Figure 7.7) and is thus not a result of carbohydrate dilution. Reductions in green leaf nitrogen concentration in response to CO_2 enrichment were also observed in *D. richardsonii* when grown as isolated plants under any tested nutrient regime (chapter 3, 4 & 5).

The reduction of leaf nitrogen concentrations under CO_2 enrichment is a common phenomena at the isolated plant level (eg. Arp & Berendse, 1993), and appears to be expressed at the community level and in the field. For community or field grown herbaceous species, leaf nitrogen concentrations of OTC grown tallgrass prairie species were reduced under CO_2 enrichment, including that of the C_4 species within the community (Owensby *et al.*, 1993a, 1994). Shoot nitrogen concentrations were also lower under CO_2 enrichment in mixed swards of *Lolium perenne* L. (perennial ryegrass) and *Trifolium repens* L. (white clover) or mixed swards of *Festuca pratensis* HUDS. (meadow fescue) and *Trifolium pratense* L. (red clover) (Overdieck, 1993), in *Avena barbata* (Jackson *et al.*, 1995) and in *Scirpus olneyi* (Curtis *et al.*, 1989b). These reductions in leaf nitrogen concentrations are probably partly related to increases in leaf carbohydrate content, and, at least in the C_3 species a decrease in leaf protein concentration in response to the improved carboxylation efficiency of Rubisco under CO_2 enrichment (chapter 1).

There are some notable exceptions to the "general rule" of decreased green leaf nitrogen concentration under CO_2 enrichment. *Poa sieberana* (Chapter 3) exhibited no change in leaf nitrogen concentration in response to CO_2 enrichment, possibly related to luxury nitrogen uptake. Nitrogen concentrations of field grown grasses and herbs in naturally CO_2 enriched areas of Italy showed no consistent response, with both strong reductions and increases in response to CO_2 enrichment (Körner & Miglietta, 1994). However, the enriched site was more fertile than the "control", which may explain the increases in nitrogen concentration under CO_2 enrichment. Under controlled conditions leaf nitrogen concentration was not changed in a model tropical plant community by growth at high CO_2 (Arnone,JA *et al.*, 1995).

Field grown swards of *D. linkii* Kunth had green leaf nitrogen concentrations between 20 and 75 mg N g^{-1} C (assuming a 40% by mass C concentration; Lodge & Whalley, 1983; Archer & Robinson, 1988). Thus if leaf nitrogen concentration is used as a measure of nitrogen stress (Greenwood, 1976), the degree of stress imposed on these microcosms is similar to, and greater than that observed in the field.

Total green leaf nitrogen on a ground area basis was reduced under CO_2 enrichment (Figure 7.8). This effect is not commonly observed in the field, as reductions in nitrogen concentration are usually compensated for by increases in above ground biomass, as with the tallgrass prairie experiments (Owensby *et al.*, 1993a, 1994). When the tallgrass prairie was fertilised, above ground nitrogen was actually increased (Owensby *et al.*, 1994). This was also the case for the swards of Overdieck (1993).

Growth under CO_2 enrichment increased leaf nitrogen productivity for net microcosm carbon gain (NP_M) to a similar extent at all N levels, at least by the end of the experiment (Figure 7.9). This demonstrates that a reduction in green leaf nitrogen, either as a result of differences in allocation between CO_2 treatments (chapter 5), or due to a reduction in nitrogen availability at high CO_2 will not necessary negate the effect of CO_2 on carbon gain, as NP_M is increased by up to 100% under CO_2 enrichment.

Senesced leaf and surface litter nitrogen

Senesced leaf nitrogen (Figure 7.10) was generally lower in the high CO_2 microcosms at mid- and high-N, while surface litter nitrogen (Figure 7.11) was lower in the high-N microcosms grown under CO_2 enrichment. These two fractions are discussed further in the following chapter.

Root nitrogen

Root nitrogen concentration (per unit root carbon) was reduced by CO_2 enrichment at all N levels (Figure 7.12). When expressed on a structural C basis this reduction was not significant at low-N (Figure 7.7). A reduction in root nitrogen concentration at high CO_2 was noted in isolated plant studies, where it could be attributed to increased plant size (chapter 5). This effect of plant size cannot be tested at the microcosm level, as the root fraction contains both live and senesced root.

Data on root growth of plant communities in response to CO_2 enrichment is sparse, and data from herbaceous plants grown as communities which include nitrogen parameters are very rare. *Scirpus olneyi* root nitrogen concentration decreased under CO_2 enrichment, while from the presented data it is evident that total root nitrogen increased at high CO_2 (Curtis *et al.,* 1990). However, as this sedge is rhizomatous it is not strictly comparable. Root nitrogen concentration in a tallgrass prairie decreased in response to CO_2 enrichment in one season, but not in another (Owensby *et al.,* 1993a). Root nitrogen concentrations under CO_2 enrichment are discussed further in chapter 8 in relation to nutrient turnover.

Total root nitrogen was lower at high CO_2 in the low- and mid-N treatments, while it was not changed at high-N. This is contrasts with the data of Owensby *et al.* (1993) who found increases in total root nitrogen under CO_2 enrichment in a tallgrass prairie community dominated by C_4 species. Root carbon, and hence nitrogen may have been lower in this experiment due to the higher θ_v reducing allocation to root. The data of Curtis *et al.* (1990) and Owensby *et al.* (1993a) are the only known published data for total herbaceous community or grassland root nitrogen to date. This is an area in which more research is needed, especially as the observed responses are so different, and decomposing root is a major source of nitrogen for continuing plant growth (Abbadie *et al.,* 1992).

In this experiment, no plant pool contained more nitrogen under CO_2 enrichment than under ambient levels of CO_2. The pools immediately associated with carbon fixation, the green leaf and the root nitrogen pools were lower in their total nitrogen content under CO_2 enrichment, implying that total live plant nitrogen was lower at high CO_2.

Soil nitrogen

Soil nitrogen was increased by CO_2 enrichment, although statistically so only at the two higher rates of nitrogen supply (Figure 7.14). As for soil carbon, this is a parameter that is seldom

reported, probably due to heterogeneity and the small treatment effects relative to the large background nitrogen content of topsoils.

As with soil carbon, the increase in soil nitrogen may result from increased root turnover and the accumulation of decomposition products, increased movement of decomposition products from the surface litter layer into the soil, or increased deposition of nitrogen into the rhizosphere by roots. There are additional sources of nitrogen accumulation, those of microbial uptake of applied fertiliser nitrogen, bypassing the role of the plant, and of the accumulation of nitrogen following dinitrogen fixation, as previously discussed.

Deposition of nitrogen into the rhizosphere may correlated well with C deposition, as many of the compounds involved are nitrogenous (Rovira, 1969). Deposition of nitrogen into the rhizosphere as exudate has been measured at between 6 and 11 g N m^{-2} in pasture species (Biondini *et al.,* 1988), and 18-33% of the total wheat plant nitrogen (Janzen, 1990). Soil solution amino-N compound concentration was increased under CO_2 enrichment (chapter 8), as was soil microbial carbon and potentially mineralisable nitrogen (chapter 8). Thus it is impossible to discern if the amino-N is the result of increased deposition of nitrogen into the rhizosphere by root, or the result of increased microbial turnover leading to higher levels amino-N of microbial origin in the soil. Increases in soil nitrogen, if maintained in the longer term, may result in increased plant growth via a larger pool of plant available nitrogen.

Summary of nitrogen accumulation and distribution after 1469 days

After 1469 days of growth, total microcosm nitrogen showed only small CO_2 effects at high-N, which were not significant at that harvest (Table 7.5). Less nitrogen accumulated in green leaf under CO_2 enrichment at all N levels, in root at low- and mid-N, in senesced leaf at mid-N and surface litter at high-N. However, more nitrogen accumulated in the soil at mid- and high-N. This is reflected in the relative distribution of nitrogen (Table 7.5), with less nitrogen present in the green leaf, surface litter, and root pools, and more nitrogen present in the soil pool under CO_2 enrichment. Thus, there was less, or the same amount of nitrogen present in the high C:N ratio plant pools, and more nitrogen in the low C:N ratio soil pool under CO_2 enrichment. All of the pools showed increases in C:N ratio under CO_2 enrichment (Table 7.6). From a mathematical viewpoint the CO_2 response in carbon accumulation was minimised by the greater proportion of nitrogen in the soil under CO_2 enrichment, the pool showing the least flexibility in C:N ratio. The C:N ratio of the total soil pool is presented, rather than that of the incremental gain from sward establishment. As the nitrogen and carbon in the soil at sward establishment

was biological active - attested to by the decrease in soil nitrogen content below levels present at sward establishment early in the experiment (Figure 7.14) - changes in the C:N ratio of the incremental gain may be confounded by changes in the form of the nitrogen and carbon present at sward establishment.

Conclusions

Microcosms from all N levels gained nitrogen from an unidentified external source over the course of the experiment. The gain of nitrogen was larger at the lower N levels than at the high-N level, suggesting that the source of the gain is sensitive to microcosm nitrogen content. The sources of this gain are probably a combination of nitrogen sorption from atmospheric ammonia, and dinitrogen fixation by free living microorganisms. There was no net CO_2 effect on this gain of nitrogen. However the levels of uncertainty surrounding the magnitude of this gain are large, and any small effect of CO_2, which would be biogeochemically significant in global terms, may well be hidden in experimental variation.

Significant gaseous losses of nitrogen from the microcosm were deduced. There was no CO_2 effect on this loss at the two lower N levels, however gaseous nitrogen loss was significantly reduced by CO_2 at the high-N level. Leachate-nitrogen loss was slightly reduced under CO_2 enrichment. This reduction in nitrogen loss at high CO_2 could have major implications in the long term for ecosystem function and carbon gain, as it is another pathway of increasing total system nitrogen, and hence carbon fixing and storage potential.

Growth at high CO_2 greatly increased the productivity of green leaf nitrogen. All plant pools had total nitrogen contents that were either reduced or not changed under CO_2 enrichment. The only microcosm pool to contain more nitrogen at high CO_2 was the soil. Thus, under CO_2 enrichment less nitrogen was present in the high C:N ratio plant pools, and more in the low C:N ratio soil pool. This may have implications for longer term nutrient availability, and hence the continued increases of carbon storage under CO_2 enrichment. This will be explored in the following chapter.

Table 7.1 Soil pH (1 soil :5 H_2O v/v) averaged over harvest and CO_2 level. LSD 0.06 within N level, 0.09 between N levels (P_L<0.05).

	Soil pH		
Soil Depth (mm)	**Low-N**	**Mid-N**	**High-N**
0-25	6.02	5.84	5.04
25-50	6.15	6.27	6.17
50-100	6.18	6.16	6.37
100-150	6.17	6.16	6.28
150-200	6.16	6.16	6.18
200 +	6.21	6.20	6.14

Table 7.2 Soil bulk density averaged over all harvests, expressed as kg m^{-3}. P levels for CO_2 and N are for the main effect. P<0.001 represented by ***.

					***P* level**		**LSD $P \leq 0.05$**
	Low-N	**Mid-N**	**High-N**	**CO_2**	**N**	**CO_2*N**	**CO_2*N**
Control CO_2	1400	1375	1354	***	***	***	25
Enriched CO_2	1518	1458	1365				

Table 7.3 Estimates of input of nitrogen to the total plant-soil system above that applied during the four years growth. Calculated as

(final total system N + total N loss) – (initial total system N + total N addition)

where nitrogen loss includes loss in leachate and gaseous loss, and nitrogen addition includes fertiliser nitrogen and background nitrogen in demineralised water used in irrigation solution. *Estimate A* applies the average (at day 1104 and 1285) gaseous loss estimate for the high-N treatment to all treatments. *Estimate B* applies each treatments estimate of gaseous loss (averaged over day 1104 and 1285). *Estimate C* applies each treatments estimate of gaseous loss. See text for further assumptions (7-160). There were no significant effects of either N level or CO_2 level on nitrogen gain (ANOVA) for A. Nitrogen gain was lower at high-N than at the other N levels (P_L<0.10) in B and C. Standard errors are based only on variation in final total system nitrogen, and do not include errors associated with estimation of losses.

		Estimate			
		A	**B**	**C**	
		---- g N m^{-2} ± SE ----			**n**
Low-N	Control CO_2	7.9 ± 2.4	9.1 ± 2.4	9.1 ± 2.4	4
Low-N	Enriched CO_2	8.0 ± 1.5	9.5 ± 1.5	9.5 ± 1.5	3
Mid-N	Control CO_2	4.5 ± 2.5	7.9 ± 2.5	7.9 ± 2.5	4
Mid-N	Enriched CO_2	5.3 ± 4.0	8.3 ± 4.0	8.4 ± 4.0	4
High-N	Control CO_2	1.5 ± 3.1	3.2 ± 3.1	3.2 ± 3.1	4
High-N	Enriched CO_2	4.1 ± 2.1	2.5 ± 2.1	2.6 ± 2.1	3

Table 7.4 Estimates of NH_3-N uptake by the swards over the entire period based on the empirical model given in Whitehead & Lockyer (1987) for atmospheric uptake by *Lolium multiflorum*. An atmospheric NH_3-N concentration of 2.5 µg m^{-3} was assumed, as was linearity of the response to this concentration. Rate (a) was calculated on the model for plants with a "low" N status and (b) for those with a high N status (Shoot nitrogen concentration of 0.89 and 1.07% by mass at an atmospheric NH_3-N concentration of 11.5 µg m^{-3}). Sorption of NH_3 was assumed to occur for 12 hours per day. LAI at harvest for each treatment was averaged over the whole experiment, and applied for the whole experimental period. Bold estimates show most appropriate model. Leaf nitrogen concentration of high-N plants was considered too far from the modelled values to estimate. See text for further details.

		LAI	**Total leaf %N by mass**	**Estimated NH_3-N uptake g m^{-2}**	
				rate a	**rate b**
Low-N	Control CO_2	0.28	1.03	1.49	**1.21**
Low-N	Enriched CO_2	0.29	0.74	**1.57**	1.28
Mid-N	Control CO_2	0.54	1.26	2.91	**2.40**
Mid-N	Enriched CO_2	0.47	0.83	**2.55**	2.09
High-N	Control CO_2	1.30	2.19	7.0	5.7
High-N	Enriched CO_2	1.16	1.51	6.1	5.2

Table 7.5 Absolute and relative distribution of nitrogen within the microcosm at the 1469 day harvest. Absolute values for soil and total microcosm are gain is that above the nitrogen present at sward establishment. Relative pool size is the fraction of the total microcosm nitrogen increment from sward establishment, expressed as a percentage. Control and enriched CO_2 are given as C and E respectively. Absolute pool sizes where the CO_2 effect is significant are indicated by (*). Probabilities are for the proportional distribution at day 1469. [†]Total microcosm N was not significantly greater under CO_2 enrichment at high-N on day 1469, although it was higher at the four preceding harvests. $P<0.001$ represented by ***, $P<0.01$ by **, $P<0.05$ by *, $P<0.1$ by +, and not significant by ns.

		Absolute pool size $g\ m^{-2}$			**Relative pool size**			***P***		
	CO_2	**LN**	**MN**	**HN**	**LN**	**MN**	**HN**	CO_2	**N**	**C*N**
green leaf	C	0.7	1.9	6.4	4.7	6.8	8.7	**	**	ns
	E	0.5*	1.2*	4.4*	3.0	4.2	5.7			
senesced leaf	C	1.6	3.1	6.6	10.6	11.1	9.1	ns	ns	ns
	E	1.5	2.7*	6.6	9.3	9.5	8.7			
surface litter	C	1.8	5.4	23.7	12.4	18.7	32.1	+	*	ns
	E	1.8	4.9	17.8*	11.4	16.9	23.3			
root	C	4.2	9.4	20.0	28.4	33.3	27.5	*	ns	ns
	E	3.2*	6.1*	19.6	20.6	21.2	25.9			
soil -	C	7.5	8.8	15.8	43.6	29.5	21.2	*	*	ns
above sowing N	E	8.9	14.5*	26.9*	54.8	47.2	35.2			
Microcosm total	C	15.9	28.9	73.5	100	100	100			
above sowing N	E	16.0	29.6	76.2[†]	100	100	100			

Table 7.6 Carbon to nitrogen ratio of major pools at the 1469 day harvest. C:N ratio for soil is for the total soil pool, including that present at sward establishment. Total microcosm C:N is presented as the C:N ratio of the increment above sward establishment levels, and as that including the C and N present in the microcosm at sward establishment. Control and enriched CO_2 are given as C and E respectively. *P*<0.001 represented by ***, *P*<0.01 by **, *P*<0.05 by *, *P*<0.1 by +, and not significant by ns.

		C:N ratio				*P*		LSD $P \leq 0.05$
	CO_2	LN	MN	HN	CO_2	N	C*N	C*N
green leaf	C	36	32	17	***	***	*	2
	E	56	46	22				
senesced leaf	C	97	87	52	***	***	*	10
	E	138	129	82				
surface litter	C	25	27	24	***	*	ns	5
	E	32	39	38				
root	C	32	28	21	***	***	+	2
	E	39	34	24				
soil	C	7.9	9.0	9.1	**	+	ns	0.4
+ sward estab.	E	8.3	9.3	9.7				
Microcosm total	C	31.2	32.7	23.5	***	***	ns	4
- sward estab.	E	36.4	37.4	29.3				
Microcosm total	C	12.5	16.3	16.8	***	***	**	0.6
+ sward estab.	E	13.9	17.8	20.4				

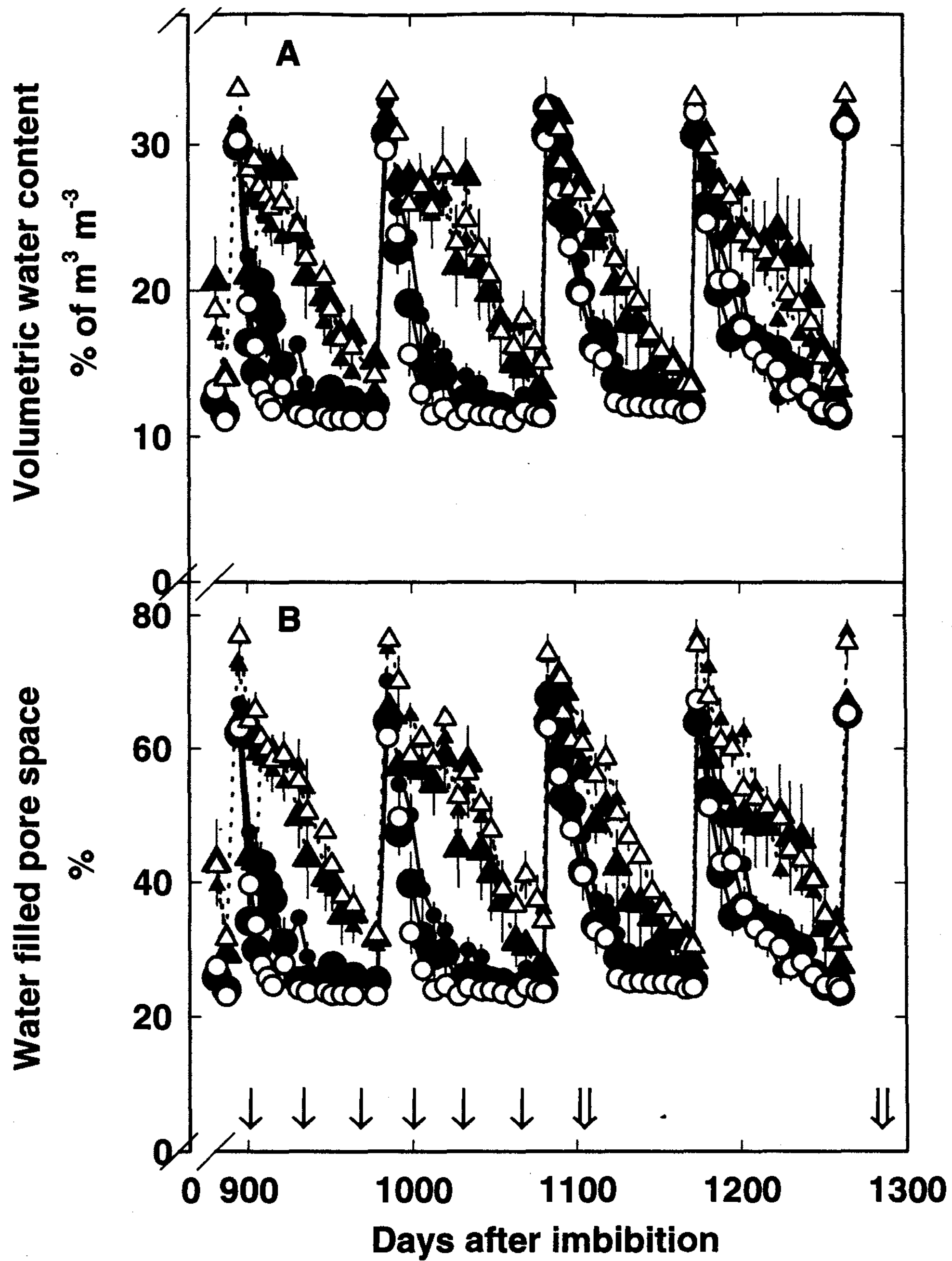


Figure 7.1 Additions of ^{15}N (↓) in relation to volumetric soil water content (θ_v, A) and water filled pore space (WFPS, B). Harvests of the ^{15}N enriched microcosms were undertaken on the days 1104 and 1285 (⇓). Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol.

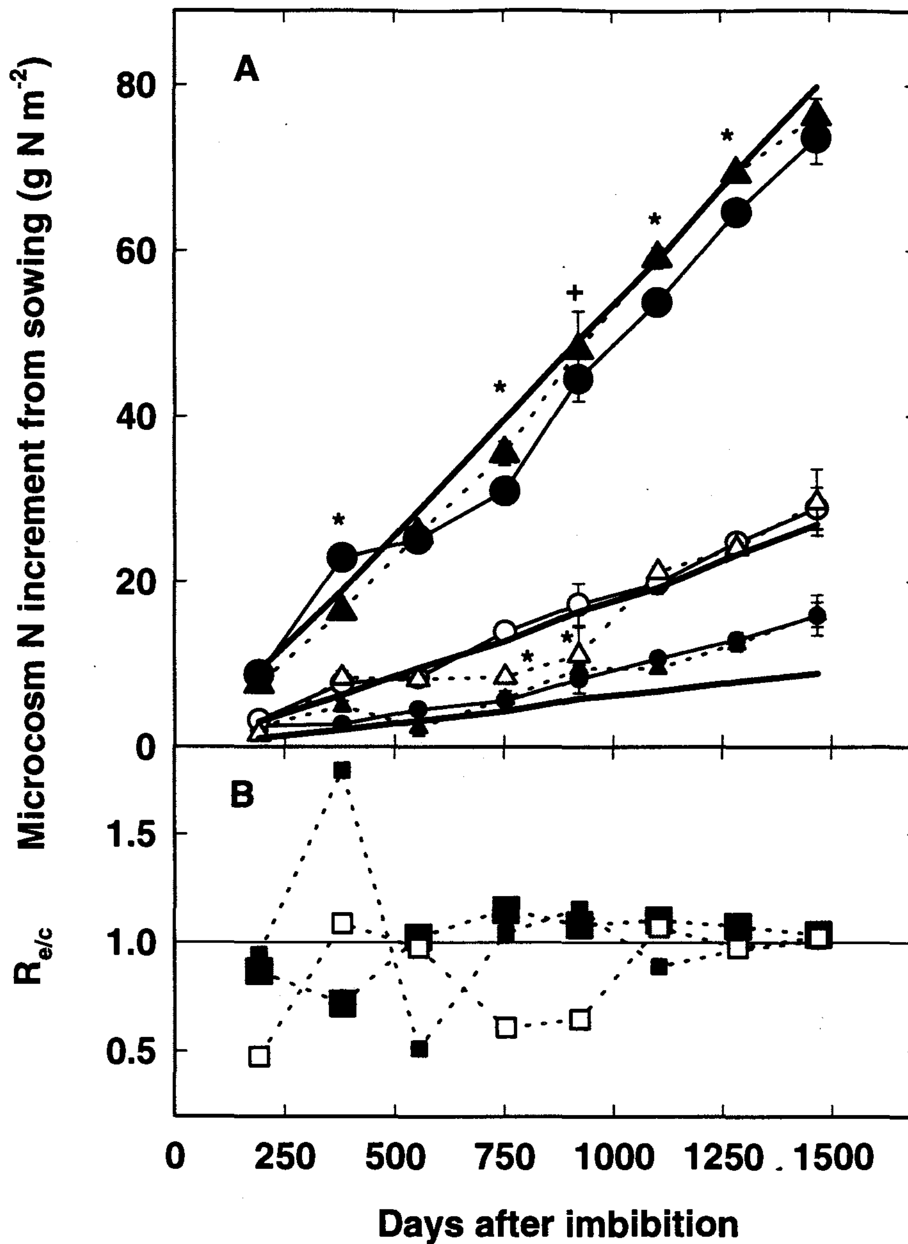


Figure 7.2 Microcosm nitrogen increment above that present at sowing. (A) Absolute values. Control low-N (●), mid-N (○), high-N (●), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. Heavy lines show N application to each N treatment (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■). The response of microcosm nitrogen increment and the response of the difference between applied and recovered nitrogen to CO_2 were dependant both on harvest and N level. ($P<0.01$). Harvest-N level combinations which exhibited a significant CO_2 effect within the N level are marked by (*) for $P_L<0.05$ and (+) for $P_L<0.1$ (same for both effects).

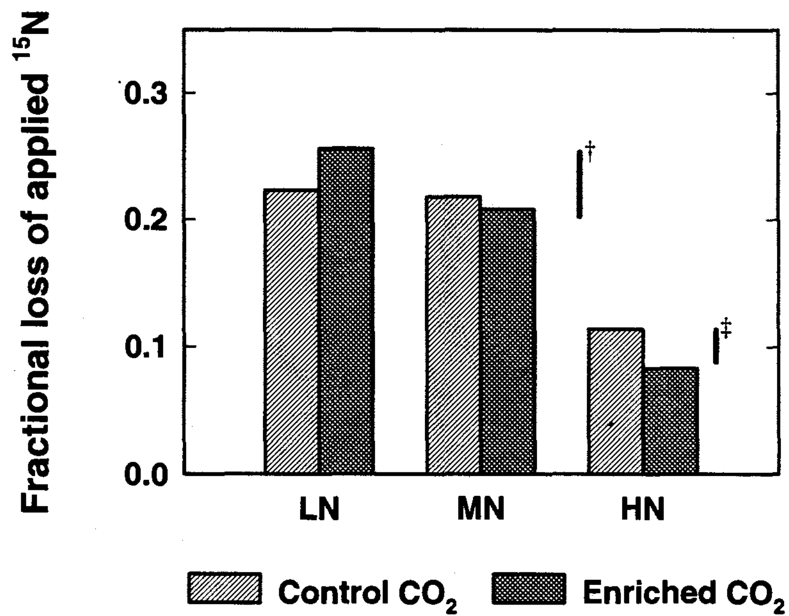


Figure 7.3 Fraction of ^{15}N applied to the microcosms which was not recovered at harvest, averaged over the 1104 and 1285 day harvest. Low-N represented by LN, mid-N by MN, high-N by HN. Vertical lines show 5% LSD for (†) analysis including all N levels and (‡) high-N only analysis.

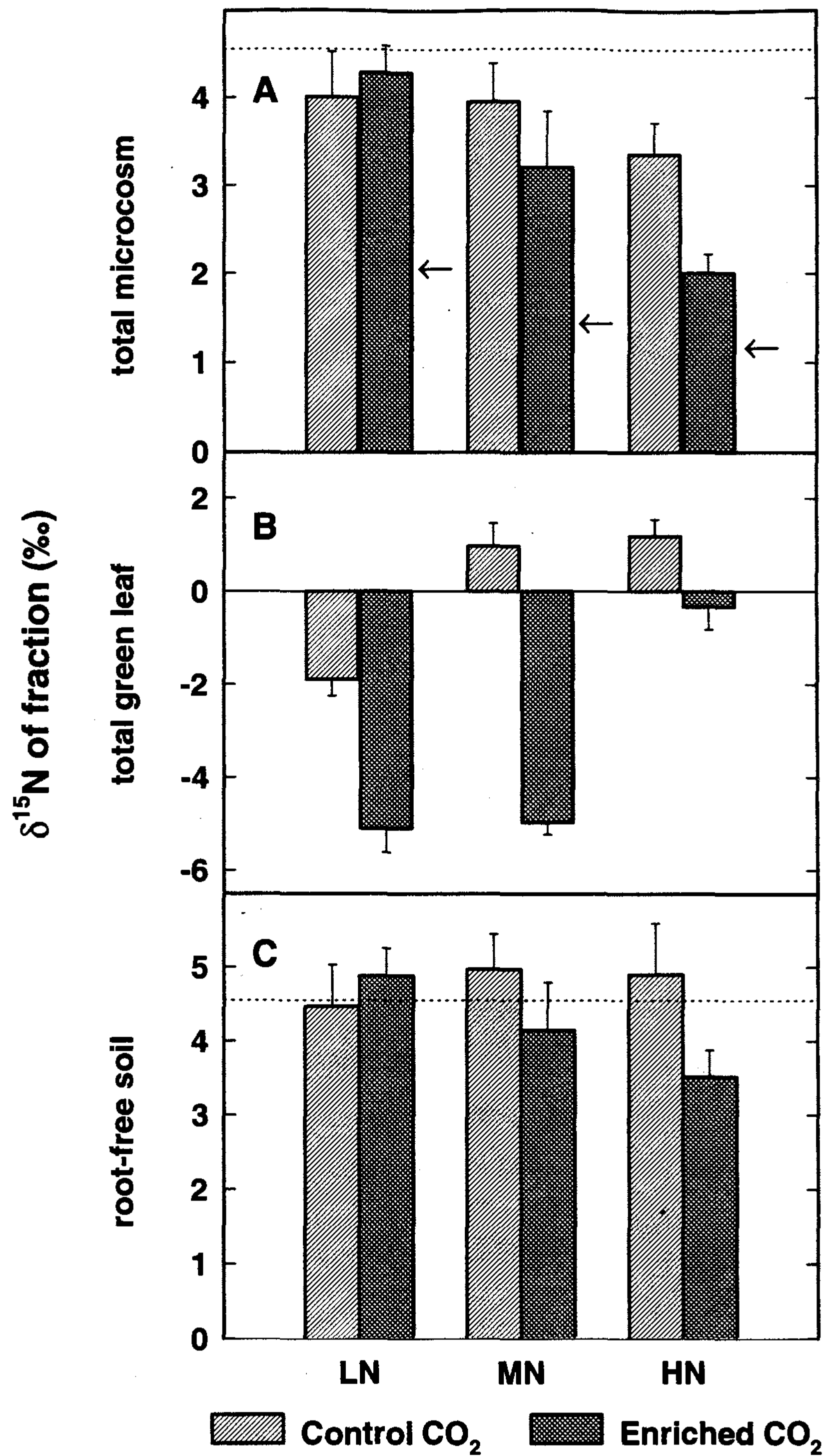


Figure 7.4 Total system $\delta^{15}N$ in non-^{15}N enriched microcosms at the 1469 day harvest of experiment one. Low-N represented by LN, mid-N by MN, high-N by HN. Dotted line in (A) and (C) shows initial soil $\delta^{15}N$ of 4.6 ± 0.1‰. Error bars are ± one standard error. Arrows in (A) indicate the upper end of the range of estimated microcosm $\delta^{15}N$ assuming no gross loss or gain of nitrogen and fertiliser $\delta^{15}N$ of -2.3 to 1.0‰. The lower end of the range of microcosm $\delta^{15}N$ estimates were -0.2, -0.5 and -2.0‰ for the low-, mid- & high-N treatments respectively.

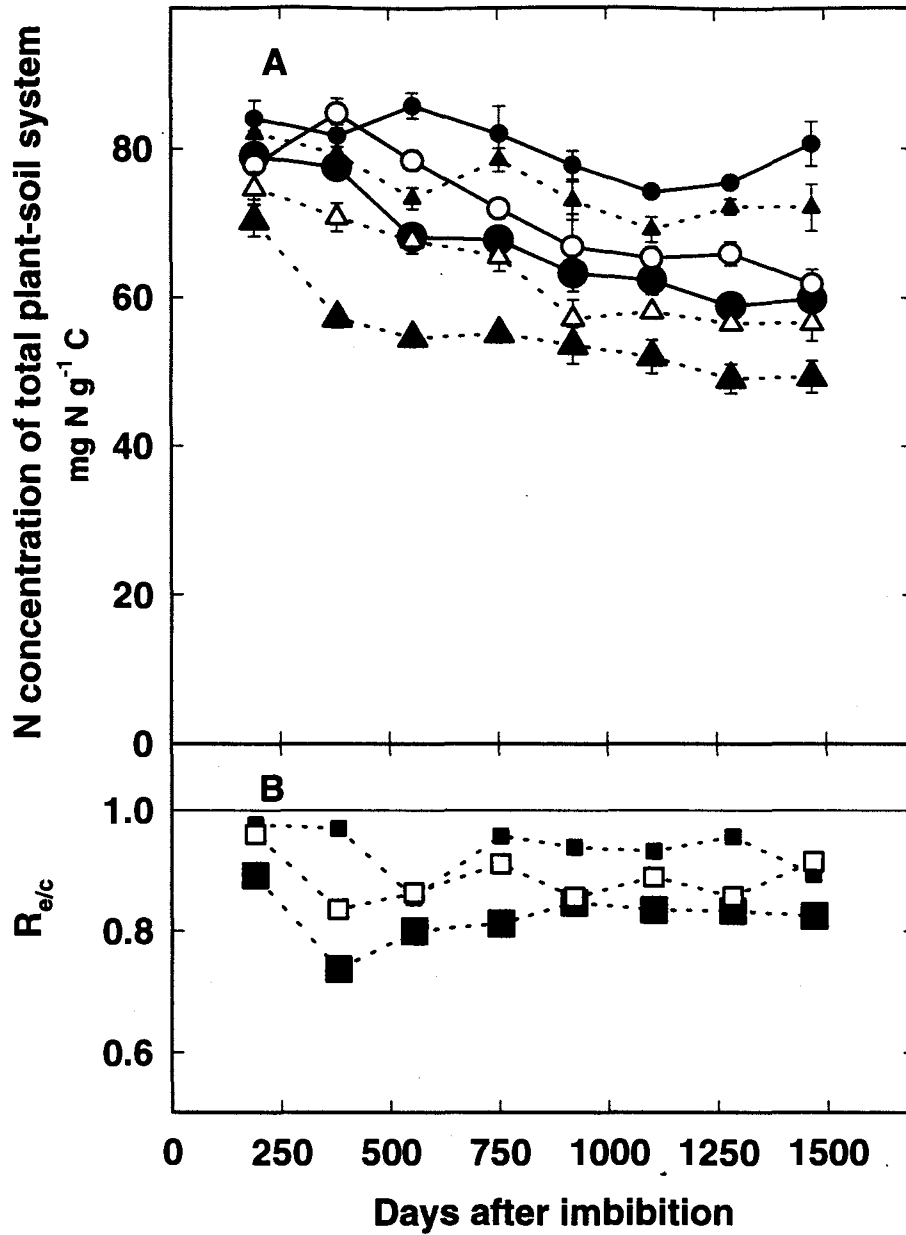


Figure 7.5 Total plant-soil system N concentration at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (●), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Initial soil N concentration was 147 mg N g^{-1} C. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■).

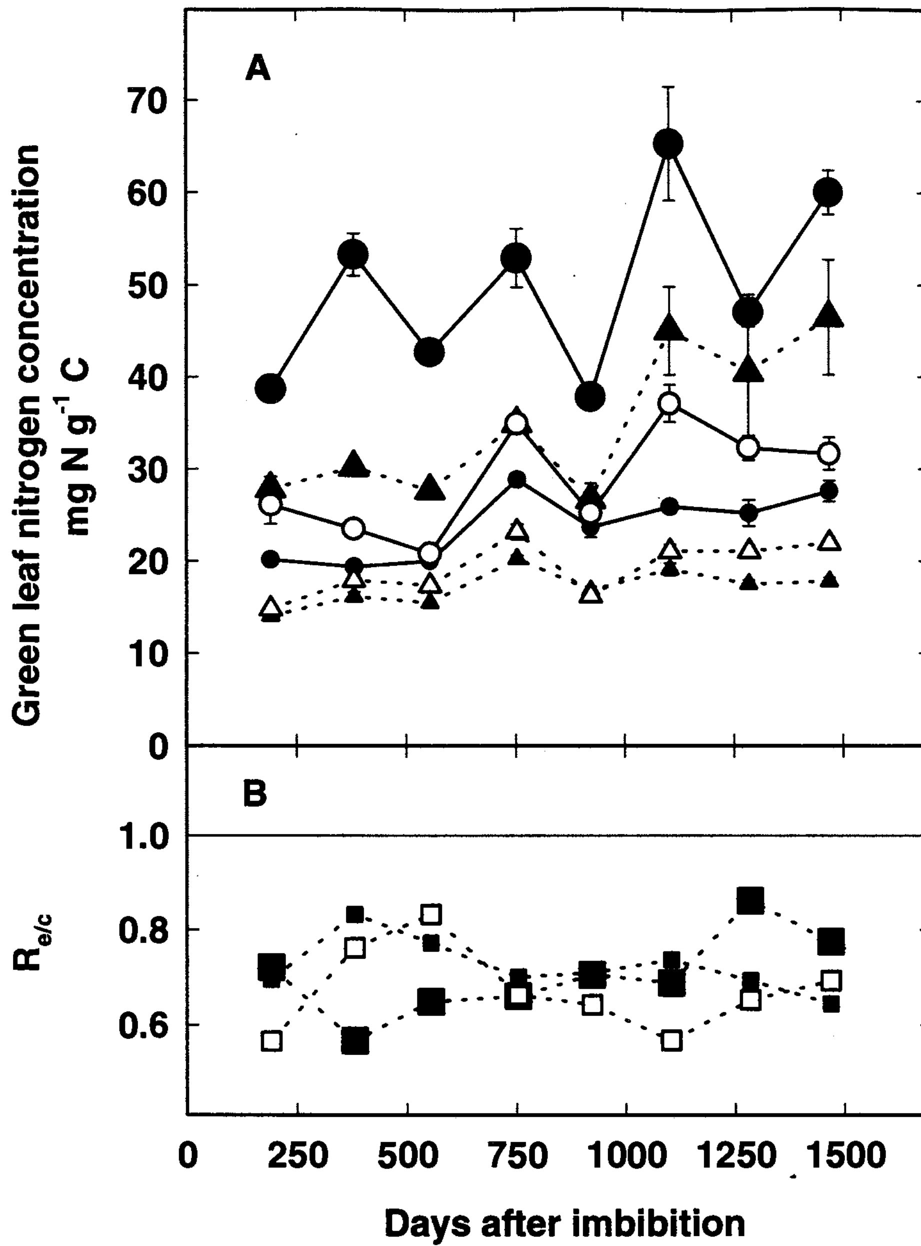


Figure 7.6 Green leaf nitrogen concentration at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■).

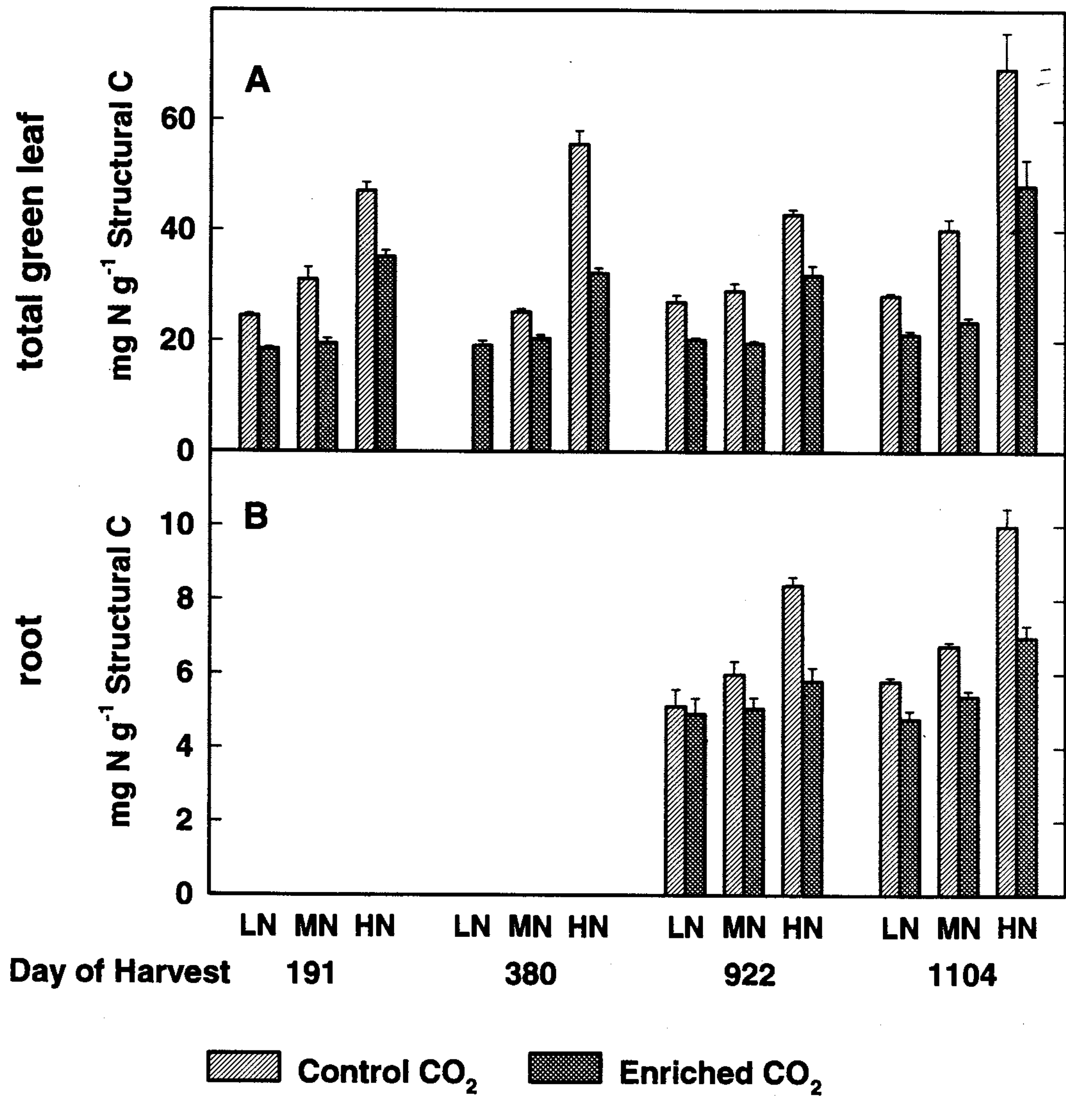


Figure 7.7 Structural nitrogen concentrations. (A) Green leaf and (B) root. Low-N represented by LN, mid-N by MN, high-N by HN. Non-structural carbohydrate concentration, and hence structural C was not determined for the control-low-N leaf at day 380 due to sample shortage.

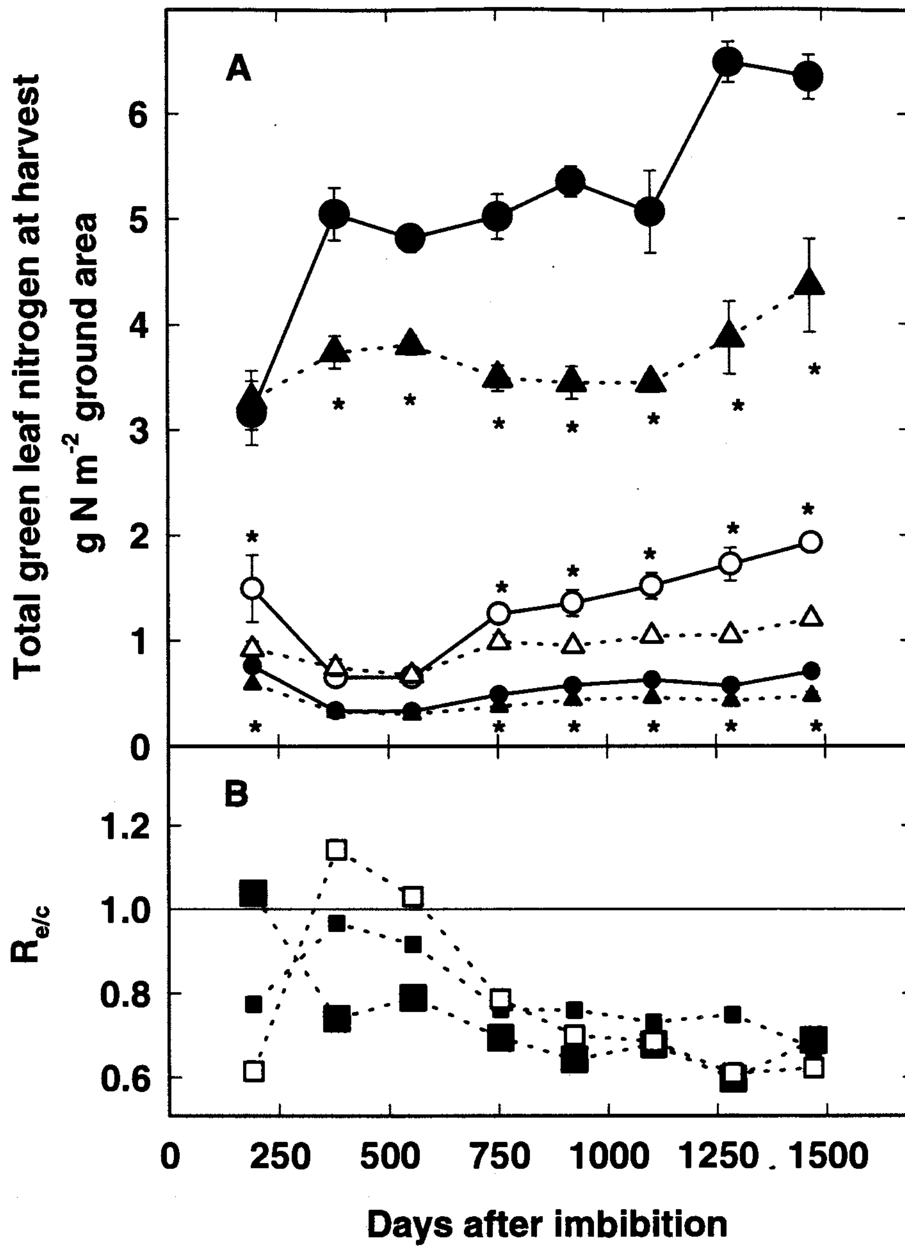


Figure 7.8 Total green leaf nitrogen per unit ground area at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■). The response of green leaf nitrogen to CO_2 was dependant both on harvest and N level. (P<0.01). Harvest-N level combinations which exhibited a significant CO_2 effect within the N level (P_L<0.05) are marked by (*).

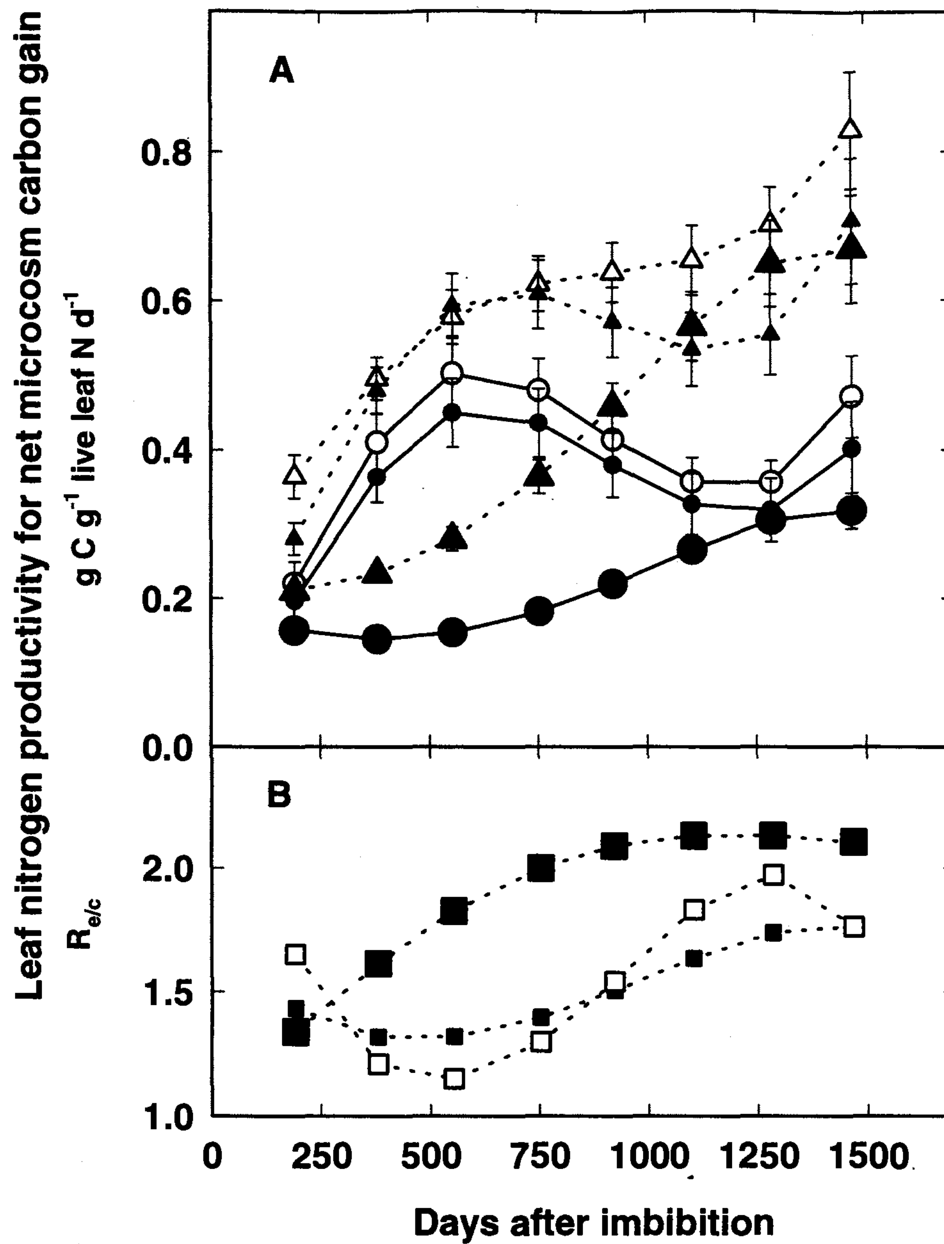


Figure 7.9 Leaf nitrogen productivity for net microcosm carbon gain (NP_M). Based on total green leaf N at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■).

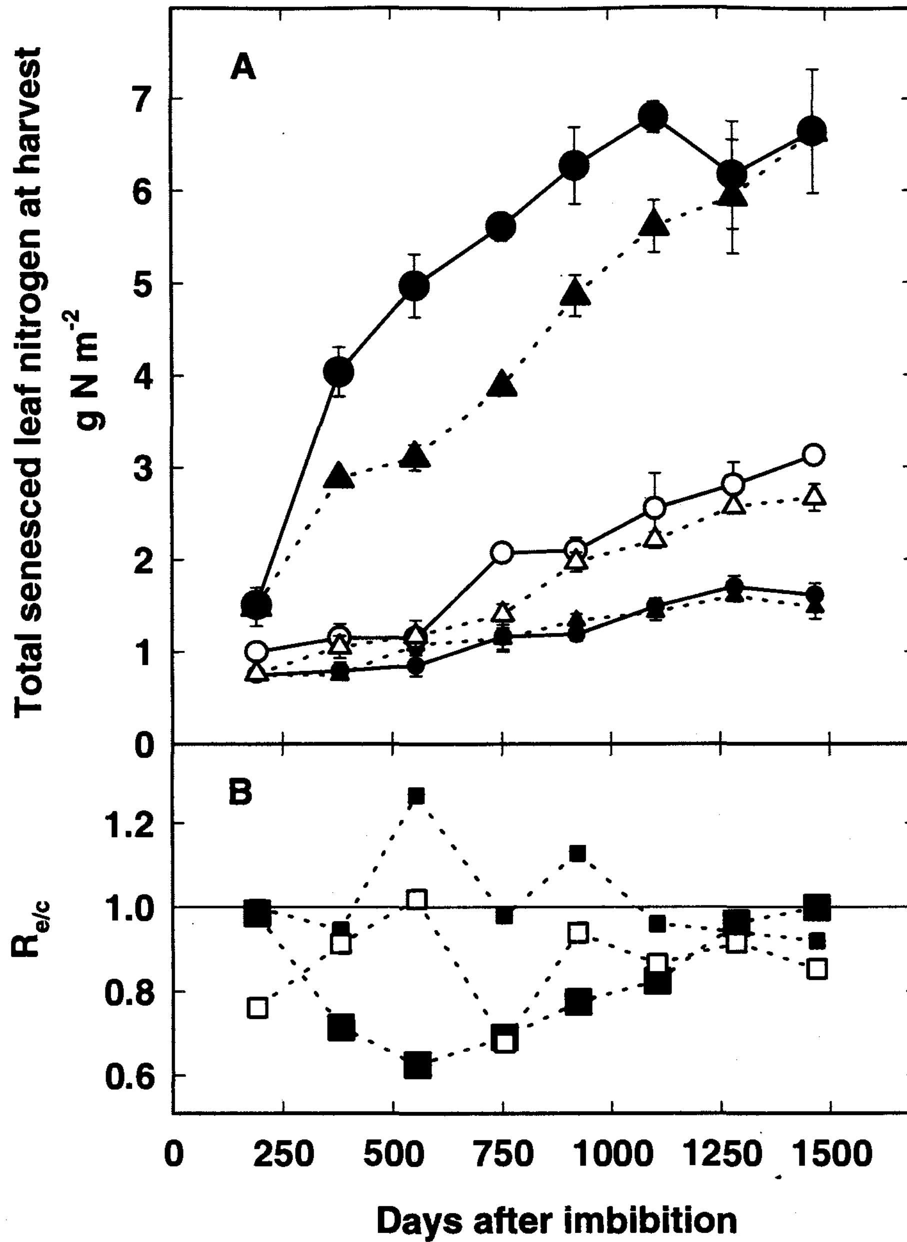


Figure 7.10 Total senesced leaf nitrogen per unit ground area at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■).

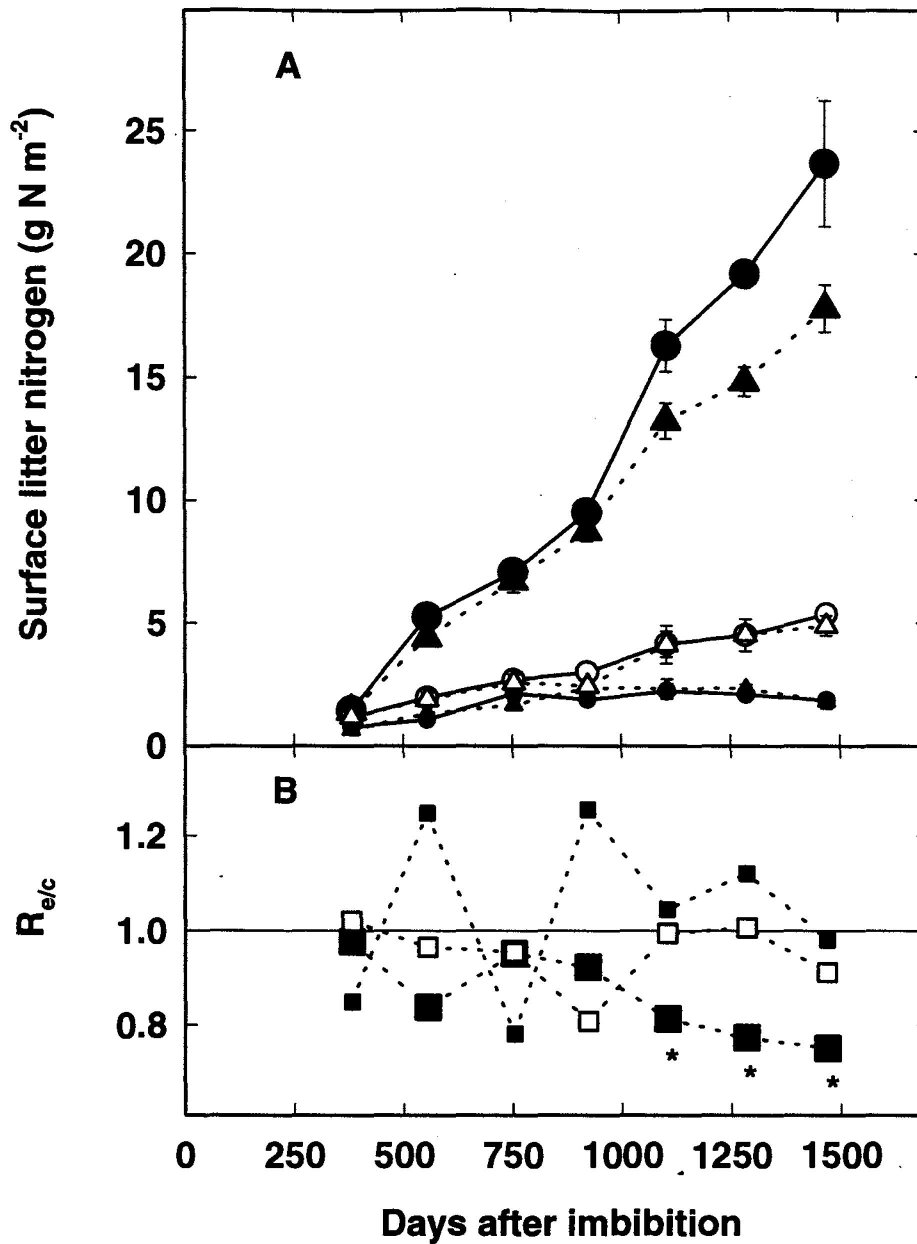


Figure 7.11 Total surface litter nitrogen at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■). The response of surface litter nitrogen to CO_2 was dependant both on harvest and N level. (P<0.01). Harvest-N level combinations which exhibited a significant CO_2 effect within the N level (P_L<0.05) are marked by (*).

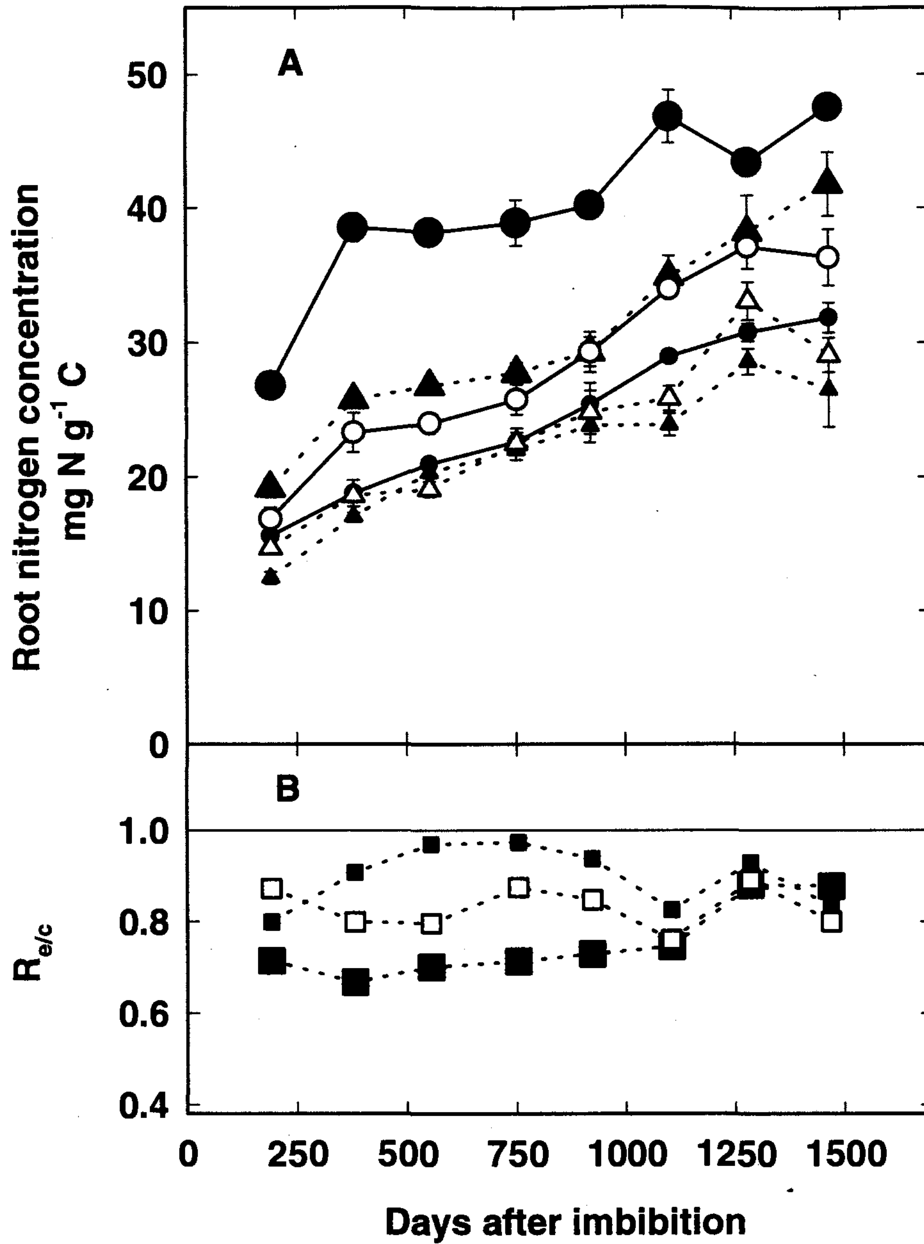


Figure 7.12 Root nitrogen concentration at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (●), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■).

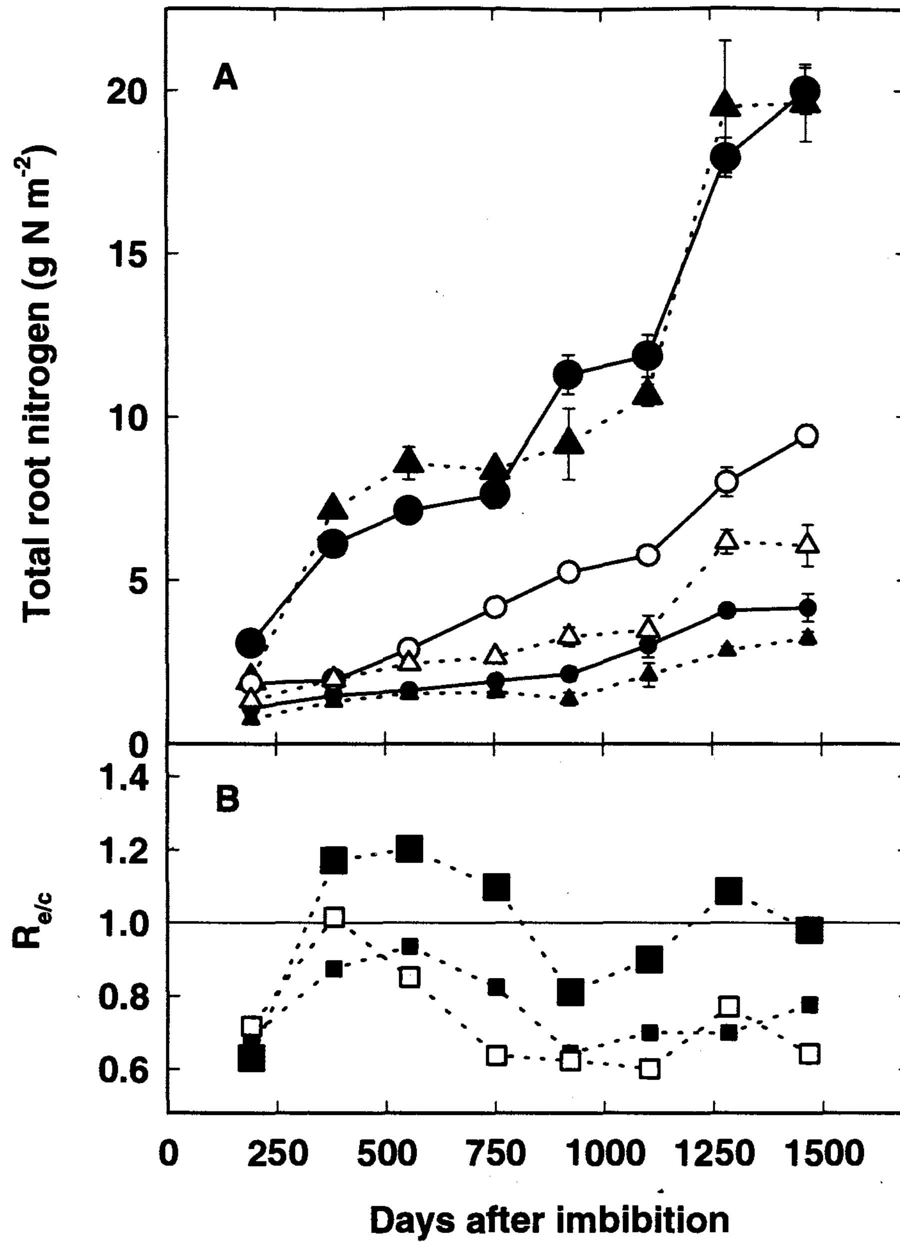


Figure 7.13 Total root nitrogen per unit ground area. (A) Absolute values. Control low-N (●), mid-N (○), high-N (●), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■).

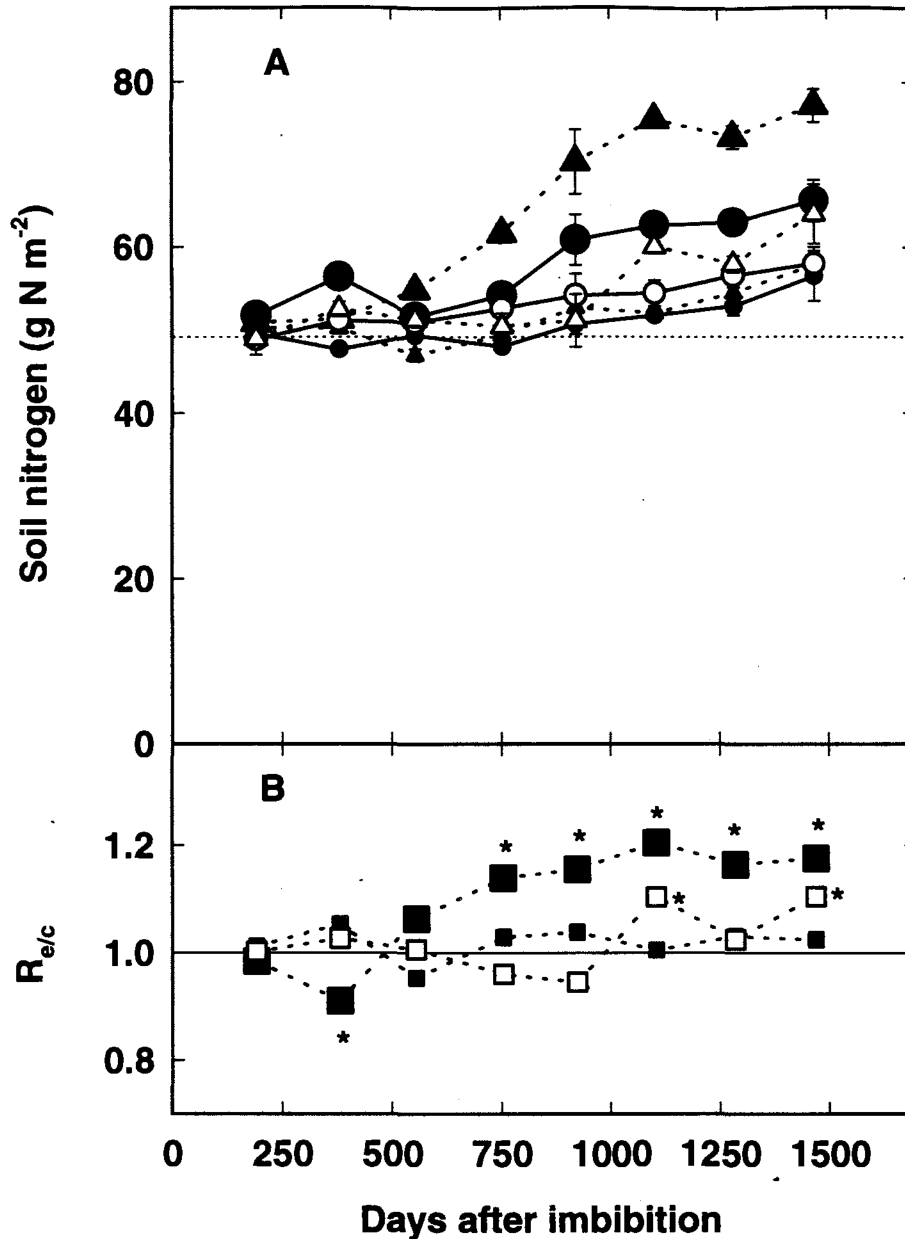


Figure 7.14 Total soil nitrogen at harvest, root free. (A) Absolute values. Control low-N (●), mid-N (○), high-N (●), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. Fine horizontal dotted line shows starting N content. (B) CO_2 response ratio. Low-N (■), mid-N (□), and high-N (■). The response of soil nitrogen to CO_2 was dependant both on harvest and N level. (P<0.01). Harvest-N level combinations which exhibited a significant CO_2 effect within the N level (P_L<0.05) are marked by (*).

Chapter 8. Decomposition and related soil parameters in *Danthonia richardsonii* swards in response to CO_2 and nitrogen supply over four years of growth

Introduction

We have observed increased net carbon sequestration by the nitrogen-limited grass microcosm under elevated atmospheric CO_2 concentrations. In this experiment, the continual addition of fertiliser-N simulates mineralisation of nitrogen from the slow cycling pools of soil organic nitrogen. Thus, the important feedback of current plant growth onto soil nitrogen mineralisation has been over-ridden. It has been hypothesised that, in the long term growth under CO_2 enrichment will alter nitrogen availability, and in turn, the potential of ecosystems to respond to CO_2 enrichment. These hypothesises are of two contrasting types. Firstly, it has been hypothesised that growth under CO_2 enrichment would *decrease* nitrogen availability as nitrogen is immobilised in high C:N ratio plant litter, which may have a slower decomposition rate (Melillo *et al.*, 1990). A similar hypothesis is that of Díaz *et al.* (1993), which proposed immobilisation of nitrogen into an enlarged microbial biomass, reducing nitrogen availability for plant growth. Alternately, the hypothesis of Zak *et al.* (1993) stated that an increase in soil microbial biomass under CO_2 enrichment should *increase* nitrogen availability via increased organic matter turnover.

In nature, rates of decomposition are determined by three broad groups of factors (Swift *et al.*, 1979). These factors are litter quality, the factor most often reported in CO_2 enrichment studies, the character and size of the decomposer population, and environmental factors, the most important of which are moisture and temperature. In this chapter the effects of growth at high CO_2 on litter quality, on its decomposition, both *in vitro* and *in vivo*, changes in the decomposer population, and other soil attributes related to the cycling of nitrogen are examined.

Materials and Methods

Nitrogen resorption efficiency

The efficiency of nitrogen resorption from green leaves as they senesce (resorption efficiency, %), was calculated as;

$$\left(1-\frac{Senesced\ leaf\ lamina\ N\ concentration}{Green\ leaf\ lamina\ N\ concentration}\right)\cdot 100$$

where nitrogen concentrations were expressed on a structural carbon basis. Green leaf lamina nitrogen concentration was averaged over all green leaf lamina in the canopy. Although many methods of calculating resorption efficiency have been proposed (del Arco *et al.,* 1991; Pugnaire & Chapin, 1992), this simple calculation gives similar results to more complex methods (del Arco *et al.,* 1991). Nitrogen resorption might be over-estimated if nitrogen was leached from the senesced leaf lamina during spraying with demineralised water (page 6-104). This effect was assumed to be uniform between treatments.

in vitro decomposition assays

Separate decomposition assays were performed for senesced leaf base, senesced leaf lamina, and root. Samples of senesced leaf base or root (~150 mg) or senesced leaf lamina (~70 mg) were chopped into pieces ~10 mm long and mixed with 10 g of fine washed sand and placed in a glass sample tube (155 mm high, 23 mm ID). A further 10 g of fine sand was then placed on top of the sand-sample mix. Samples of senesced leaf base and root were prepared from every pot at the 922 day harvest. Due to sample shortage senesced leaf lamina from the mid- and high-N treatments only was assayed. Pretreatments of this material included both chopping and grinding to a fine powder in a puck mill as a comparison of macro-structural effects.

Inoculum was prepared for the leaf base and leaf lamina assays by wetting, in a beaker, 40 g of surface litter with 20 mL of distilled water. The beaker was covered with parafilm and left to stand at room temperature (~20°C) for 3 days. Following this, 500 mL of distilled water was added to the beaker and the mix stirred well. The solution was allowed to settle for 1 hour and the supernatant decanted through a 0.5 mm sieve. The supernatant was made up to 1 L. Four mL of this solution was added to each sample tube as inoculum. For the senesced leaf assays all samples were inoculated with inoculum prepared using surface litter from the CHN treatment. Another set of sample tubes was prepared for the leaf base material from the enriched treatments, which were inoculated with inoculum prepared using surface litter from the EHN treatment. Inoculum for the root assay was prepared in the same way using soil from the CHN treatment, which was initially wetted to 20% θ_m and let stand for 3 days before dilution. In the root assay, as well as root from each treatment being inoculated with inoculum prepared from CHN soil, another set of sample tubes were prepared which were inoculated with inoculum prepared using soil from its original treatment. Inoculum blanks were included for each inoculum type used. Preliminary experiments indicated that cumulative respiration was insensitive to inoculum volume, as demonstrated by Franzluebbers *et al.* (1995).

Cotton wool plugs were placed in the top of each tube to slow drying. The sample tubes were weighed weekly and the sample mixture returned to 20% θ_m with distilled water. Sample tubes were incubated in the dark at 20°C.

Carbon evolution from the microbial complex was initially determined daily. The period between measurements was increased over time as respiration rates decreased and became more uniform. Carbon evolution was determined by placing a Suba-seal (William Freeman, Sth. Yorkshire, UK) in the tube and withdrawing a 6 mL sample of air with a syringe for analysis. On insertion the syringe contained 6 mL of CO_2-free air, which was thoroughly mixed with the atmosphere in the tube prior to the 6 mL sample extraction. The CO_2 concentration was immediately determined as outlined in Diagram 8-1. The tubes were let stand at incubation temperature for a period long enough for internal atmospheric CO_2 concentration to approximately double before another sample was collected, as outlined above, and the Suba-seal finally removed.

Respiration rate (*RR*) at time *x* was calculated as;

$$RR_x = \frac{\left(C_{t_2} - C_{t_1}\right)}{\left(t_2 - t_1\right)} \cdot \frac{1}{C_j}$$

where $$x = t_1 + \frac{\left(t_2 - t_1\right)}{2}$$

and *C* was the mass of CO_2-C in the tube after the initial sample collection (t_1) or at the final sample collection (t_2), and C_j was the original mass of sample carbon in the tube. Masses of carbon were calculated from the universal gas law, rearranged to;

$$C_{t_{1,2}} = \frac{P_{t_{1,2}} V_{t_{1,2}}}{RT_{t_{1,2}}} \cdot 12.01$$

where *R* was the universal gas constant, *T* was the ambient temperature at t_1 and t_2 ($t_{1,2}$) respectively, P was the partial pressure of CO_2 in the sample tube at t_1 and t_2 respectively, 12.01 was the molar mass of carbon, and *V* was the headspace volume of the sample tube at t_1 and t_2 respectively. The headspace volume was the sum of the syringe volume, tube headspace, and sand pore space at t_2, but only the headspace volume and sand pore space of the tube at t_1, as the mass of CO_2-C remaining in the tube was required at t_1, not the total mass of CO_2-C, as at t_2.

Respired carbon was cumulated (CRC) to time x_n by calculating the area under the respiration rate curve as;

$$CRC = \sum_{i=1}^{n} \left(\frac{\left(RR_{x_i} + RR_{x_{i-1}}\right)}{2} \cdot \left(x_i - x_{i-1}\right) \right)$$

Respiration rate at time zero (i=0, inoculation) was not different from zero after blank correction, and thus was assumed to be zero in all assays. In this calculation there was assumed to be no difference between replicate tubes, and calculations were undertaken on a source pot basis.

Taylor & Parkinson (1988) outlined the statistical inadequacies of cumulative respiration curves. As each sequential point on the cumulative curve is dependant on the previous points, assumptions of independence required for regression analysis are violated. Hence statistical inference is not drawn from curve fitting to cumulative respiration data in this experiment. However, cumulated respired carbon at the end of the incubation was tested by ANOVA for treatment effects (Taylor & Parkinson, 1988; Cotrufo *et al.,* 1994; Cotrufo & Ineson, 1995; Franzluebbers *et al.,* 1995). Cumulated respired carbon from leaf base tissue was regressed against litter quality parameters to determine which were exerting the most influence on cumulated respiration. Rates of carbon evolution from the decomposing senesced leaf base complex were compared by calculating average respiration rates using linear regression techniques between days 1-10, 10-20, 20-30, 30-50, 50-100, and 100-297 of incubation. Natural logarithm transformations were applied to the data before analysis, and back-transformed means and standard errors are presented.

For the experiment with senesced leaf base, 5 replicate tubes within each pot / inoculum type were used, while 2 replicate tubes were used in each treatment combination in the root and senesced leaf lamina assays. Respiration rates were calculated on 1 replicate only at each measurement time. A replicate was harvested periodically from the senesced leaf base experiment. As much litter as possible was recovered from the sand and dried. Its total carbon content was determined, and compared with the cumulated respired carbon. Carbon loss from the litter approximated cumulated respired carbon, according to the equation;

$$CRC = 0.038\,(\pm 0.011) + 0.702\,(\pm 0.066) \times mass\ loss \quad P < 0.001 \quad r^2 = 0.54$$

However the mass loss estimate was significantly higher than the respired carbon estimate. This was considered to be due to loss of litter in the harvesting process, and transfer of carbon to the dissolved organic carbon fractions.

Results

Litter quality

Senesced leaf nitrogen concentration

Senesced leaf nitrogen concentration was decreased by growth at high CO_2 when averaged over all other treatments (Figure 8.1; $P<0.001$) and CO_2 level interacted with N supply ($P<0.05$). The effect of CO_2 was significant at all levels of N supply ($P_L<0.001$), however the response increased as N level increased from an $R_{e/c}$ of 0.79 at low-N to 0.70 and 0.60 at mid- and high-N respectively.

C:N ratios of tissue used in decomposition assays

Carbon to nitrogen ratios of tissue used for *in vitro* decomposition assays is given in Table 8.1. C:N ratios of senesced leaf base, senesced leaf lamina and root were increased at high CO_2 ($P<0.001$).

Nitrogen resorption efficiency

Nitrogen resorption efficiency was not affected by CO_2 enrichment (Table 8.2). Resorption efficiency was higher at day 1104 than day 922 ($P<0.05$).

Defoliated leaf nitrogen concentration

The nitrogen concentration of defoliated leaf (green + senesced) was reduced under CO_2 enrichment (Figure 8.2; $P<0.001$), with an $R_{e/c}$ of 0.64. Defoliated leaf nitrogen concentration increased with N level ($P<0.001$).

Surface litter nitrogen concentration

Surface litter nitrogen concentration was decreased when grown under CO_2 enrichment ($R_{e/c}$=0.66; Figure 8.3; $P<0.001$). While there was a significant main effect of N level ($P<0.001$), the absolute effect of N level was small compared to the CO_2 effect.

Root-free soil nitrogen concentration

Root-free soil nitrogen concentration was decreased by growth at high CO_2 (Figure 8.4; $P<0.01$).

Non-structural carbohydrates

Non-structural carbohydrate (NSC) concentration was higher in senesced leaf lamina than in senesced leaf base (Figure 8.5; $P<0.001$). Leaf lamina and leaf base responded to CO_2 differently ($P<0.001$). Senesced leaf NSC concentration was reduced by growth at high CO_2 in

leaf lamina but not significantly so in leaf base (P_L<0.05, $R_{e/c}$=0.84 & 0.96 respectively). As N supply increased, NSC concentration decreased (P<0.001), and there was an interaction between CO_2 level and N supply rate (P<0.05). The CO_2 effect was significant at all N levels (P_L<0.05). However it was greater at low-N than at mid- and high-N ($R_{e/c}$=0.85, 0.93, 0.90 for low-, mid- & high-N respectively).

Hemicellulose-pectin fraction

There were no significant treatment effects on the proportion of structural carbon accounted for by the hemicellulose - pectin fraction (Table 8.3).

Cellulose

Cellulose concentrations in senesced leaf lamina and senesced leaf base (Figure 8.6) increased in response to CO_2 enrichment ($R_{e/c}$=1.07 & 1.10 respectively; P<0.05). Cellulose concentration in senesced leaf base decreased as N supply increased (P<0.01). No treatment significantly affected root cellulose concentration, although it did tend to increase in response to CO_2 enrichment (Figure 8.6).

Lignin

Lignin concentrations in senesced leaf lamina, senesced leaf base, and root were not affected by growth at high CO_2 (Figure 8.7). Lignin concentration in senesced leaf base increased as N supply rate increased (P<0.01), while leaf lamina and root lignin concentration was unaffected by N level.

Lignin concentrations in green leaf lamina (not presented) were increased by growth at high CO_2 at day 922 ($R_{e/c}$=1.09; P<0.01). No treatment effected lignin concentration in green stubble (not presented) , although the trend was for an increase in concentration in response to CO_2.

Polyphenolics

The concentration of extractable polyphenolics was reduced by CO_2 enrichment in senesced leaf base when expressed as a fraction of structural carbon ($R_{e/c}$=0.87; Table 8.4; P=0.05). Extractable polyphenolic concentration decreased as N level increased (P<0.05). Extractable polyphenolic concentrations in leaf lamina and root were not affected by the treatments.

Decomposition

***in vitro* assays potential decomposition rate assays**

Senesced leaf base: standard inoculum

After 297 days of incubation, cumulative respired carbon from senesced leaf base (Figure 8.8A) was lower from tissue originating from the high CO_2 treatments incubated with inoculum from CHN litter ($R_{e/c}$=0.76; P<0.01). The growth N level also affected cumulative respired carbon (P<0.05). Senesced leaf base from the high-N treatments respired more carbon than those from low or mid-N (P_L<0.05), between which there was no difference.

Cumulative respired carbon was regressed against litter quality determinants of decomposition rate, and results are summarised in Table 8.5. Respiration rates averaged over periods within the incubation are summarised in Table 8.6. Both CO_2 and N level effects on respiration rates were transitory, although the respiration rates of material produced under CO_2 enrichment were always lower than that produced under ambient CO_2 concentrations.

Senesced leaf base: enriched CO_2-high-N inoculum

Inoculum type had no statistical effect on cumulative respired carbon (Table 8.7), and differences between N treatments evident with a common inoculum were still present.

Senesced leaf lamina

Senesced leaf lamina grown under CO_2 enrichment respired less C than that grown under ambient CO_2 concentrations (Figure 8.9; P<0.05). There was no difference between the chopped or ground pretreatments.

Root: standard inoculum

After 150 days cumulative respired carbon from root (Figure 8.8B) was lower from tissue originating from the high CO_2 treatments incubated with standard inoculum ($R_{e/c}$=0.86; P<0.05). The growth N level also affected cumulative respired carbon (P<0.05). Root from the high-N treatments respired more carbon than those from low- or mid-N (P_L<0.05), between which there was no difference. There was an interaction between CO_2 and N level (P=0.10), with no effect of CO_2 treatment at low-N on cumulative respired carbon.

Root: inoculum from own treatment

There was no effect of inoculum type on cumulative respired carbon (Table 8.7), and all treatment differences noted with a common inoculum were evident when inoculum from the source treatment was used.

In situ decomposition

There were no CO_2 effects on the difference between carbon recovered at harvest and carbon addition to the surface litter layer as defoliated leaf (Figure 8.10). There was a significant N effect ($P<0.001$), with the low-N treatment gaining carbon initially, the mid-N showing no significant effect, and the high-N treatment having a significant negative balance ($P_L<0.05$). The N levels showed different trends over time ($P<0.001$), with the low- and mid-N treatments moving towards a negative balance late in the experiment, as net accumulation of carbon in the surface litter layer ceased. The ratio of carbon recovered to carbon added to the surface litter layer was unaffected by CO_2 enrichment (Figure 8.11).

A small effect of CO_2 on litter nitrogen balance was evident when averaged over all treatments (Figure 8.12; $P<0.07$), with high CO_2 swards having a greater recovery of nitrogen than those at ambient CO_2 ($R_{e/c}$ 1.22), although both CO_2 treatments showed a positive balance ($P_L<0.05$). The N levels showed different responses ($P<0.001$), and all N levels were statistically different from each other ($P_L<0.05$). Mid-N had the largest positive balance, with high-N the lowest, although all showed a positive balance ($P_L<0.05$).

Microbial Biomass Carbon

Soil microbial biomass carbon

Soil microbial biomass carbon in the surface 50 cm of (root free) soil was determined at day 753 and at each harvest thereafter (Figure 8.13). Growth at high CO_2 increased microbial carbon when averaged over the other treatments ($R_{e/c}$=1.5; $P<0.001$). However there was an interaction with N supply rate ($P<0.01$). Microbial carbon was unaffected by N level in the control CO_2 treatment, while it increased with N level at high CO_2. At low-N there was no CO_2 effect, while at mid- and high-N the average $R_{e/c}$ was 1.4 and 2.1 respectively ($P_L<0.001$). At harvests between days 922 and 1104 microbial carbon was also determined in the 50-100 mm soil fraction (not presented). There were no significant CO_2 effects at this depth. Soil collected from near the collection site of the soil used in this experiment had a microbial carbon concentration of ~450 μg C g^{-1} soil (at 25-50 mm depth) mid-winter in 1995. Microbial C concentrations in the ambient experimental treatments ranged from 200-600 μg C g^{-1} soil (0-50 mm).

Microbial carbon as a proportion of root-free soil carbon (C_{mic}:C) was unaffected by N level (Figure 8.14). The CO_2 effect on C_{mic}:C was dependant on the level of N supply ($P<0.01$).

There was a non-significant depression at low-N, a non-significant increase at mid-N and an increase at high-N (high-N $R_{e/c}$=1.31; P_L<0.01).

Surface litter microbial biomass carbon

Microbial carbon in the surface litter pool was determined at day 1285 and 1469 (Table 8.8), and was not affected by CO_2 level. As N supply increased, surface litter microbial carbon increased (*P*<0.001). Under CO_2 enrichment C_{mic}:C was lower ($R_{e/c}$=0.54; *P*<0.001). As N supply rate increased C_{mic}:C declined (*P*<0.001).

Potential nitrogen mineralisation

Soil potentially mineralisable nitrogen

Potentially mineralisable nitrogen in the surface 50 mm of soil was increased by growth at high CO_2 when averaged over all treatments (Table 8.9; *P*<0.001). However, there was an interaction with N supply (*P*<0.01), and the effect was only present at mid- and high-N ($R_{e/c}$=1.78 & 2.45 respectively; P_L<0.05).

Surface litter potentially mineralisable nitrogen

Potentially mineralisable nitrogen from the surface litter (Table 8.8) was decreased by growth at high CO_2 ($R_{e/c}$=0.41; *P*<0.05). As N level increased, potentially mineralisable N increased (*P*<0.001).

Extractable nitrogen

Amino-nitrogen levels were higher under CO_2 enrichment in the surface 50 mm of soil (Table 8.9; *P*<0.001). High CO_2 increased total mineral-N and ammonium-N at the high rate of N supply, but not at the lower N levels (Table 8.9; *P*<0.05). Mineral N was higher at high-N than at the lower N levels due to increases in both the ammonium and nitrate pools.

Soil carbon and nitrogen pools

Slow cycling soil carbon, defined in this experiment as the difference between total soil carbon and microbial carbon, was increased by CO_2 enrichment (Table 8.10; *P*<0.001). Slow cycling soil nitrogen in the surface 50 mm of soil, defined in this experiment as the difference between total nitrogen pool and the sum of potentially mineralisable N, mineral N and amino-N was increased under CO_2 enrichment ($R_{e/c}$=1.18; Table 8.9; *P*<0.01).

Microbial C:N ratio index

The effect of growth at elevated CO_2 on microbial C:N ratio index was dependant on the rate of N supply (Table 8.11; $P<0.05$). The index was lower under CO_2 enrichment at mid- and high-N, but not at low-N when averaged over harvest ($P_L<0.05$). The index was lower at low-N under control CO_2 than at mid and high-N, while under CO_2 enrichment the index higher at low-N than at mid or high-N when averaged over harvest ($P_L<0.05$).

Arbuscular mycorrhizae infection

Root infection by arbuscular mycorrhiza was not affected by any treatment (Table 8.12).

Discussion

The nitrogen concentration (per unit carbon) of the total system was lower at high CO_2. This was the result of an increase in total system nitrogen productivity at all N levels in response to high atmospheric CO_2 (Figure 7.9). The increase in total microcosm carbon at high CO_2 resulted from increases in the C:N ratio (decreasing nitrogen concentration) of most of the plant and soil fractions, including root free soil, and a small increase in total microcosm nitrogen at high-N. These decreases in nitrogen concentration show a more efficient use of nitrogen and are commonly observed in plant material grown at high CO_2 (Arp & Berendse, 1993).

This increase in C:N ratio of senesced leaf and root grown at high CO_2 indicates a reduction in litter quality, one of the three groups of factors controlling litter decomposition (Swift *et al.*, 1979). The reduction in litter quality was confirmed by *in vitro* decomposition assays, and was attributed to the increased C:N ratio, as other measured litter quality parameters showed little sensitivity to CO_2 or N level. However, this decrease in litter quality did not translate to differences in *in vivo* litter disappearance, probably due to changes in the other factors controlling decomposition - the micro-environment and the decomposer population.

Growth under CO_2 enrichment increased average soil moisture contents (θ_v; Table 6.4, Figure 6.2, Figure 7.1), and thus possibly the moisture content of the surface litter layer. As decomposition rates are increased at higher moisture contents, at least over the range of increase observed in this study, this may have a positive feedback on decomposition rate.

Soil microbial biomass carbon was higher under CO_2 enrichment at the two higher N supply rates, while there was no CO_2 effect in the surface litter. If microbial activity were well correlated with microbial biomass in this experiment, this may also have a positive feedback on

decomposition rates. These three elements of the decomposition process will now be discussed in turn.

Litter quality

Litter quality parameters

Mass loss of tissue undergoing decomposition processes - a similar index of decomposability to cumulative respired carbon which was used in this study - has been widely reported. Mass loss, or rates of mass loss of senesced leaf tissue, have shown significant correlations with various litter quality parameters, such as N and P concentration, C:N ratio, indices of non-structural carbohydrate content, lignin content, cutin content, lignin:N ratio, pholyphenolic content, polyphenol:N ratio, (lignin + polyphenolic):N, cutin:N, cutin:P, and the "ligno-cellulose index" (lignin/(lignin + cellulose)) (eg, Waksman & Tenney, 1928; Daubenmire & Prusso, 1963; King & Heath, 1967; Melillo *et al.,* 1982; Aber *et al.,* 1989; Taylor *et al.,* 1989; Fox *et al.,* 1990; Palm & Sanchez, 1991; Gallardo & Merino, 1993; Constantinides & Fownes, 1994)

Similar studies have been undertaken for root tissue (Weaver, 1947), correlating mass loss or rates of mass loss with N, P, S, C:N ratio, total carbohydrate concentration and lignin concentration (Herman *et al.,* 1977; Berg, 1984; Dormaar & Willms, 1993). The relationship;

$$\frac{(C{:}N)*(\%\ Lignin)}{\sqrt{(\%\ NSC + \%\ Cellulose + \%\ Hemi\text{–}Pec)}}$$

has been proposed as an index of root indecomposability, and may apply equally well to leaf material. The relationship incorporates terms related to the substrates nitrogen content, its lignin content, which represents the recalcitrant fraction, and its carbohydrate, or energy content (Herman *et al.,* 1977). Many of these litter quality parameters were measured in this experiment. Changes in these parameters due to treatment, and there relationship with *in vitro* decomposition are discussed below.

Nitrogen concentrations

The nitrogen concentration of senesced leaf is greatly reduced by growth at high CO_2 (Figure 8.1). This is not attributable to non-structural carbohydrate concentrations of the senesced material being higher at high CO_2 (Figure 8.5). Nitrogen resorption efficiency was not influenced by N or CO_2 level (Table 8.2). Thus, surprisingly, the reduction of nitrogen concentration in green leaf at high CO_2 was not offset by reductions in nitrogen resorption efficiency during senescence (Field *et al.,* 1992; Arp & Berendse, 1993). Although nitrogen resorption efficiency is often unchanged or enhanced under nutrient stress (Aerts & de Caluwe,

1989; Field *et al.,* 1992; Pugnaire & Chapin, 1992), Arp and Berendse (1993) presented arguments for a reduction in resorption efficiency at high CO_2 due to assumed changes in within-leaf nitrogen partitioning to less labile pools. However, this does not appear to be the case in this study. Nutrient resorption efficiency was higher at the harvest on day 1104 than at 922. Leaves senescing under a lower radiation load (day 1104 harvest) had a higher resorption efficiency than those senescing under a higher radiation load (day 922 harvest). This agrees with the findings of Pugnaire and Chapin (1992), who found greater resorption efficiencies when sink strength was strong relative to the supply of carbohydrate.

The reduction in nitrogen concentration of live tissue is a very common response to CO_2 enrichment, as exhibited by isolated plants of *D. richardsonii* and other grasses in this study (chapter 3, 4 & 5). Less data is available for senesced material, especially that from herbaceous plants. Senesced leaf C:N ratios have been observed to increase in tree leaves under CO_2 enrichment (Coûteaux *et al.,* 1991; Cotrufo *et al.,* 1994; Boerner & Rebbeck, 1995). Senesced plant material from the salt marsh sedge *Scirpus olneyi* (C_3) was found not to differ in C:N ratio between CO_2 levels (Curtis *et al.,* 1989b). Little or no difference in C:N ratio of senesced leaf has also been observed in the C_3 grass *Bromus mollis,* although it was not completely senesced (Larigauderie *et al.,* 1988), *Quercus alba* (white oak) (Norby *et al.,* 1986) and soybean (Reddy *et al.,* 1989).

The response of root nitrogen concentration to CO_2 enrichment is not well understood. Root nitrogen concentration was reduced by growth at high CO_2 in this study (Figure 7.12), although the effect was small at low-N. This difference at low-N was explained by increases in NSC concentration (Figure 7.7). Evidence in the literature is mixed. Root nitrogen concentrations are sometimes decreased by growth at high CO_2 (chapter 4 & 5; Curtis *et al.,* 1990; Arp & Berendse, 1993; Gorissen et al., 1995). However, root nitrogen concentration has often been observed to be insensitive to CO_2 enrichment (chapter 4 & 5). In field grown tallgrass prairie, root nitrogen concentration was reduced by high CO_2 one year, but not in another (Owensby *et al.,* 1993a). In *Trifolium repens* L. (white clover) that was fixing N_2 it was not changed by CO_2 enrichment. Fine root C:N ratio of *Castanea sativa* Mill. (sweet chestnut) was not increased by CO_2 enrichment, although the C:N ratio of coarse roots did increase (Rouhier *et al.,* 1994). Other species have also shown little change in root nitrogen concentration in response to CO_2 enrichment (Chu *et al.,* 1992; Arp & Berendse, 1993; Cotrufo & Ineson, 1995; Pregitzer *et al.,* 1995), possibly in response to increased total plant nitrogen uptake at high CO_2 (Cotrufo & Ineson, 1995). Thus, the effect of CO_2 on root nitrogen concentration is complex. However, as

shown in chapter 5 reductions of root nitrogen concentration under CO_2 enrichment in isolated plants of *D. richardsonii* could be fully explained by changes in plant size. Thus, if ontogenetic effects were allowed for, the response of root nitrogen concentration to CO_2 enrichment may be more consistent.

Non-structural carbohydrate, hemicellulose-pectin and cellulose concentrations

In this study, CO_2 level had no major effects on NSC or hemicellulose-pectin concentrations, while cellulose concentrations were increased slightly. Surprisingly, non-structural carbohydrate levels in senesced leaf tended to be slightly *reduced* by CO_2 enrichment (Figure 8.5), while root NSC concentrations were unaffected by treatment (page 6-110). In the literature, soluble carbohydrate concentrations have been observed to not change (Cotrufo *et al.*, 1994) or increase (Cotrufo *et al.*, 1994; Boerner & Rebbeck, 1995) in senesced leaf at high CO_2. This is a very variable fraction as it is subject to leaching (Swift *et al.*, 1979). The amorphous hemicellulose-pectin fraction showed no response to treatment (Table 8.3). Cellulose concentration was higher at high CO_2 in both senesced leaf fractions, and tended to decrease with increasing nitrogen supply in senesced leaf base (Figure 8.6). No treatment affected senesced root cellulose concentration. Holocellulose (total insoluble polymer carbohydrates, Berg *et al.*, 1984) in senesced tree leaf showed no response to CO_2 concentration (Cotrufo *et al.*, 1994).

Lignin concentration

Lignin concentrations showed only small treatment effects (Figure 8.7). No change in lignin concentration was noted in any senesced tissue in response to CO_2 enrichment, while lignin concentration increased with N level in senesced leaf base, but not in root or senesced leaf lamina. Lignin concentrations of green and senesced leaf in this experiment were similar to those reported in the literature for Gramineae (Iiyama & Wallis, 1990; Bilbro *et al.*, 1991; Thomas,RJ & Asakawa, 1993; Kemp *et al.*, 1994). There are few reports of the effect of growth at high CO_2 on lignin concentrations of grasses. Lignin concentrations in senesced tree leaf were observed to increase at high CO_2 in *Fraxinus excelsior* L. (ash), *Betula pubescens* Ehrh. (birch), *Acer pseudoplantanus* L. (sycamore), *Picea sitchensis* (Bong.) Carr. (Sitka spruce) (Cotrufo *et al.*, 1994), and *Liriodendron tulipifera* L. (yellow poplar) (Boerner & Rebbeck, 1995). Lignin concentration in *Poa pratensis* L. (Kentucky bluegrass), *Andropogon gerardii* Vitman (big bluestem, C_4) and *Sorghastrum nutans* (L.) Nash. (Indiangrass, C_4) also showed little sensitivity to CO_2 level or nitrogen supply (Kemp *et al.*, 1994).

Polyphenolic concentration

Polyphenolic concentration was reduced by CO_2 enrichment in senesced leaf base (Table 8.4), and declined as N level increased. However, polyphenolic concentrations were unaffected by the treatments in senesced leaf lamina and root. Polyphenolic concentrations in senesced leaf base and lamina may be under-estimated as the samples were oven dried at 80°C, which has been observed to reduce recovery of phenolics (Lindroth & Pajutee, 1987). The polyphenolic concentrations were similar to those noted in senesced leaf of Gramineae (Thomas,RJ & Asakawa, 1993).

Polyphenolics are a diverse range of compounds. They have been associated with a reduction in palatability and pathogen attack (Swift *et al.,* 1979). High concentrations of polyphenolics in litter have been linked to rapid formation of humus due to the ability of polyphenolics to precipitate proteins and "mask" cellulose, protecting it from microbial degradation (Swift *et al.,* 1979). Polyphenolic concentrations tend to be higher under nutrient stress (Swift *et al.,* 1979), and are more resistant to degradation than lignin (Minderman, 1968).

Little attention has been focused on the role of polyphenolics on decomposition of litter produced at high CO_2. A body of work has developed in relation to phenolic concentration at high CO_2 and insect performance. These reports are related to live foliage of tree species, which have a higher concentration of polyphenolics than were observed in *D. richardsonii.* Condensed tannin concentration was observed to increase in response to CO_2 in *Betula papyrifera* and *Pinus strobus* (Roth & Lindroth, 1994). Total extractable polyphenolics were also noted to increase in *Lindera benzoin* (L.) Blume leaf and stem tissue in response to CO_2 enrichment (Cipollini *et al.,* 1993). Thus the response of polyphenolic concentration in *D. richardsonii* to CO_2 enrichment may differ from that observed in tree species.

The physical characteristics of the senesced material can also affect decomposition rates, with leaf toughness being a very good predictor (better than C:N) of mass loss for a range of leaf litters (Gallardo & Merino, 1993). Physical characteristics of senesced material were not directly examined in this study, however tissue pre-treatments of grinding and chopping prior to *in vitro* decomposition assays were used to elucidate any effect, and are discussed below.

In conclusion, many litter quality parameters may be sensitive to CO_2 enrichment. However, in this experiment the only differences between CO_2 levels were in C:N ratio, polyphenolic concentration in senesced leaf base, and cellulose concentration.

in vitro decomposition

Cumulative respired carbon from the plant litter-microbial complex can be used as an index of decomposition (Coleman,DC, 1973; Flanagan & Veum, 1974; Bunnell *et al.,* 1977b). Microbial populations on senesced leaf base (Figure 8.8) and senesced leaf lamina (Figure 8.9) respired less carbon per unit initial carbon from tissue grown at high CO_2. A similar effect was noted for root tissue (Figure 8.8), although the CO_2 effect was absent at low-N. Root tissue was a mixture of live and dead root. This may not reduce the utility of the results, as nitrogen is thought not to be resorbed from roots on senescence (McClaugherty *et al.,* 1984; Nambiar, 1987; Norby, 1994). As roots are often detached from the plant prior to death by soil animals in the field (Stanton, 1988), the use of a mixture of live and dead root may be appropriate. Both senesced leaf base and root tissue produced at the high-N supply rate accumulated more respired carbon during *in vitro* incubation than did the corresponding tissue produced at the two lower N supply rates, however no such effect was noted for senesced leaf lamina tissue. Both the CO_2 and nitrogen effect on respiration rate appear to be transitory, with the effect being reduced later in the decomposition assay (Table 8.6). However, this may be confounded by the greater amount of substrate remaining in those treatments with a slower decomposition rate.

Measurements of decomposition as either mass loss or accumulated respiration have shortcomings. The microbial biomass (live and dead) can accumulate a large proportion of the original substrate carbon (Clark,FE & Paul, 1970; Swift, 1973; Flanagan & Veum, 1974; Paul & Juma, 1981; Clark,FE & Woodmansee, 1992), resulting in underestimates of carbon loss from the substrate, both as mass loss and as cumulated respiration. Additionally, respiration data do not account for resistant, microbial by-products which may not be recovered in mass loss data, or carbon respired as ethylene or methane, further underestimating loss (Flanagan & Veum, 1974). The determination of decomposition rates with the method used in this study may also underestimate the maximal rates of decomposition as the microbial population had no source of nitrogen other than that present in the substrate (Fog, 1988). However, the effect of an external source of nitrogen on decomposition rate is variable, and has been observed to reduce decomposition rates (Fog, 1988; Prescott, 1995), especially late in the decomposition process (Fog, 1988; Coûteaux *et al.,* 1995).

Decreases in decomposition of tree leaf (Coûteaux *et al.,* 1991; Cotrufo *et al.,* 1994; Boerner & Rebbeck, 1995), tree root (Cotrufo & Ineson, 1995) and grass root (Gorissen *et al.,* 1995) substrate grown at high CO_2 have also been noted with simple microflora inoculum (Coûteaux *et al.,* 1991).

Determinants of decomposition rate

Significant relationships between cumulative respired carbon from senesced leaf base substrate and a number of initial litter quality parameters were found (Table 8.5). In descending order of variance explained these were, C:N ratio, lignin:N ratio, (polyphenolic + lignin):N, the relationship of Herman *et al.* (1977), the relationship of Herman *et al.* (1977) including polyphenolics as a factor contributing to a reduction in decomposition rate, and polyphenolic:N ratio. The best predictor amongst those tested was the simple C:N ratio, although only half of the variance was explained. The more complex relationships were no better. The lack of an effect of lignin on improving prediction is expected when, as here, there is little variation in its concentration, and when lignin concentrations are low (Taylor *et al.*, 1989).

Addition of CO_2 or N level to all of the models resulted in a significant improvement in the amount of variance explained (Table 8.5). This suggests that there where treatment effects on parameters that were not determined which affected cumulative respiration, possibly a parameter related to physical structure (Swift *et al.*, 1979; Gallardo & Merino, 1993). Physical structure of dicotyledonous plant leaves have been noted to change at high CO_2 (Thomas,JF & Harvey, 1983) and it is possible that morphological changes also occur in the leaves of C_3 monocots, as suggested by changes in specific leaf area (eg. Table 5.8). However, the ground senesced leaf lamina did not show a higher level of decomposition than the chopped (Figure 8.9). This suggests that the factor reducing decomposition at high CO_2 was not increased resistance of microbial entry into the structure of the tissue, which might have been overcome by grinding.

In summary, plant tissue grown at high CO_2 generally exhibited a lesser degree of decomposition in *in vitro* decomposition assays. The only exception to this was root from the low-N treatment, which showed no response to CO_2. The CO_2 effect on decomposition of senesced leaf base could be largely explained by the increase in C:N ratio. However the total variance explained was low, and there appeared to be other unknown treatment-induced changes affecting decomposition. The growth CO_2 and nitrogen level effect on respiration rates of the senesced leaf base-microbial complex were transitory, with no statistical effect remaining after the first 100 days of incubation.

in vivo decomposition - litter carbon & nitrogen balance

Estimates of *in vivo* decomposition were made from the balance between known carbon input to the surface litter layer, and recovery of carbon from the surface litter layer at harvest. No effect

of CO_2 was noted on the estimated carbon balance of the surface litter layer (Figure 8.10), while the balance became more negative as N level increased. Note that this is an absolute flux. When the rate of carbon (and N) loss from the litter layer per harvest interval was calculated as a fraction of surface litter carbon (or N), the same treatment effects were noted. However those calculations involve additional assumptions of homogeneity between pots within the sward, and hence are not presented. Another method of accounting for the differences in quantity of addition of defoliated leaf to the surface litter layer is to compare the ratio of recovered carbon from the surface litter layer to the quantity of carbon added as defoliated leaf (Figure 8.11). No CO_2 effects on this ratio were noted. This ratio is very noisy due to contamination of the surface litter layer with soil - which was relatively large at the lower N levels.

The positive carbon balance values at low- and mid-N are surprising, considering the small differences in nitrogen concentration of the surface litter, at least within CO_2 levels (Figure 8.3). The carbon and nitrogen balance values are estimates only, as carbon and nitrogen also accumulated in surface litter from leaf fall, moss growth, root growth and soil contamination at harvest, probably contributing to the positive carbon and nitrogen balance at low- and mid-N. Smaller additions of water to this layer at low- and mid-N may have contributed to the positive balance (page 6-104), and the larger surface litter load at high-N (Figure 6.9) may have provided a more favourable microenvironment for decomposition. Loss of carbon from this fraction could be attributed to microbial respiration and leaching of dissolved organic carbon and small particulate matter into the soil. These figures, along with those of the *in vitro* decomposition assays highlight the loss of carbon from the system via microbial respiration, and that gross carbon uptake by the sward is larger than the net increment measured in this experiment (Figure 6.3). It should be noted the carbon and nitrogen additions to the sward litter layer were a mixture of green and senesced leaf, having a combined nitrogen concentration as shown in Figure 8.2. Thus the non-structural carbohydrate content and nitrogen concentration was higher in this tissue than in that used in the *in vitro* assay.

A lack of a negative CO2 effect on sward or soil decomposition rates has been noted in other studies (Kemp *et al.,* 1994; Ross *et al.,* 1995). In the study of Ross *et al.* (1995) initial C:N ratios in the plant material were not changed greatly by growth CO2 concentration, and cumulative CO2 production early in the incubation was higher in the tissue grown under CO2 enrichment. In the study of Kemp *et al.* (1994), C:N ratios were increased at high CO2 in the C3 grass, but not the C4. While there were transitory treatment effects in mass loss from a litter bag study, after 18 months no within species treatment effects remained (Kemp *et al.,* 1994).

All treatments in this experiment showed a positive nitrogen balance in the surface litter layer. This is a common phenomenon (eg. Kemp *et al.,* 1994), most likely due to scavenging and immobilisation of nitrogen by microbes from fertiliser or soil (Swift *et al.,* 1979), and dinitrogen fixation on the high C:N ratio substrate (Swift *et al.,* 1979; Roper, 1985). Contributions to the nitrogen gain may also be attributed to leaf fall, root fragments and contamination with soil nitrogen. Nitrogen concentration of the surface litter (Figure 8.3) as a whole is high enough, at least in the CHN treatment, to expect some net mineralisation of nitrogen (Swift *et al.,* 1979), perhaps contributing to the growth of root in the surface litter.

There is great diversity in field decomposer populations (Swift *et al.,* 1979). Most decomposition studies, including those carried out in this study, have restricted decomposer populations to microflora, excluding micro-arthropods and other invertebrates. Micro-arthropods were present in the grass swards, although no attempt was made to quantify their numbers or diversity. The presence of micro-arthropods can be important in decomposition process, both directly, and in the reduction of particle size, stimulating microbial degradation (Swift *et al.,* 1979). This may be especially important in litter types with a high physical resistance to attack. In the ground-breaking study of Coûteaux *et al.* (1991) it was found that when the decomposer population consisted only of microflora, plant tissue (senesced chestnut leaf) produced at high CO_2 decomposed less than that produced at ambient CO_2 concentrations, as expected from the differences in initial C:N ratio. However when the diversity of the decomposer population increased (+ nematodes; + Collembola; + Isopoda) this difference was steadily reduced, and reversed when the full organism complex was involved in decomposition. Thus the use of simple, *in vitro* assays may be of limited use in predicting field decomposition rates.

Environmental factors influencing decomposition

Environmental factors, such as soil moisture, temperature and pH can have a large effect on decomposition rate (Swift *et al.,* 1979). Temperature will not be discussed, as it was not a variable in this experiment, although soil surface temperature may differ slightly between treatments due to differences in LAI, θ_v, and litter-load.

Soil moisture content is very important for microbial growth and hence litter decomposition (Wilson & Griffin, 1975; Bunnell *et al.,* 1977a, b; O'Connell, 1990; Andrén *et al.,* 1992; Grant & Rochette, 1994). Bacteria cease activity at water potentials below -1.0 to -0.5 MPa, while fungi and actinomycetes remain active at much lower soil water potentials (Wilson & Griffin,

1975; Swift *et al.,* 1979). The sensitivity of bacteria to moisture content may be related to restricted mobility as the soil dries (Wilson & Griffin, 1975). The activity of fungi is severely restricted at mass water contents below about 10%, as free water is often necessary for the diffusion of extracellular enzymes (Swift *et al.,* 1979).

Climatic factors become more important as determinants of decomposition rates as substrate quality improves (Heal & French, 1974). Dormaar and Willms (1993) concluded that temperature and moisture effects often override the effects of litter quality on root decomposition rates. The analysis of Meentemeyer (1978) and Dyer *et al.* (1990) also showed a very strong influence of the environment on decomposition rates. Thus soil moisture content can exert a large effect on decomposition rates. The extent of this influence may be substrate specific, and the relative effects tend to change from biotic control of decomposition in temperate regions to abiotic control in arid regions (Coûteaux *et al.,* 1995). This may be the result of more uniform soil moisture conditions in temperate regions. Berg *et al.* (1993) in a study spanning 39 sites from the sub-arctic to the subtropical regions showed actual evapotranspiration to be the major determinant of mass loss. Litter quality parameters did improve predictions of decomposition rates, although the effect of these was always strongly dependant on actual evapotranspiration. Thus the relationship between abiotic and biotic control of decomposition is complex.

Bunnell *et al.* (1977b) proposed a model of microbial respiration rate R as a function of temperature T and moisture content M for any stage of decomposition where;

$$R(T,M) = \frac{M}{a_1 + M} \cdot \frac{a_2}{a_2 + M} \cdot a_3 \cdot a_4^{\frac{T-10}{10}}$$

and a_1 is the moisture content at which microbial activity is at half its maximum value, which is a measure of water availability. a_2 is the moisture content at which gas exchange within the substrate complex is at half its maximum value, and is a measure of O_2 availability. a_3 is the substrate quality factor *viz:* the respiration rate at 10°C when oxygen and water are not limiting. a_4 is the Q_{10} for the respiration rate when moisture and oxygen are not limiting. This model showed a very good fit to ten different substrates under varying environments (Bunnell *et al.,* 1977b), and demonstrates the interactive nature of substrate and environmental quality on decomposition processes. Although this model was developed for use in the tundra, it has been tested extensively as a predictor of decomposition rate and is useful in regions where temperature maxima of microbial respiration are not reached (O'Connell, 1990).

For a comparison of the CO_2 effect on litter decomposition in the microcosm, the effect of increases in actual evapotranspiration noted in field studies can be though of as increases in soil moisture content in the microcosm. The higher soil moisture content under CO_2 enrichment in this experiment was measured as an average over the soil profile (Table 6.4, Figure 6.2, Figure 7.1). However, as water is applied from the soil surface it could be assumed that the enriched treatments had a higher soil moisture content in the surface soil. This may have contributed to higher moisture contents in the surface litter layer, although this was not determined. The loss of carbon in the high nitrogen treatment also appears to be related to the commencement of routine spraying of the canopy with demineralised water (page 6-104, Figure 8.10). Thus it is probable that the higher average soil moisture contents in this experiment contributed to the equal rates of disappearance of surface litter at high CO_2, even though litter quality was reduced.

Microbial parameters

Estimates of soil microbial carbon were higher at high CO_2 at the two highest N levels, but not at low-N. This is largely a measure of bulk soil microbial carbon, rather than rhizosphere microbial carbon. A large proportion of rhizosphere soil would be included in this fraction as the soil was brushed from the root during sieving. The estimate of microbial carbon in the surface litter layer was little different at high CO_2.

No calibration was undertaken with this soil or surface litter between the flush of ninhydrin reactive compounds and carbon mineralised from the fumigation-incubation method (Jenkinson & Powlson, 1976), the accepted standard for microbial carbon estimates. The factor used to calculate microbial carbon from the flush of ninhydrin reactive compounds was 21 (Amato & Ladd, 1988). The generality of that calibration has recently been questioned (Wardle & Ghani, 1995). The factor has been shown to vary between soil types (Sparling *et al.,* 1993), land use (Joergensen, 1996), and possibly with soil depth (Ross & Tate, 1993), however 21 is considered appropriate for soils of the Canberra region (V.V.S.R. Gupta, personal communication).

Might treatment affect the relationship between the flush of ninhydrin reactive compounds and microbial carbon in this experiment? That question was not addressed in this study. A possible reason for the CO_2 effect is the large difference in substrate nitrogen concentration, especially in the surface litter layer (Figure 8.3), and microbial populations have been noted to exhibit flexibility in C:N ratios (Swift *et al.,* 1979). However this method of estimating microbial carbon with a single conversion factor has been validated for substrates (soil, humus and leaf

litter) with a range of C:N ratios from 12 to 80, and varying pH and soil bulk densities collected from a similar soil type and was found to give results comparable with those of other methods (Sparling *et al.,* 1994). In that dataset the microbial C:N ratio varied between 3.5 in a woodland litter to 7.7 in a pasture soil (Sparling *et al.,* 1994). This method has also been shown to have a constant conversion factor across soils recently amended with straw, relative to substrate induced respiration and ATP methodologies for determining microbial biomass (Ocio & Brookes, 1990).

The estimates of soil microbial biomass carbon concentrations in ambient CO_2 treatments (200-550 $\mu g\ g^{-1}$ soil) of this experiment are similar to those observed in Australian field soils (Sparling & Zhu, 1993; Sparling *et al.,* 1993, 1994). The estimates of microbial carbon in the EHN treatment (<1250 $\mu g\ g^{-1}$ soil) were lower than that observed in some forest soils from New Zealand (Ross & Tate, 1993) and similar to an arable field soil from Texas USA (Franzluebbers *et al.,* 1995). Estimates of microbial carbon in the surface litter layer of this experiment were similar to those observed in the <6 mm fraction of litter collected from sandy soils in WA (Sparling *et al.,* 1994). Thus the estimates of microbial carbon from this experiment are similar to those observed in the field.

As the proportion of total soil carbon that was microbial (C_{mic}:C; Figure 8.14) was higher at high CO_2, the increase in soil microbial carbon under CO_2 enrichment was not merely a reflection of increased soil carbon levels (Figure 6.12). However, the ratio of microbial carbon to soil carbon is much higher than that reported in the literature, as presented in review (Insam *et al.,* 1989; Insam, 1990) or presented (Brandenburg & Sparling, 1994; Gupta *et al.,* 1994) or calculated from Australian data (Sparling & Zhu, 1993; Sparling *et al.,* 1993). The maximum C_{mic}:C observed in those studies was approximately 70 mg g^{-1}, relative to maximal concentrations in this experiment of ~200 mg g^{-1}. The initial soil carbon concentration in this experiment was 0.16%, so this ratio will be much more sensitive to changes in microbial carbon than for most soils.

The ratio of microbial to total (organic) carbon is correlated with moisture availability, (Insam *et al.,* 1989; Insam, 1990), and has been empirically modelled as;

$$y = 64.1 - 109.5x + 55.7x^2$$

where y is the concentration of microbial carbon as a proportion of soil organic carbon (mg g^{-1}) and x is the ratio of precipitation to evaporation (Insam, 1990). Deviations from this relationship are attributed to vegetation type, soil type and non-equilibrium soil carbon concentrations (Insam *et al.,* 1989; Insam, 1990; Sparling, 1992). If this relationship were to

hold for this experiment, which was below the range of precipitation:evaporation ratios from which the model was determined, the microbial carbon concentration would be ~60 mg microbial C g^{-1} organic C. This relationship was developed from cropped soils, which may differ from grassland soils. However, a deviation above the predicted value indicates increasing soil carbon levels, (Insam, 1990), as is occurring in this experiment (Figure 6.12). The higher ratio at high CO_2 may indicate a greater equilibrium soil carbon concentration at high CO_2, at least at the two higher rates of N supply (Sparling, 1992).

Contrary to the results for soil, surface litter microbial carbon (g C_{mic} m^{-2}) did not differ between CO_2 levels, and a lower proportion of litter carbon was microbial at high CO_2 (Table 8.8). Surface litter C_{mic}:C was high in this experiment. Visually the surface litter was heavily covered by spores. Microbial carbon has been observed to form up to 70% of litter carbon (Clark,FE & Paul, 1970; Swift, 1973; Paul & Juma, 1981). The high C_{mic}:C observed here may partially be the result of a lack of disturbance from animals and invertebrates, which would reduce the physical size of this decaying material and force much of it into the soil fraction, mainly via burial by soil fauna (Clark,FE & Woodmansee, 1992). If correction were made to the surface litter carbon balance for this proportional microbial carbon content a difference between the CO_2 treatments may emerge. However, the data on surface litter microbial carbon must be considered with caution. In the week prior to, and during harvest, microcosm water content was adjusted manually by pouring water onto the surface of the pot, resulting in a wetter surface litter layer through this period than in normal growth. However, day of harvest or water added in this period were not significant as covariates in ANCOVA.

Potentially mineralisable nitrogen exhibited similar response patterns to experimental treatments as did microbial carbon estimates. The nitrogen pool that is mineralised shows a strong correlation with the pool of plant extractable nitrogen (Keeney & Bremner, 1966a, b; Stockdale & Rees, 1994). Potentially mineralisable nitrogen, as assayed in this experiment, has also been shown to correlate well with microbial nitrogen estimates by the fumigation-incubation method, both in magnitude and in the source of the extracted nitrogen, as determined by ^{15}N techniques (Myrold, 1987; Stockdale & Rees, 1994). Thus an index of microbial C:N ratio in this experiment can be defined as the ratio of the microbial carbon to potentially mineralisable nitrogen, as shown in Table 8.11.

The index of microbial C:N ratio probably overestimates actual microbial C:N ratio, as the potentially mineralisable nitrogen method was observed to extract only 87% of biomass nitrogen determined from fumigation-incubation, averaged over 17 soil types (Stockdale &

Rees, 1994). However, it is evident that the CO_2 and N treatments in this experiment may be changing these relationships. No CO_2 effects were noted at low-N on the index of microbial C:N, as with the other microbial parameters. At mid- and high-N, growth at high CO_2 resulted in a reduction in the index of soil microbial C:N ratio, which is the opposite effect to that noted in any other nitrogen pool. This was the result of an higher C:N index at the higher N levels at ambient CO_2, and a lower C:N index at the higher N levels under CO_2 enrichment. Data are not presented for surface litter as it was very variable. This C:N ratio change may indicate a change in microbial population structure at high CO_2, although microbial C:N ratio is flexible, and reflects changes in the substrate being utilised for growth (Swift *et al.,* 1979). For instance, *in vitro* the nitrogen content of fungal mycelium has been noted to vary almost twenty-fold in response to varying substrate nitrogen content (Levi & Cowling, 1969). High atmospheric CO_2 may increase the deposition of readily available carbon into the rhizosphere (Norby *et al.*, 1987; van Veen *et al.,* 1991; Rattray *et al.,* 1995). This may favour bacterial growth, lowering the microbial C:N ratio (Anderson,JPE & Domsch, 1980).

No consistent change in microbial C:N ratio was noted in a *Lolium perenne* L. *Trifolium repens* L. sward (Ross *et al.,* 1995), however microbial C:N ratios did tend to be lower at high CO_2, and this may be influenced by the potentially large input of atmospheric nitrogen by the legume. Populations of bacterial grazing nematodes were observed to increase at high CO_2 in similar swards, suggesting an increase in bacterial populations (Yeates & Orchard, 1993). Microbial C:N ratio was not determined in the study of Zak *et al.* (1993), however analysis of soil phospholipid fatty acid profiles indicated that CO_2 had no effect on microbial structure (Zak *et al.,* 1996). The authors strongly emphasised the fact that their study was a short term study (152 days) and that the only carbon entering the soil was root derived, suggesting that the finding may not hold when nutrient cycling was fully established (Zak *et al.,* 1996). From the available evidence the cause of the shift in the soil microbial C:N index in this study cannot be determined, however it is probably the result of a decreased substrate C:N ratio, and/or a change in microbial population structure.

A number of reports of the effect of CO_2 on microbial populations (direct counts, or physiological based estimates of biomass) have now been published, and CO_2 effects on the microbial population are variable (Norby *et al.,* 1986; O'Neill *et al.,* 1987; Owensby *et al.,* 1993b; Zak *et al.,* 1993, 1996; Rice *et al.,* 1994; Newton *et al.,* 1995; Ross *et al.,* 1995; Schenk *et al.,* 1995; Tingey *et al.,* 1995; Schortemeyer *et al.,* 1996). It has been hypothesised that at high CO_2 more nitrogen would become associated with the microbial fraction, reducing its

availability for plant growth (Díaz *et al.,* 1993). However, the amount of plant available nitrogen, and the rate of nitrogen cycling is often correlated with microbial carbon (Franzluebbers *et al.,* 1995). In the study of Díaz *et al.,* (1993) microbial carbon and nitrogen was increased at high CO_2, and while the plant community biomass was not significantly increased at high CO_2, there was a positive trend. A counter hypothesis was proposed by Zak *et al.* (1993): *viz.* the size of the microbial biomass pool will increase following greater carbon inputs to the soil in response to CO_2 increase, resulting in greater carbon turnover and higher levels of nitrogen availability. The increase in nitrogen availability is expected in response to a larger microbial carbon pool, or as a result of a greater rate of turnover of the pool, increasing the amount of ammonium and dead microbial nitrogen in the soil (Zak *et al.,* 1993). The findings of this study lend some support to both hypotheses. At high CO_2, the microbial carbon and nitrogen pools were larger, and there were increases in the size of the soil amino-nitrogen, and potentially mineralisable nitrogen pools, at least at the higher N levels. Live aboveground carbon was not increased by CO_2 enrichment, nor was total root carbon. However, leaf, and possibly root turnover rates were increased under CO_2 enrichment, and total microcosm carbon gain was increased.

As there is a positive relationship between microbial and total (organic) soil carbon (Insam, 1990), these data, and that from the literature suggest that total system carbon may increase as atmospheric CO_2 concentrations increase. However this response may not be seen as increases in live biomass in the short term, but rather as an increase in the rate of carbon cycling, and higher residual levels of carbon in the soil.

Arbuscular mycorrhizal infection

Arbuscular mycorrhizae (AM) are a large group of fungi present in almost all soils (Abbott & Robson, 1991). Arbuscular mycorrhizae infection of Gramineae and other herbaceous plants is common, and infection can provide benefit for the host via improved nutrient uptake, of both P (Plenchette *et al.,* 1983) and N (Barea *et al.,* 1987; Johansen *et al.,* 1992), improved water uptake (Subramanian *et al.,* 1995), and AM have been implicated in nutrient transfer between plants (Johansen & Jensen, 1996). AM infection was not affected by treatment, and infection rates were high, at about 60% of root length infected. This is within the range of infection noted for field grown Gramineae (Abbott & Robson, 1991).

There are few studies (knowingly) incorporating AM infection in CO_2 studies. No interaction between AM infection and CO_2 level was observed in *Lolium perenne* for leaf area or biomass production in grasses grown on a $\frac{1}{8}$ or $\frac{1}{2}$ strength Hoagland solution (Marks & Clay, 1990).

Those studies that have reported infection levels have found an increase (Whitbeck, 1993; Monz *et al.,* 1994; Morgan *et al.,* 1994) or no change in infection at high CO_2 (Whitbeck, 1993; Monz *et al.,* 1994). However, if root length is increased at high CO_2, as with the *Liriodendron tulipifera* L. (yellow poplar), an increase in AM tissue would result with no change in infection level, which may be integral to the CO_2 response (O'Neill *et al.,* 1991; O'Neill, 1994).

Soil carbon and nitrogen pools

The fraction of total soil carbon and nitrogen gain from sowing that was present in slow-cycling pool (Table 8.10 & Table 8.9) was increased by CO_2 enrichment. Thus, the extra carbon and nitrogen accumulation in the soil under CO_2 enrichment occurred both in fast- and slow-cycling pools. The fraction of soil carbon referred to in this study as slow soil carbon probably contains some labile carbon associated with the amino-N fraction. This quantity of carbon is assumed to be to small to influence these conclusions. Concentrations of other exudates may be expected to be low in the soil, as they are rapidly metabolised by the microbial population. The C:N ratio of this carbon gain in the slow pool tended to be higher under CO_2 enrichment (Table 8.10). Although that increase in C:N ratio was not significant, the nitrogen concentration (per unit carbon) of the total root-free soil pool was decreased (ie. C:N ratio increased) under CO_2 enrichment (Figure 8.4), suggesting that such changes may be lost in experimental variability. Thus, growth under CO_2 enrichment has increased carbon and nitrogen accumulation in the more stable soil pools.

Conclusions

Growth at high CO_2 reduced nitrogen concentrations in senesced leaf base, senesced leaf lamina and root. Reductions in root nitrogen concentration at the low-N level may result from carbohydrate dilution, while at the higher N levels, and in the shoot tissues, the CO_2 effect remained when expressed on a structural carbon basis. Other litter quality parameters measured showed little change in response to CO_2 enrichment.

As nitrogen concentrations were reduced, and hence C:N ratios increased at high CO_2, *in vitro* decomposition, measured as accumulated respired carbon, were lower in material grown under CO_2 enrichment, except in root grown at the low-N level. This was largely due to lower respiration rates from the high CO_2 microbial-substrate complex immediately following the initial respiratory flush. Respiration rates after about 100 days showed no treatment effects, either due to growth N level or growth CO_2 level.

Litter balance calculations at the sward level showed no effect of CO_2 level on carbon loss from the surface litter. There was a strong N effect, with losses higher at high-N. No net loss of nitrogen occurred from the surface litter layer. The difference between *in vitro* and in sward measurements is attributed to changes in the decomposer population and in soil moisture content.

Soil microbial carbon was higher under CO_2 enrichment at the two higher N levels, probably resulting from higher inputs of carbon from the plant. Soil microbial C:N ratio decreased at high CO_2 at mid- and high-N, potentially resulting from increases in rhizosphere nitrogen deposition or changes in microbial population structure. The soil C_{mic}:C ratio was higher under CO_2 enrichment at the two higher rates of N supply, suggesting a higher equilibrium soil carbon concentration at high CO_2. This was further supported by increases in the slow soil carbon pool. Potentially mineralisable N was increased under CO_2 enrichment at the two higher rates of N supply. Thus, plant available N may be higher at high CO2 at mid- and high-N, independently of external N supply. This indicates that the maintenance of increased total system carbon storage in response to CO2 enrichment may continue in the long term, even if application of fertiliser N ceased, and plant available nitrogen became totally dependant on within microcosm processes.

Table 8.1 Carbon to nitrogen ratio of fractions used for *in vitro* decomposition assays. All material was collected at the 922 day harvest. Control and enriched CO_2 are given as C and E respectively. $P<0.001$ is ***, $P<0.01$ is **, $P<0.05$ is * and $P<0.1$ is +, ns is not significant.

		C:N ratio			P			LSD P£0.05
	CO2	**LN**	**MN**	**HN**	**CO2**	**N**	**C*N**	**C*N**
senesced leaf	C	100	76	65	***	***	ns	17
lamina	E	146	134	91				
senesced leaf base	C	115	107	56	***	***	**	6
	E	134	136	89				
root	C	40	34	25	***	***	*	2
	E	42	41	34				

Table 8.2 Nitrogen resorption efficiency (%), calculated from N concentrations expressed on a structural carbon basis. Nitrogen resorption efficiency was higher at the 1104 day harvest ($P<0.05$). There was no CO_2 effect.

Harvest DOE	CO2	Low N	Mid N	High N
922	Con CO2	66.3 ± 1.6	54.7 ± 2.4	59.2 ± 1.4
	Enr CO2	67.7 ± 0.5	62.2 ± 3.4	60.0 ± 2.2
1104	Con CO2	71.1 ± 1.3	70.5 ± 3.2	75.3 ± 1.0
	Enr CO2	69.9 ± 1.0	73.9 ± 1.5	78.3 ± 0.3

Table 8.3 Hemicellulose - Pectin C, as a fraction of structural C at the 922 day harvest. Treatment mean followed by one standard error. There were no significant treatment effects.

		mg Hemi. + Pec. C g^{-1} Struct. C		
		Low N	**Mid N**	**High N**
Senesced leaf lamina	Con CO_2	438 ± 8	394 ± 8	395 ± 10
	Enr CO_2	428 ± 14	403 ± 8	398 ± 14
Senesced leaf base	Con CO_2	481 ± 6	475 ± 11	485 ± 3
	Enr CO_2	469 ± 10	469 ± 8	492 ± 7
Root	Con CO_2	526 ± 6	548 ± 6	514 ± 10
	Enr CO_2	545 ± 14	524 ± 22	511 ± 13

Table 8.4 Extractable polyphenolic concentrations, as tannic acid equivalents from root, senesced leaf base and senesced leaf lamina at the 922 day harvest. *P* levels for CO_2 and N are for the main effect. $P<0.05$ represented by ∗, $P<0.1$ by +, and not significant by ns.

		mg poly. C g^{-1} struct. C			*P* levels		
		Low N	**Mid N**	**High N**	**CO_2**	**N**	**CO_2*N**
Sen. leaf lamina	Con CO_2	11.9 ± 0.6	11.3 ± 0.2	11.0 ± 0.2	ns	ns	ns
	Enr CO_2	11.4 ± 0.3	10.9 ± 0.4	10.8 ± 0.1			
Sen. leaf base	Con CO_2	8.6 ± 0.4	6.7 ± 0.3	6.4 ± 0.3	+	*	ns
	Enr CO_2	7.2 ± 0.2	6.4 ± 0.2	5.5 ± 0.1			
Root	Con CO_2	6.1 ± 0.6	4.6 ± 0.2	4.9 ± 0.2	ns	ns	ns
	Enr CO_2	nd	4.1 ± 0.1	4.5 ± 0.2			

Table 8.5 Determinants of cumulated respired C of senesced leaf base after 297 days of incubation. Variance explained is that explained by linear regression of cumulative respired carbon on the predictor, with *P* as the level of significance. (-) indicates no relationship. Columns headed CO_2 and N show the level of significance added by including treatment as a factor in the stepwise model, and significance shows that extra variance can be explained by treatment after that accounted for by the determinant. $P<0.001$ represented by ***, $P<0.01$ by **, $P<0.05$ by *, $P<0.1$ by +, and not significant by ns. †From Herman *et al.* (1977), see text.

	Variance explained (%)	***P***	**CO_2**	**N**
Lignin concentration	7.1	*ns*	***	**
Cellulose concentration	8.2	+	**	**
Hemicellulose + Pectin + other conc.	-	*ns*		
C:N ratio	54.3	***	*ns*	*
Total non-structural carbohydrate conc.	-	*ns*		
lignin:N ratio	44.4	***	*	*
Lignin:Cellulose ratio	12.7	+	***	**
Lignin:(Hemi-Pectin + Cellulose) ratio	12.1	+	***	**
Polyphenolic concentration	-	*ns*		
Polyphenolic:N ratio	20.4	*	**	*
(Polyphenolic + Lignin):N ratio	44.4	***	*	*
† $\frac{(C{:}N) * (\%\ Lignin)}{\sqrt{(\%\ NSC + \%\ Cellulose + \%\ Hemi\text{–}Pec)}}$	42.6	***	*	*
$\frac{(C{:}N) * (\%\ Lignin + \%\ Polyphenolic)}{\sqrt{(\%\ NSC + \%\ Cellulose + \%\ Hemi\text{–}Pec)}}$	42.6	***	*	*

Table 8.6 Average instantaneous respiration rate of microbial-senesced leaf base complex over periods within the 297 day incubation. *P* levels for CO_2 and N are for the main effect. $P<0.001$ represented by ***, $P<0.01$ by **, $P<0.05$ by *, $P<0.1$ by +, and not significant by ns.

	Average instantaneous respiration rate over range of days of incubation ---------- ng C g initial C s^{-1} ----------					
Main effects	**0-10**	**10-20**	**20-30**	**30-50**	**50-100**	**100-297**
Control CO_2	217	95	64	44	36	18
Enriched CO_2	148	62	40	29	30	15
Low N	171	71	48	35	34	17
Mid N	144	70	43	33	30	14
High N	232	92	61	40	33	19
P - CO_2	***	***	**	*	ns	ns
P - N	***	*	+	ns	ns	ns

Table 8.7 Effect of inoculum type on cumulative respired carbon during *in vitro* decomposition rate assay. Common inoculum was derived from the CHN treatment, while other inoculum was derived from EHN surface litter for senesced leaf base and from the individual treatment's own soils for the root. There were no significant effects of inoculum type (ANOVA).

			Common Inoculum	**Other Inoculum**
Senesced leaf base	Low N	Enr CO_2	0.31 ± 0.02	0.26 ± 0.04
	Mid N	Enr CO_2	0.26 ± 0.01	0.25 ± 0.03
	High N	Enr CO_2	0.36 ± 0.03	0.30 ± 0.02
Root	Low N	Con CO_2	0.29 ± 0.01	0.29 ± 0.02
		Enr CO_2	0.29 ± 0.02	0.27 ± 0.01
	Mid N	Con CO_2	0.33 ± 0.01	0.31 ± 0.01
		Enr CO_2	0.26 ± 0.01	0.27 ± 0.01
	High N	Con CO_2	0.39 ± 0.02	-
		Enr CO_2	0.33 ± 0.03	0.31 ± 0.01

Table 8.8 Microbial carbon of surface litter, microbial carbon as a proportion of surface litter carbon (C_{mic}:C) and potentially mineralisable nitrogen of surface litter (P_{min}-N), averaged over harvests on day 1285 and 1469. $P<0.001$ represented by ***, $P<0.01$ by **, $P<0.05$ by *, $P<0.1$ by +, and not significant by ns. Analysis of microbial carbon and P_{min}-N undertaken on *ln* transformed data, and presented as back-transformed means and standard errors. C_{mic}:C presented as mean, LSD=0.08 (CO_2*N; $P<0.05$).

		N level			*P* level		
		Low	**Mid**	**High**	**CO_2**	**N**	**C*N**
Microbial C	Con CO_2	0.70 ± 0.07	2.57 ± 0.25	14.6 ± 1.4	ns	***	ns
($g\ m^{-2}$)	Enr CO_2	0.70 ± 0.07	2.41 ± 0.23	13.0 ± 1.2			
C_{mic}:C	Con CO_2	0.49	0.35	0.15	***	***	ns
($g\ g^{-1}$)	Enr CO_2	0.30	0.14	0.06			
P_{min}-N	Con CO_2	1.0 ± 0.3	3.6 ± 1.1	16.0 ± 4.7	*	***	ns
($mg\ m^{-2}$)	Enr CO_2	1.0 ± 0.3	2.0 ± 0.6	5.4 ± 1.6			

Table 8.9 Components of total soil nitrogen averaged over harvests on day 1285 and day 1469. Mineral N (NH_4^+, NO_3^-), total mineral N ($NH_4^+ + NO_3^-$), potentially mineralisable N (P_{min}-N), extractable amino-N (amino-N), fast-cycling soil nitrogen;

fast soil nitrogen = [Pmin-N + amino-N]

slow cycling soil nitrogen;

slow soil nitrogen = ([total root-free soil N] - [fast soil nitrogen] - [total mineral N]) and total root-free soil N. All data are for the surface 50 mm of soil and are expressed as g m^{-2}. *P* levels for CO_2 and N are for the main effect. $P<0.001$ represented by ***, $P<0.01$ by **, $P<0.05$ by *, $P<0.1$ by +, and not significant by ns.

		N level			*P* level			LSD $P \leq 0.05$
		Low	**Mid**	**High**	**CO_2**	**N**	**C*N**	**C*N**
		------ **g m^{-2}** -----						
NH_4^+-N	Con CO_2	0.15	0.18	0.42	ns	***	**	0.18
	Enr CO_2	0.10	0.15	0.61				
NO_3^--N	Con CO_2	0.04	0.07	0.19	ns	*	ns	0.15
	Enr CO_2	0.03	0.02	0.26				
total mineral N	Con CO_2	0.18	0.25	0.60	ns	***	*	0.14
	Enr CO_2	0.12	0.17	0.87				
P_{min}-N	Con CO_2	1.4	1.4	1.1	***	*	**	0.5
	Enr CO_2	1.4	2.5	2.7				
amino N	Con CO_2	0.12	0.14	0.15	***	**	+	0.03
	Enr CO_2	0.15	0.25	0.27				
fast soil N	Con CO_2	1.5	1.5	1.2	***	*	**	0.6
	Enr CO_2	1.5	2.7	3.0				
slow soil N	Con CO_2	11.5	13.4	14.5	**	***	*	1.9
	Enr CO_2	13.0	14.0	19.7				
Total soil nitrogen	Con CO_2	13.2	15.2	16.3	***	***	**	1.7
	Enr CO_2	14.6	16.9	23.5				

Table 8.10 Components of total soil carbon averaged over harvests on day 1285 and day 1469. Total root-free soil carbon, microbial or fast soil carbon, and slow cycling soil carbon;

slow soil C = ([total C] - [microbial C])

and the C:N ratio of the slow pool. All data are for the surface 50 mm of soil and are expressed as g m^{-2}. *P* levels for CO_2 and N are for the main effect. $P<0.001$ represented by ***, $P<0.01$ by **, $P<0.05$ by *, $P<0.1$ by +, and not significant by ns.

		N level			***P* level**			**LSD $P \leq 0.05$**
		Low	**Mid**	**High**	**CO_2**	**N**	**C*N**	**CO_2*N**
		------ **g m^{-2}** -----						
Microbial C	Con CO_2	20	22	17	***	**	**	3
	Enr CO_2	20	30	36				
slow soil C	Con CO_2	100	133	169	***	***	***	7
	Enr CO_2	118	159	245				
Total soil carbon	Con CO_2	119	155	186	***	***	***	5
	Enr CO_2	138	189	282				
slow C:N	Con CO_2	8.7	9.9	11.8	ns	ns	ns	3.5
	Enr CO_2	10.8	11.5	12.1				

Table 8.11 Index of soil microbial C:N ratio (g g^{-1}), averaged over the 1285 and 1469 day harvests. Calculated as the ratio of the estimate of microbial C to potentially mineralisable N (see text). *P* levels for CO_2 and N are for the main effect. $P<0.001$ represented by ***, $P<0.01$ by **, $P<0.05$ by *, $P<0.1$ by +, and not significant by ns.

					***P* level**		**LSD $P\leq0.05$**
	Low N	**Mid N**	**High N**	**CO_2**	**N**	**CO_2*N**	**CO_2*N**
Control CO_2	14.0	16.5	16.4	*	ns	*	2.7
Enriched CO_2	15.7	12.2	13.3				

Table 8.12 Arbuscular mycorrhizal infection (AM) of root in the surface 50 mm of soil at the 1285 day harvest. ns is not significant.

		N level			*P* level			LSD $P \leq 0.05$
		Low	**Mid**	**High**	CO_2	**N**	CO_2*N	CO_2*N
AM (% infection)	Con CO_2	57	56	60	ns	ns	ns	14
	Enr CO_2	58	67	63				

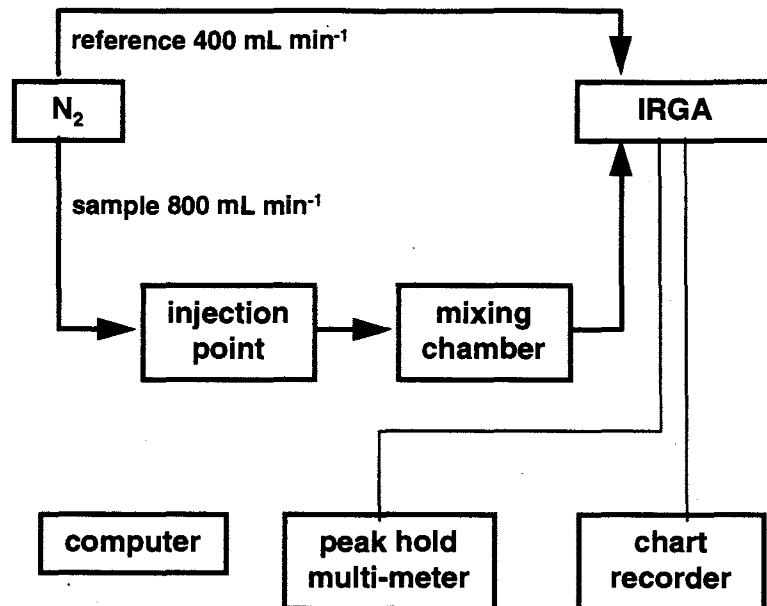


Diagram 8-1 Apparatus for measuring CO_2 evolution from the decomposing litter complex. The sample pulse was introduced through a Suba seal at the injection point, passed through the mixing chamber in the N_2 flow and then into the IRGA. Output of the multi-meter was manually entered into the computer which automatically logged the time of data entry. The mixing chamber prolonged the peak, giving a more accurate determination of CO_2 concentration. The chart recorder allowed retrieval of data for which the multi-meter was not reset. Approximately one determination per minute could be made. The system was calibrated using gases of a known CO_2 concentration generated using Wösthoff mixing pumps.

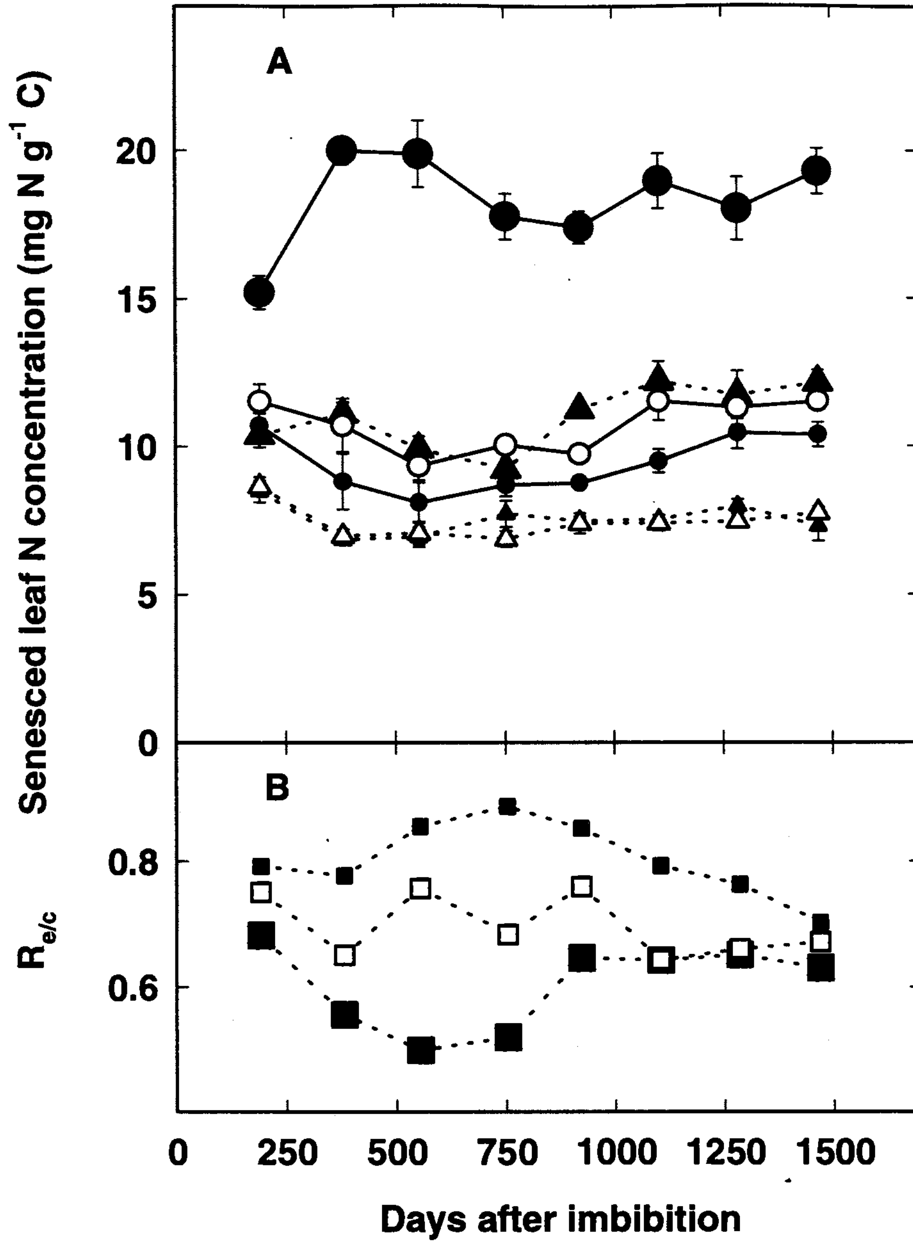


Figure 8.1 Nitrogen concentration of senesced leaf at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low N (■), mid N (□), and high N(■).

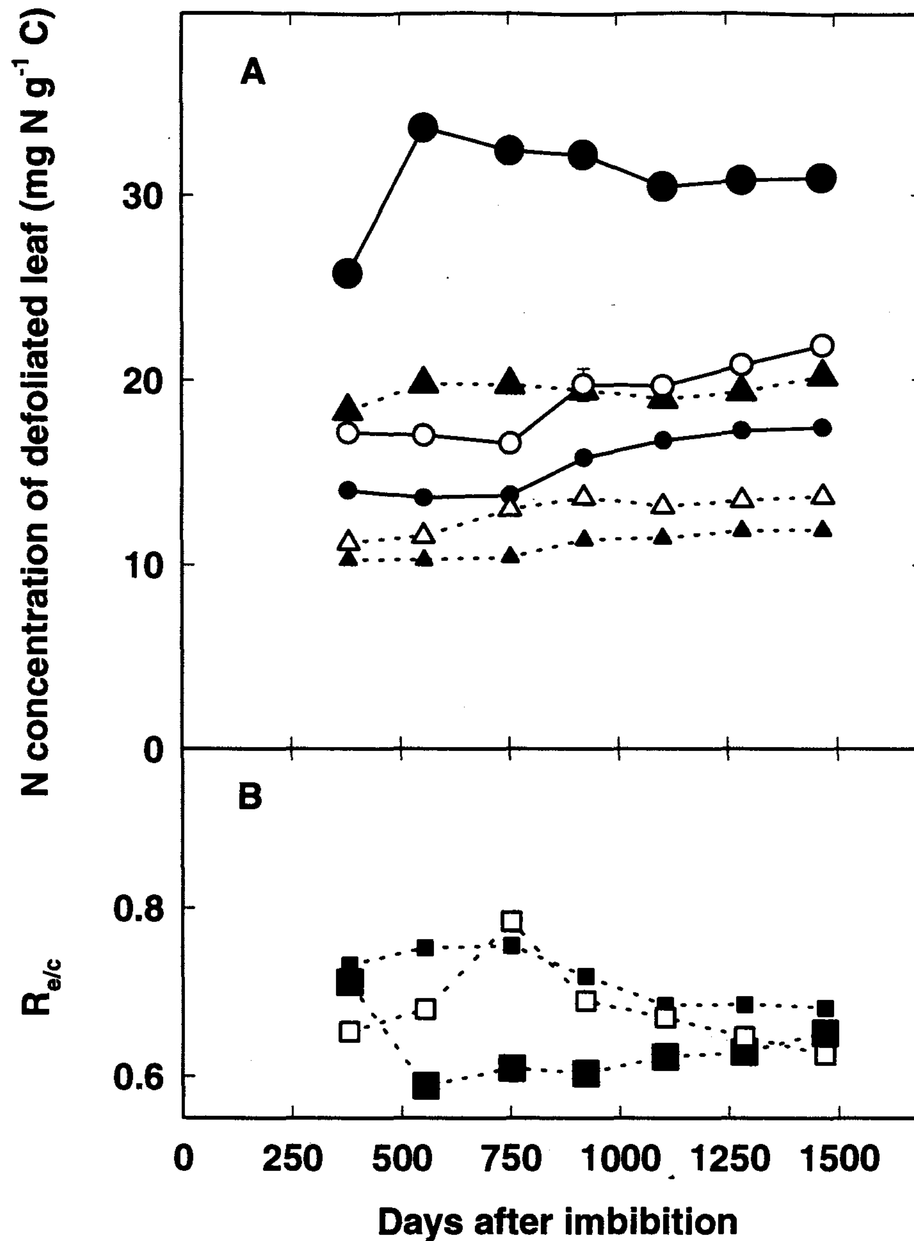


Figure 8.2 Nitrogen concentration of defoliated leaf at harvest (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low N (■), mid N (□), and high N(■).

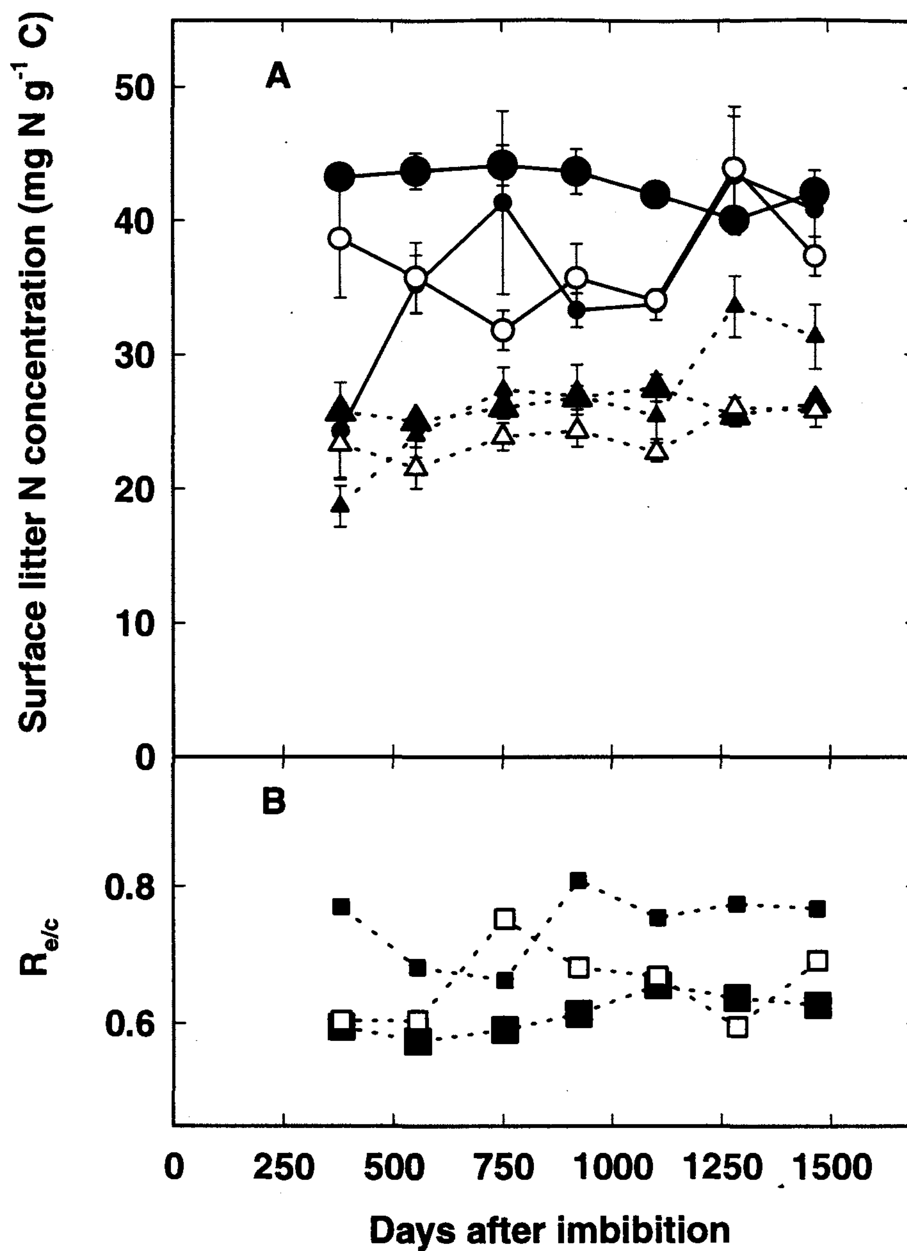


Figure 8.3 Nitrogen concentration of surface litter at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low N (■), mid N (□), and high N(■).

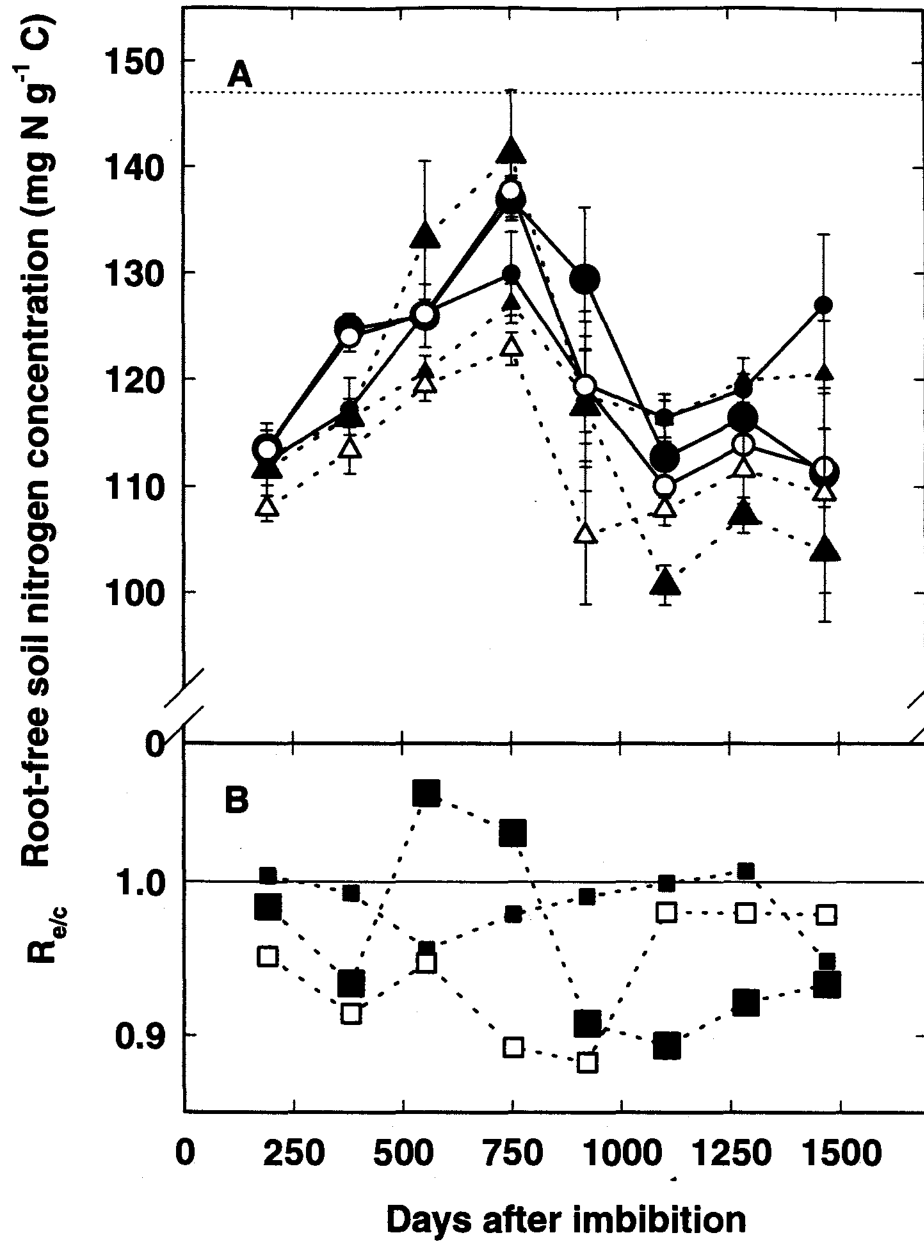


Figure 8.4 Nitrogen concentration of root free soil at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted joining line. Error bars are ± one standard error, which may be concealed by the symbol. Horizontal dotted line shows starting N concentration. (B) CO_2 response ratio. Low N (■), mid N (□), and high N(■).

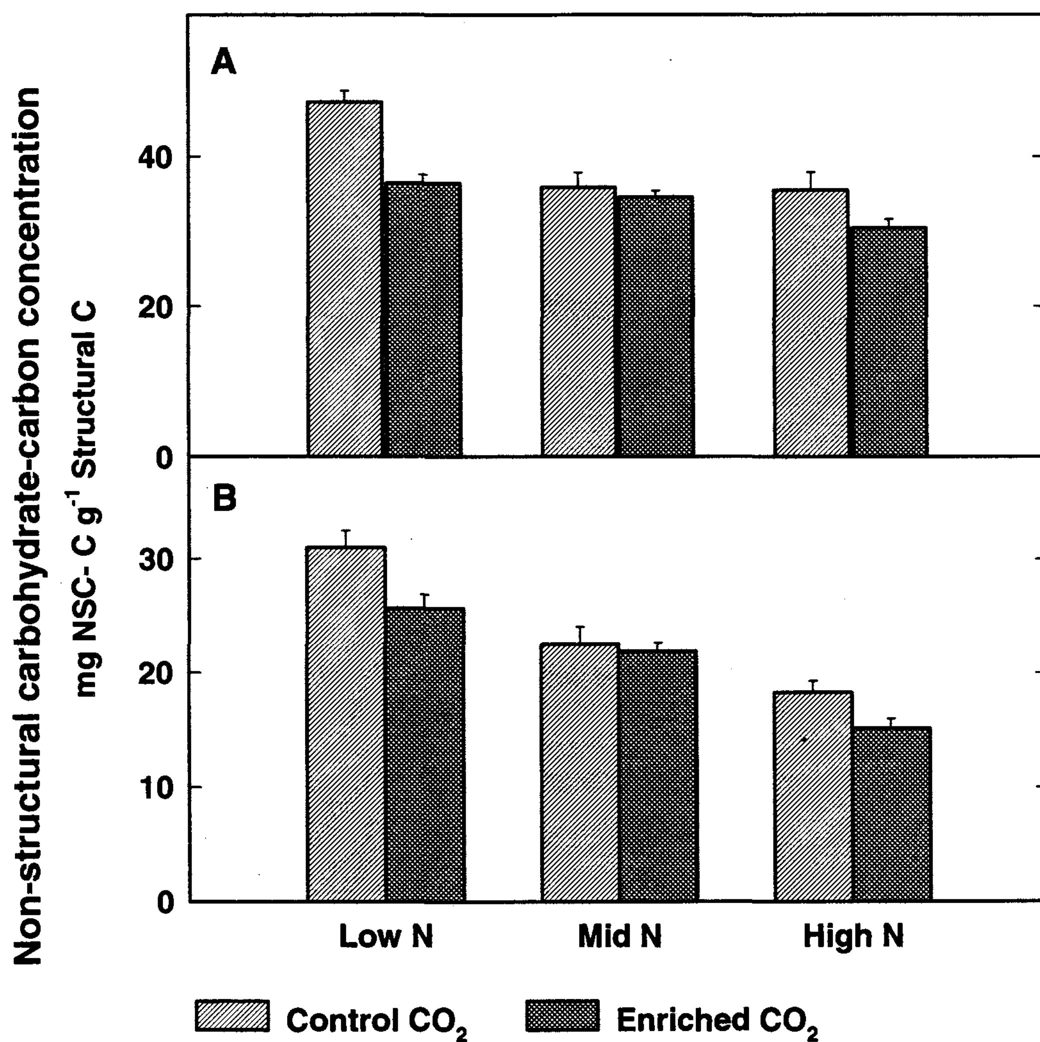


Figure 8.5 Non-structural carbohydrate-carbon concentration at the 922 day harvest of (A) senesced leaf lamina and (B) senesced leaf base.

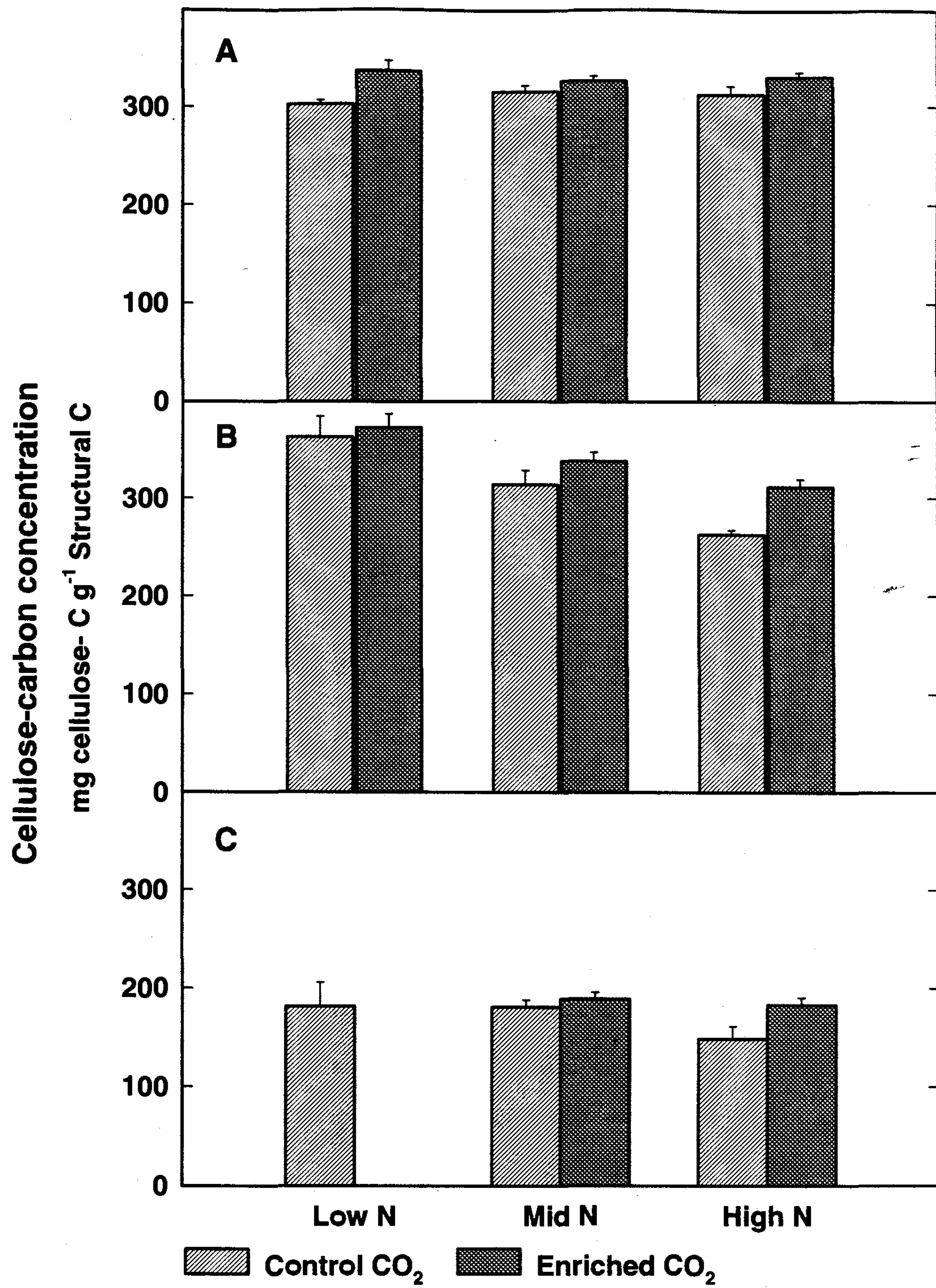


Figure 8.6 Cellulose-carbon concentration on a structural carbon basis at the 922 day harvest for (A) senesced leaf lamina, (B) senesced leaf base and (C) root. Cellulose concentration not determined in root of the control low-N treatment due to lack of sample.

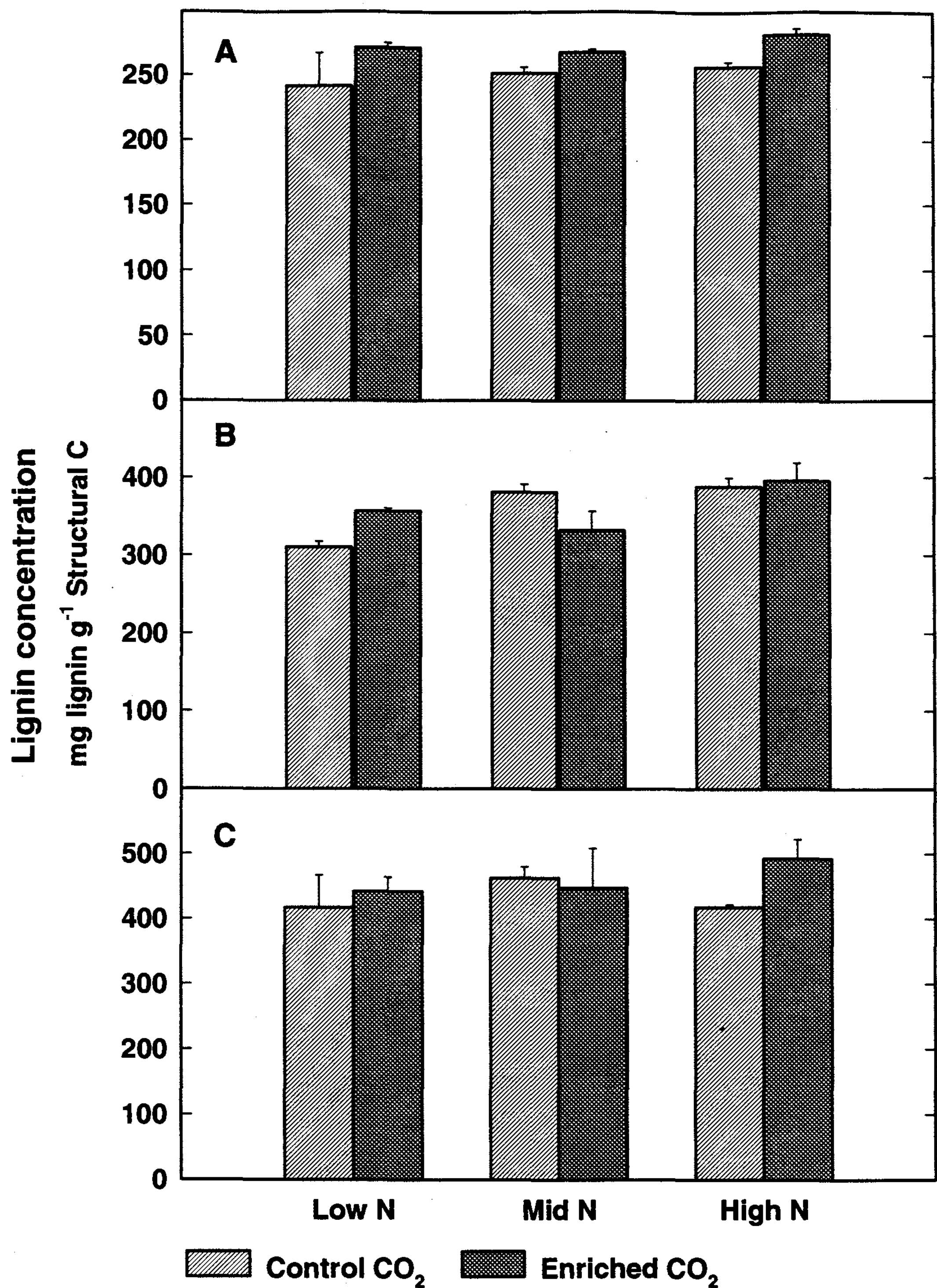


Figure 8.7 Lignin concentration on a structural carbon basis at the 922 day harvest of (A) senesced leaf lamina, (B) senesced leaf base, and (C) root.

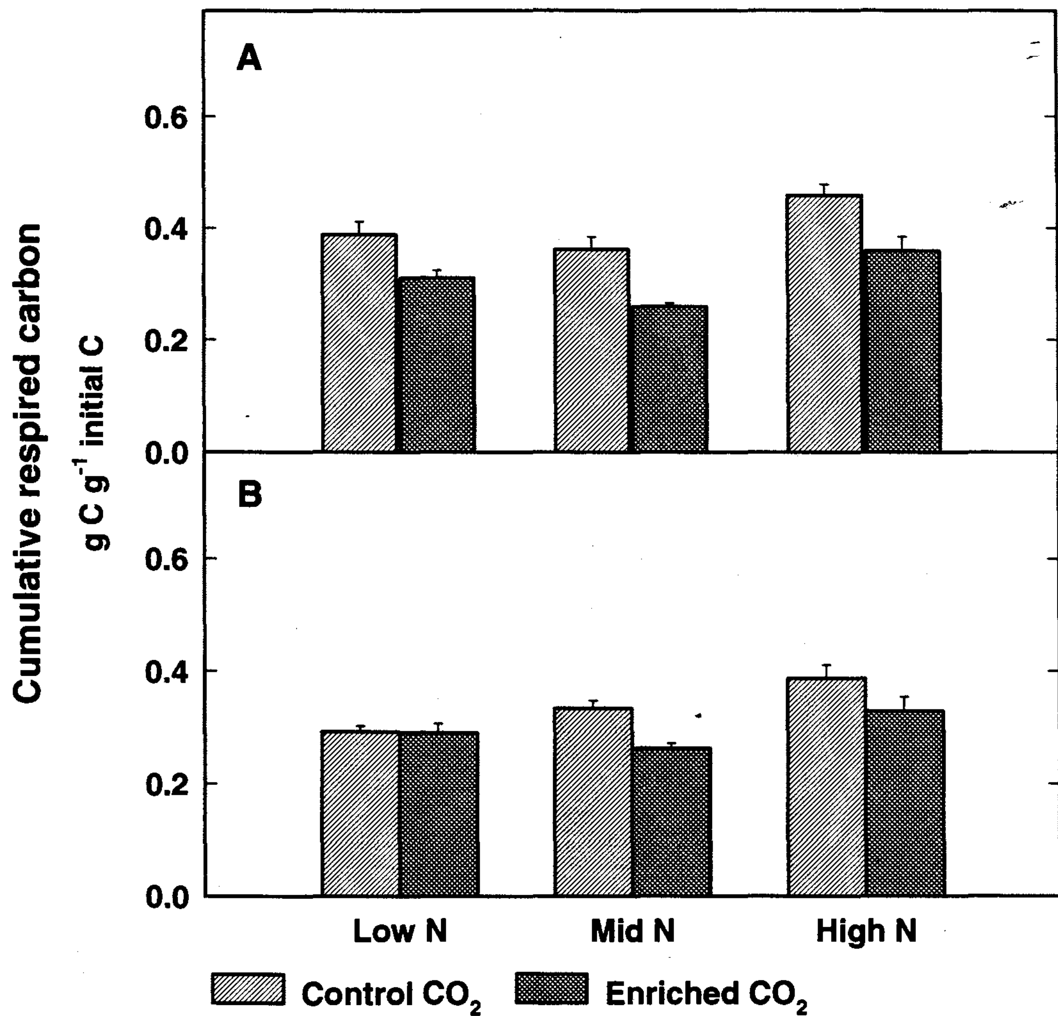


Figure 8.8 Cumulative respired carbon from the microbial-litter complex during decomposition assays. (A) Microbial-senesced leaf base complex over the first 297 days of incubation. (B) Microbial-root complex over the first 150 days of incubation. Error bars are ± one standard error.

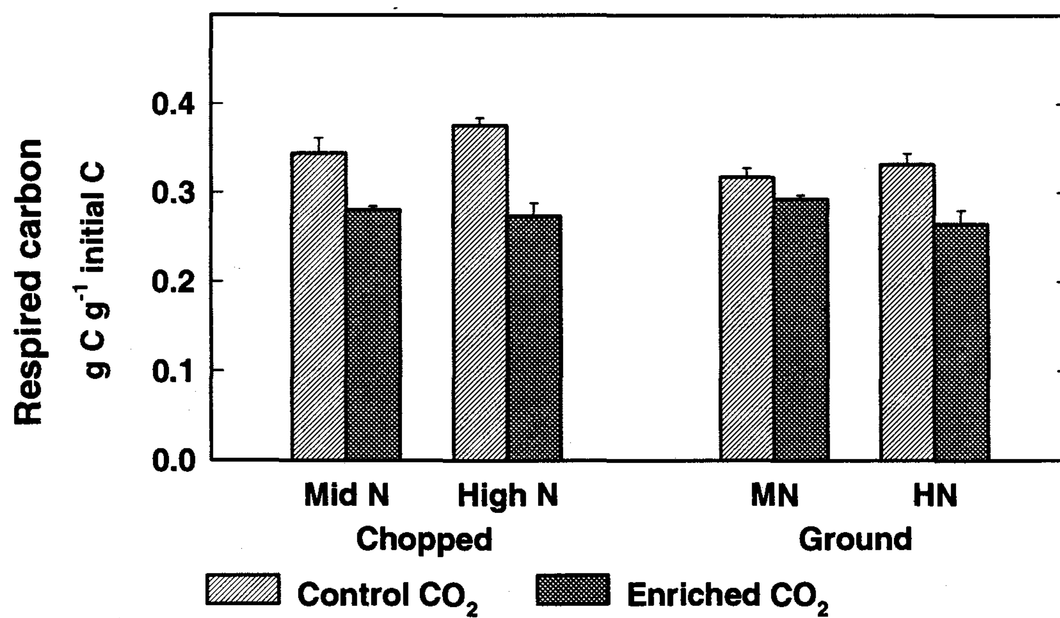


Figure 8.9 Cumulative respired carbon from the microbial-senesced leaf lamina complex over the first 107 days of incubation. Not determined on low-N senesced leaf lamina due to insufficient sample. Error bars are ± one standard error.

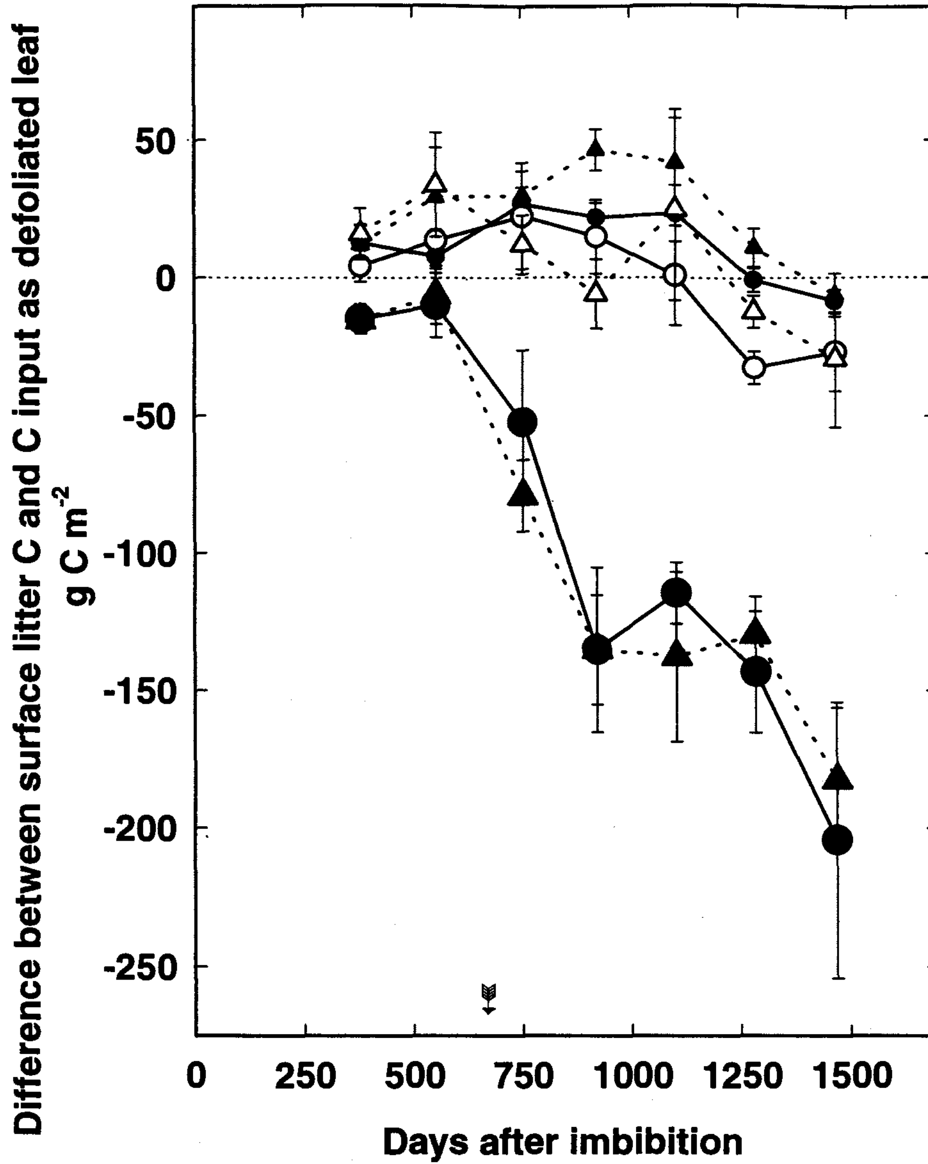


Figure 8.10 Difference between surface litter C and cumulative C addition to surface litter layer as defoliated leaf at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted line. Error bars are ± one standard error, which may be concealed by the symbol. Arrow (➸) shows commencement of decomposition spraying.

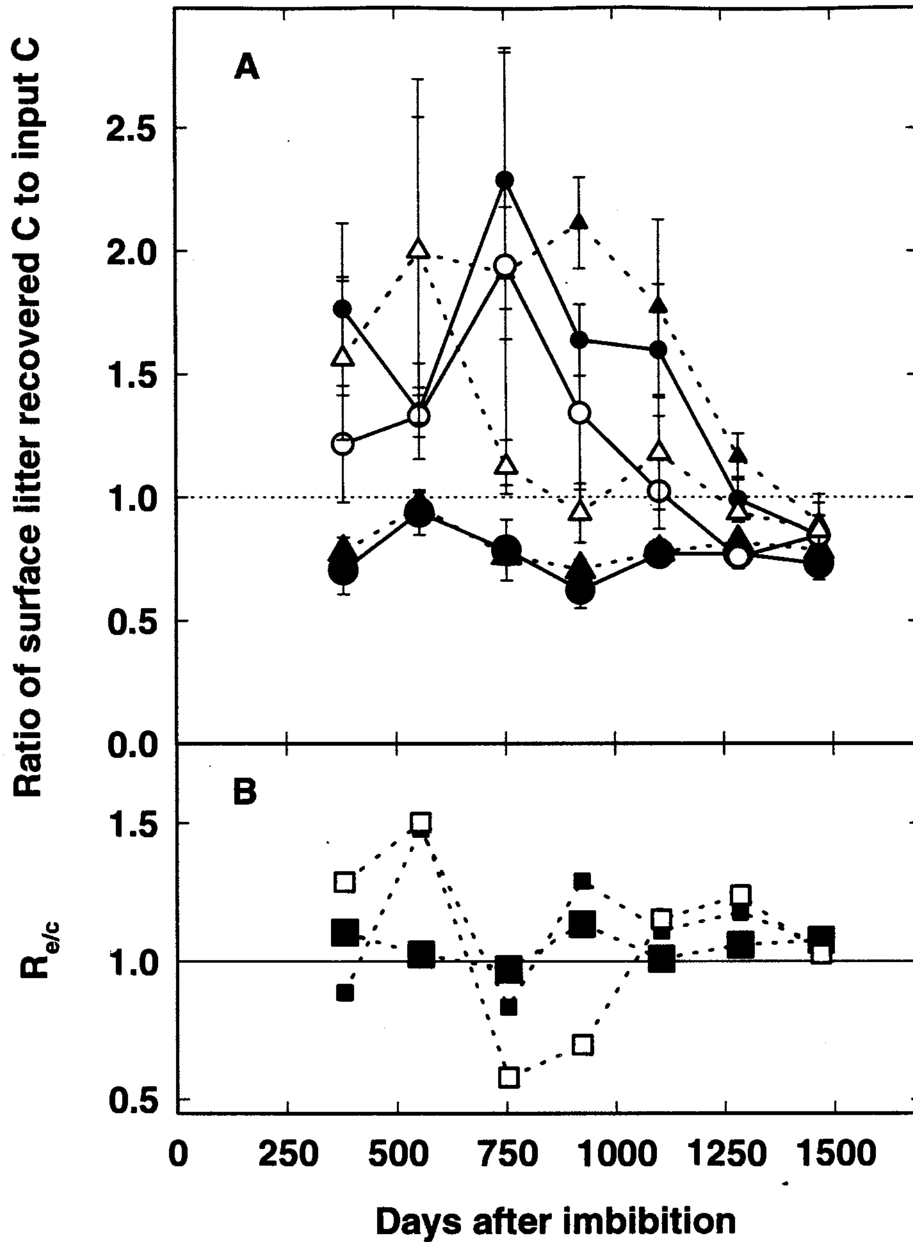


Figure 8.11 Ratio of carbon recovered from surface litter layer to cumulative carbon addition as defoliated leaf to litter layer. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low N (■), mid N (□), and high N(■).

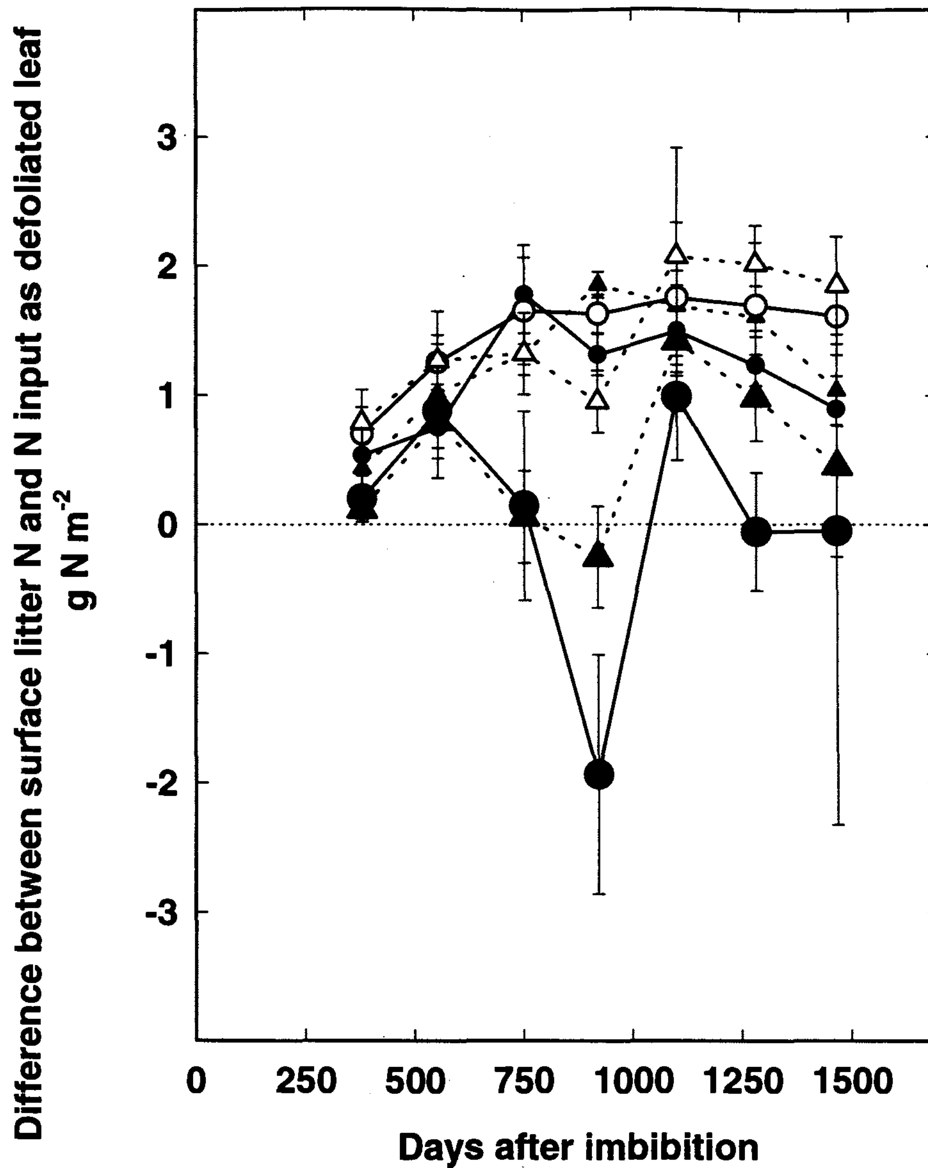


Figure 8.12 Difference between surface litter N and cumulative N addition to surface litter layer as defoliated leaf at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted line. Error bars are ± one standard error, which may be concealed by the symbol.

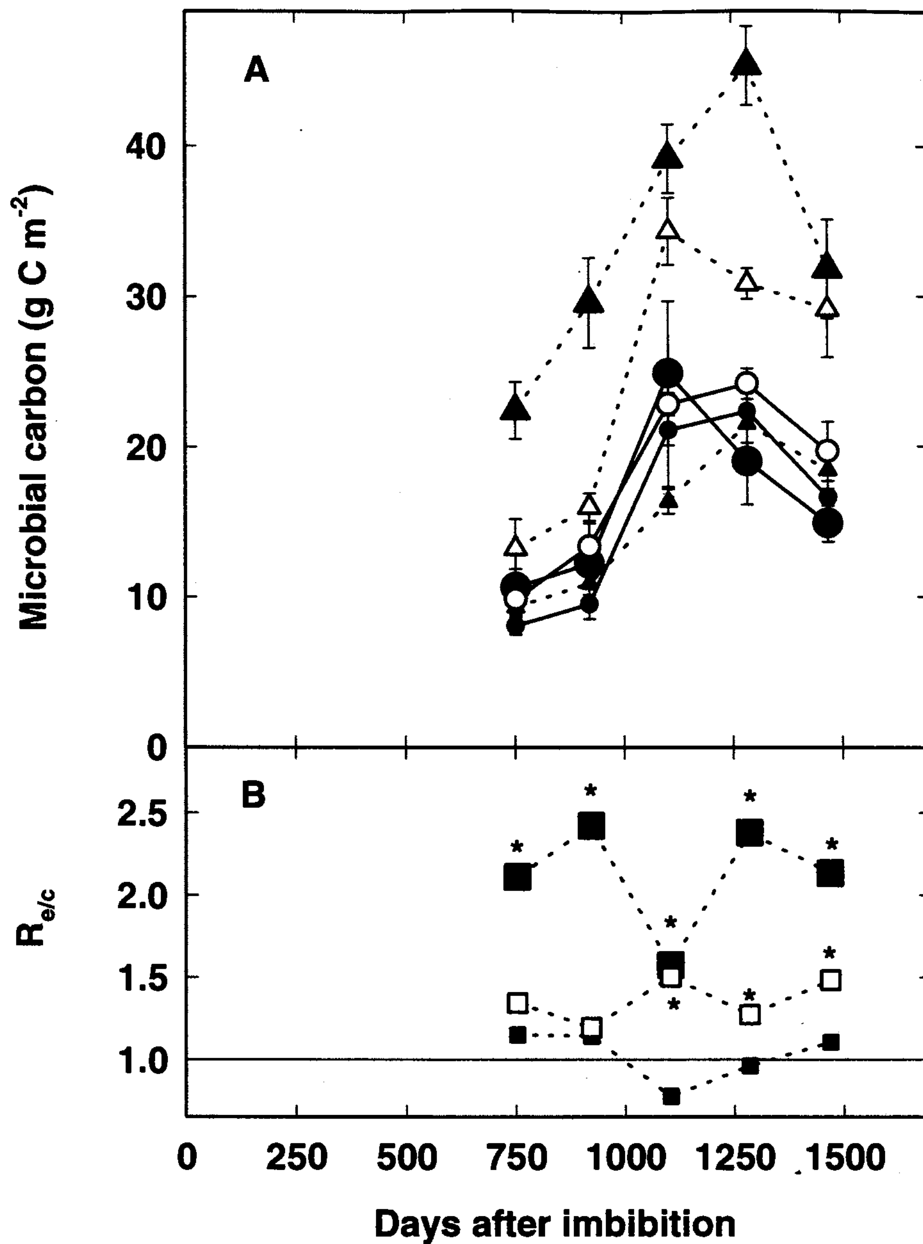


Figure 8.13 Microbial carbon in root free soil from 0-50 mm below the soil surface at harvest. (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low N (■), mid N (□), and high N(■). The response of microbial carbon to CO_2 was dependant both on harvest and N level ($P<0.05$). Harvest-N level combinations which exhibited a significant CO_2 effect within the N level ($P_L<0.05$) are marked by (*).

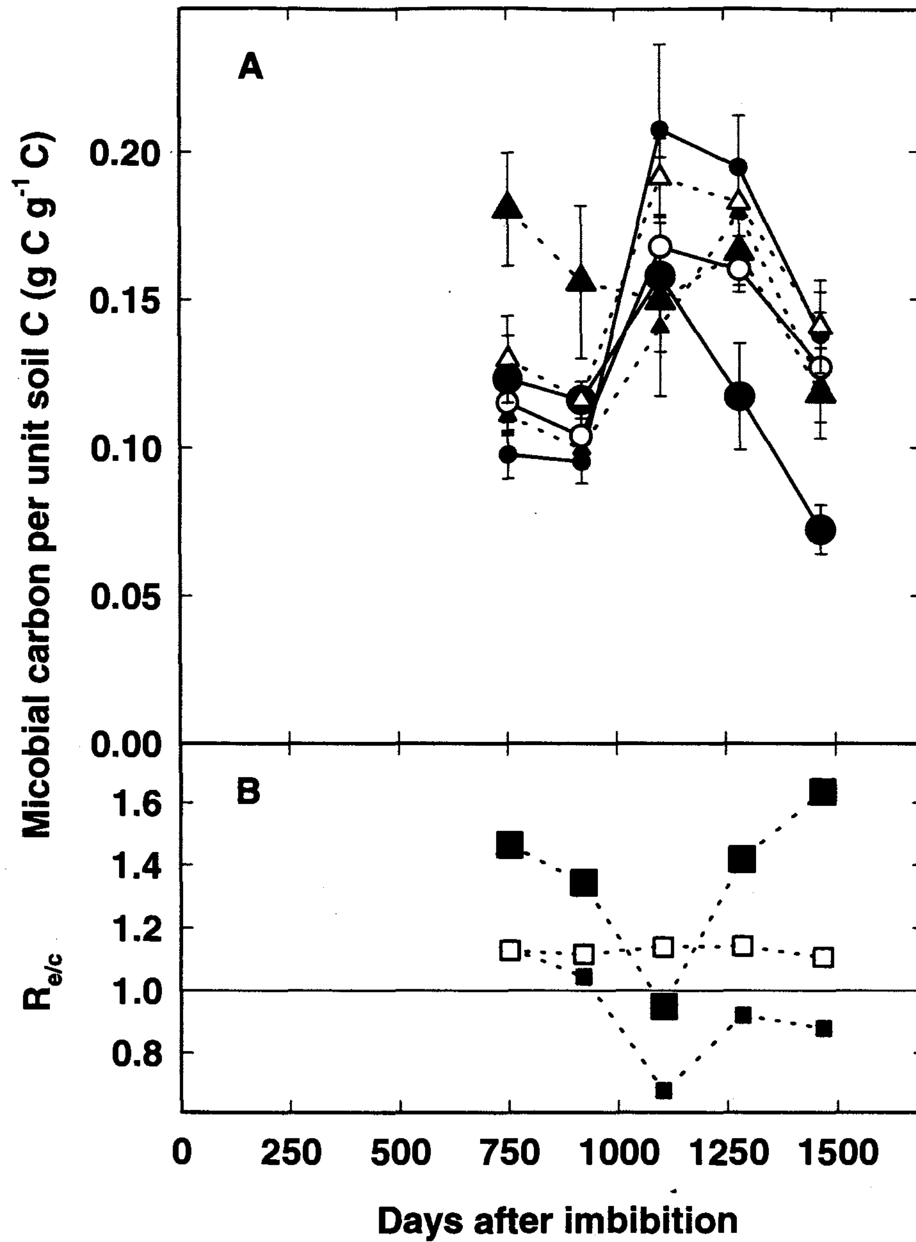


Figure 8.14 Relationship between microbial and total soil C in the surface 50 mm of soil (A) Absolute values. Control low-N (●), mid-N (○), high-N (⬤), with solid joining line. Enriched low-N (▲), mid-N (△), high-N (▲) with dotted line. Error bars are ± one standard error, which may be concealed by the symbol. (B) CO_2 response ratio. Low N (■), mid N (□), and high N(■).

Chapter 9. Synthesis of findings

Determination of changes in carbon and nitrogen pool size, rather than fluxes was the main methodology employed in this study. Gross fluxes of nitrogen between the microcosm and the environment were estimated. Data were discussed in detail in the experimental chapters. A brief summary of findings, based on the simple conceptual model of Diagram 1-1, and the final harvest at day 1469 and associated isolated plant are now presented.

Summary of results

Carbon acquisition and accumulation - Processes and pool size

Danthonia richardsonii, when grown as an isolated plant exhibited growth responses to CO_2 enrichment similar to that of other species. This growth response was achieved via increases in net assimilation rate, leaf nitrogen productivity and plant nitrogen productivity.

The increases in net assimilation rate and nitrogen productivity under CO_2 enrichment were expressed at the microcosm level as an increase in carbon accumulation over the four years of the experiment, with an average enhancement ratio of 1.25. This increase in carbon accumulation is within the range needed to account for the terrestrial carbon sink, if it were applicable to all ecosystems in the field (Gifford *et al.,* 1996c). This increase in carbon accumulation was achieved without an increase in leaf area index or green leaf carbon. Thus the determination of above-ground biomass under CO_2 enrichment is not an adequate measure of carbon sequestering potential.

Respiratory carbon losses were not measured in the microcosm study. However, as the microbial carbon pool was higher under CO_2 enrichment at the higher N levels, it was assumed that soil respiration would not be lower under CO_2 enrichment, and the increase in carbon accumulation was attributed primarily to increased photosynthetic carbon gain.

Nitrogen acquisition, accumulation and loss - Processes and pool size

No CO_2 effect on net nitrogen absorption rate (rate of increase in total plant nitrogen per unit root surface area) was noted in isolated plants. Increases in nitrogen accumulation in isolated plants were noted at high CO_2 at high rates of nitrogen supply, although this was explained by increases in total plant carbon.

The increase in microcosm carbon accumulation under CO_2 enrichment was attained with the same level of microcosm nitrogen at the two lower N levels, and with slight increases in microcosm nitrogen at high-N. Thus the increase in microcosm carbon under CO_2 enrichment could largely be attributed to widening C:N ratios.

In the microcosm experiment, all N treatments lost significant quantities of applied nitrogen to the environment, as derived from a balance of applied ^{15}N. The loss was lower under CO_2 enrichment at high-N. The nitrogen loss was assumed to be the result of gaseous processes, as leachate loss of ^{15}N was low. Microcosm mass balance of nitrogen suggests that all N levels must have gained nitrogen from the environment, which was attributed to dry deposition and dinitrogen fixation. There was no evidence of a CO_2 effect on the total acquisition of nitrogen from the environment.

Carbon and nitrogen allocation and distribution

Allocation - isolated plants

Root carbon ratio of isolated plants increased slightly under CO_2 enrichment. This increase was explained by increases in total plant carbon, thus allocation patterns of carbon on a whole plant basis were not altered by CO_2 enrichment. Changes in allometric relationships indicated a reduction in leaf area at high CO_2 for a given total plant carbon. Reductions in LAI at high CO_2 were also exhibited by the microcosms. The root surface area to leaf surface area ratio of isolated plants was increased at high CO_2 when nitrogen limited growth, indicating a functional shift away from carbon acquisition towards nutrient acquisition. This was attained via a decrease in specific leaf area at high CO_2. Nitrogen allocation to root was also increased under CO_2 enrichment. Thus growth at high CO_2 had real impacts on plant nitrogen use.

Distribution - microcosms

Allocation patterns were not determined in the microcosm study.

Green leaf, senesced leaf, surface litter

The absolute size of the green leaf carbon pool was no different under CO_2 enrichment, although this effect developed over time. Trends in the CO_2 enhancement ratio suggest that green leaf carbon may stabilise at a lower level under CO_2 enrichment. That is, green leaf carbon may be lower at high CO_2 than under ambient CO_2 concentrations. The proportion of total microcosm carbon in this pool was lower under CO_2 enrichment. The green leaf nitrogen pool was substantially smaller under CO_2 enrichment, as was the proportion that it formed of

total microcosm nitrogen. This resulted in a higher C:N ratio of the green leaf pool, which was also evident when expressed on an structural carbon basis.

The senesced leaf carbon pool was larger under CO_2 enrichment, while it formed a similar proportion of total microcosm carbon. Senesced leaf nitrogen was little affected by CO_2 treatment. Thus, senesced leaf C:N ratio was greater under CO_2 enrichment.

Root

Root carbon (g m^{-2} ground area) was lower under CO_2 enrichment at the two lower N levels, while at the highest N level root carbon was increased by CO_2 enrichment. The root pool contained a smaller proportion of total microcosm carbon at all N levels under CO_2 enrichment. At the two lower N levels the total root-nitrogen pools were smaller at high CO_2, while the root nitrogen pool consisted of a smaller proportion of total system nitrogen at all N levels. Root C:N ratio was increased by CO_2 enrichment, although at low-N this could be attributed to increased non-structural carbohydrate concentrations.

Soil

The total soil carbon and total soil nitrogen pools were increased under CO_2 enrichment, both in absolute terms and as a fraction of the respective total microcosm pool. This increase was most apparent at high-N after day 554. About this time the watering strategy changed, resulting in longer periods of low soil moisture contents, and a weekly spray of the canopy with demineralised water commenced to aid decomposition. Thus growth under CO_2 enrichment resulted in higher levels of soil carbon, without the associated increase in soil nitrogen completely feeding back and reducing carbon accumulation.

At day 1285 and 1469 the carbon and nitrogen pools were further segregated in the surface 50 mm of soil. The size of the mineral nitrogen pool was not affected by CO_2 enrichment except at high-N. The fast cycling carbon pool was defined as the microbial carbon pool, and the fast cycling nitrogen pool as the sum of the potentially mineralisable nitrogen pool and the amino-nitrogen pool. These pools were both increased under CO_2 enrichment. The slow cycling pools of carbon and nitrogen, defined as the difference between the total soil pool and the fast pool, were both increased by CO_2 enrichment. Thus growth at high CO_2 resulted in a greater accumulation of carbon and nitrogen in the slow cycling soil organic matter pool. This is an important finding, and suggests that long-term carbon storage in the soil may be increased under CO_2 enrichment. The increase in carbon and nitrogen in the fast pool also suggests a

greater level of plant available nitrogen in the longer term, if the relationship between pool size, microbial turnover and nitrogen availability is not changed under CO_2 enrichment.

Growth at high CO_2 resulted in a greater proportion of microcosm nitrogen in the soil pools. This may have minimised carbon storage in the short term, owing to the low C:N ratio of this pool. In the longer term it may result in a higher plant-soil system nitrogen content, as above ground nitrogen is more susceptible to loss via herbivory (on a local scale) and fire.

Other processes

Leaf turnover, root carbon and nitrogen loss

The size of the senesced leaf carbon pool in the microcosms increased at high CO_2, without a corresponding increase in the size of the green leaf pool. This implies a faster rate of leaf turnover under CO_2 enrichment. Similar determinations could not made on root tissue. Increases in soil carbon and nitrogen imply the input into the soil was increased under CO_2 enrichment. The source of this extra input was not determined, but is probably a combination increased deposition by root and increased root turnover owing to increased soil moisture contents, with smaller contributions from the surface litter layer. The effect of CO_2 on grass root growth and turnover is an area of research which needs more attention, as the largest proportion of plant biomass in grass systems is generally below-ground.

Decomposition

Litter quality, with respect to decomposition was reduced under CO_2 enrichment in both senesced leaf and in root owing to higher C:N ratios. This effect was substantiated by *in vitro* decomposition assays in all fractions other than root produced at the low level of nitrogen supply. These decreases in *in vitro* senesced leaf decomposition were not expressed at the microcosm level, possibly owing to higher soil moisture contents.

Evapotranspiration

The effect of CO_2 enrichment in decreasing microcosm water use may be a very important factor in microcosm, and were it transferable to the field, ecosystem response to CO_2 enrichment. A decrease in the rate of water use, at least in natural grasslands would probably not result in large increases in run-off or leaching as grasslands are largely water limited (Ripley, 1992). Rather, the length of time during which soil water contents favour plant growth and active carbon gain may be extended. This would also have important implications for nutrient cycling, with increased soil moisture contents favouring decomposition (Swift *et al.*, 1979) and the return of nutrients from plant litter for further plant growth. This may have been

a major factor contributing to the equal quantity of litter decomposition *in vivo* between CO_2 levels in the high nitrogen microcosms.

Applicability of results

Fertiliser nitrogen supply

In this experiment, the effect of nutrient turnover on microcosm productivity was partially over-ridden by the continual input of mineral nitrogen. This was an unavoidable requirement of using a homogenous, low carbon and nitrogen soil to maximise the "signal to noise ratio" of the experimental data, while still maintaining a gradient of nitrogen availability. The effect of removal of this mineral nitrogen supply on the CO_2 responsiveness of the plant-soil system is not known. It would be expected that productivity would be reduced. The CO_2 effect may not be reduced as the labile nitrogen pool is higher under CO_2 enrichment, and thus the supply of internally cycled microcosm nitrogen may be higher under CO_2 enrichment - at least in the short term. If this were the case, the CO_2 response may be enhanced in the short term, until this nitrogen passed through the live plant pools and became temporarily immobilised in detritus. The determination of these effects, either experimentally or via modelling was outside of the scope of this study. It does, however remain a critically important factor in considering the applicability of these results to the field.

Stable atmospheric CO_2 concentration

The global annual average CO_2 concentration is increasing almost continually, apart from the occasional year when no increase occurred (Schimel *et al.,* 1995). A continual increase in atmospheric CO_2 concentration may elicit a different response from soil-plant communities to that observed from a step change, or from continual growth under a constant concentration. The primary response of plants to CO_2 increase, an increase in carbon fixation operates on a shorter time scale than that of the potential feedbacks on nutrient availability. Thus, system response to CO_2 increase may be greater than that observed in this study as there are continual, small increases in CO_2 concentration, which would act to offset small, potential reductions in nutrient availability resulting from previous growth.

Exclusion of fauna

There were no meso- or macrofauna present in this experiment. Microfauna were also largely excluded during microcosm construction. The exclusion of fauna from the experiment may have lowered decomposition rates, as fauna are involved in the breakdown and transport of

plant litter (Swift *et al.,* 1979; Stanton, 1988). The effect of fauna on decomposition rates may differ between CO_2 levels, as the inclusion of fauna in decomposition assays has been noted to overcome litter quality decrease resultant from growth under CO_2 enrichment (Coûteaux *et al.,* 1991).

Other implications

Forage quantity and quality

Lower quantities of green leaf, and lower quantities of above ground nitrogen may have implications for herbivore production. If these results were transferred to the field, in systems where nitrogen intake is the prime determinant of herbivore consumption and/or demand, the carrying capacity of grassland may be reduced.

Importance of long term experiments

This project has highlighted the importance of long-term experiments in investigating the effects of CO_2 increase on plant-soil systems. The response of LAI and green leaf carbon to CO_2 enrichment changed sign over the experimental period, and the CO_2 effect on soil carbon developed over time, and was not consistently noted until after 2 years of growth. Thus many of the responses of short-term experiments may be misleading. Equally, the effects observed in this experiment may not adequately reflect even longer term trends in the field, as soil carbon levels may take hundreds, or thousands of years to equilibrate to change (Parton *et al.,* 1988). However, microcosm carbon content was increased under CO_2 enrichment, including carbon storage in the soil pools. The ratio of microbial carbon to total soil carbon was high, indicating that soil carbon levels were still increasing. This ratio was increased under CO_2 enrichment at the two higher nitrogen levels, which indicated that "equilibrium" soil carbon contents may be maintained at a higher level under CO_2 enrichment. Hence, these data, when viewed in concert with other experimental data from the literature indicate that grasslands may account for a proportion of the terrestrial carbon sink of global carbon cycle models.

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Appendices

Appendix 1 *F* probabilities for leaf area index (LAI), total carbon increment from sowing, and major carbon fractions for microcosm experiment one. Carbon dioxide level represented by C, nitrogen level by N, harvest by H, and exposure (core/guard) by E.

	LAI	**Increment from sowing**	**Green leaf**	**Senesced leaf**	**Surface litter**	**Root**	**Soil**
C	0.048	<0.001	0.054	<0.001	0.001	0.677	<0.001
N	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
C*N	0.233	0.015	0.769	0.299	0.992	<0.001	0.008
H	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
E	0.817	0.984	0.665	0.401	0.616	0.173	0.405
C*E	0.630	0.566	0.932	0.375	0.432	0.602	0.781
N*E	0.153	0.874	0.327	0.598	0.560	0.754	0.341
C*N*E	0.478	0.711	0.465	0.567	0.584	0.864	0.585
H*C	0.029	0.001	<0.001	0.181	0.868	<0.001	<0.001
H*N	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
H*C*N	0.168	0.075	0.183	0.206	0.551	0.133	<0.001
H*E	0.080	0.477	0.814	0.623	0.709	0.801	0.626
H*C*E	0.531	0.163	0.452	0.147	0.808	0.409	0.909
H*N*E	0.395	0.472	0.676	0.258	0.443	0.274	0.216
H*C*N*E	0.998	0.426	0.290	0.456	0.046	0.374	0.947

Appendix 2 *F* probabilities for total nitrogen increment from sowing, difference between N application and recovery, and total nitrogen and content of major fractions for microcosm experiment one, all expressed as g m^{-2}. Carbon dioxide level represented by C, nitrogen level by N, harvest by H, and exposure (core/guard) by E.

	Increment from sowing	**Difference from application**	**Green leaf**	**Senesced leaf**	**Surface litter**	**Root**	**Soil**
C	0.942	0.937	<0.001	0.009	0.006	<0.001	0.003
N	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
C*N	0.126	0.127	0.494	0.064	0.005	0.004	0.023
H	<0.001	0.113	<0.001	<0.001	<0.001	<0.001	<0.001
E	0.660	0.637	0.896	0.639	0.467	0.026	0.724
C*E	0.846	0.839	0.385	0.551	0.370	0.393	0.984
N*E	0.871	0.875	0.427	0.400	0.585	0.322	0.693
C*N*E	0.766	0.770	0.588	0.466	0.306	0.758	0.887
H*C	0.465	0.463	<0.001	0.659	0.030	<0.001	0.007
H*N	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
H*C*N	0.004	0.004	0.010	0.160	0.007	0.144	0.002
H*E	0.514	0.484	0.980	0.714	0.576	0.788	0.546
H*C*E	0.842	0.840	0.789	0.445	0.335	0.332	0.922
H*N*E	0.986	0.985	0.933	0.164	0.115	0.115	0.918
H*C*N*E	0.408	0.405	0.861	0.78	0.120	0.494	0.248

Appendix 3 *F* probabilities for nitrogen concentration (g N g^{-1} C) of major carbon fractions for microcosm experiment one. Carbon dioxide level represented by C, nitrogen level by N, harvest by H, and exposure (core/guard) by E.

	Total plant-soil system	**Green leaf**	**Senesced leaf**	**Surface litter**	**Root**	**Soil**
C	<0.001	<0.001	<0.001	<0.001	<0.001	0.005
N	<0.001	<0.001	<0.001	0.021	<0.001	0.066
C*N	0.005	0.031	0.027	0.116	0.005	0.210
H	<0.001	<0.001	0.009	0.008	<0.001	<0.001
E	0.695	0.083	0.765	0.746	0.997	0.905
C*E	0.583	0.074	0.627	0.315	0.906	0.884
N*E	0.938	0.054	0.688	0.393	0.343	0.831
C*N*E	0.795	0.108	0.755	0.690	0.884	0.800
H*C	0.049	0.149	0.462	0.729	0.024	0.470
H*N	0.001	<0.001	0.026	<0.001	0.002	<0.001
H*C*N	0.324	0.139	0.227	0.996	0.074	0.288
H*E	0.573	0.105	0.745	0.606	0.972	0.026
H*C*E	0.734	0.529	0.279	0.156	0.622	0.482
H*N*E	0.300	0.165	0.472	0.655	0.107	0.163
H*C*N*E	0.148	0.170	0.624	0.158	0.297	0.781

Appendix 4 Environmental conditions over the whole of microcosm experiment two (493 days). Data in parenthesis show one standard deviation.

	Control	**Enriched**
Atmospheric CO_2 (daytime av; μL L^{-1})	360 (12)	725 (40)
Dew point (°C)	12.3	12.3
Av air temperature (°C)	21.1	21.0
Temperature sum (air, degree days base 0°C)	10416	10397
Av below pot temperature (°C)	21.3	21.3
Total evaporation (mm)	6094	5980
Total short wave radiation (MJ m^{-2})	4833	

Appendix 5 Summary of carbon data from microcosm experiment two after 493 days of growth. All values are g m^{-2} unless otherwise stated, and are followed by one standard error. CO_2: Con ~350 μL L^{-1}, Enr ~700 μL L^{-1}. *P* levels (ANOVA) are $P<0.001$ (***), $P<0.01$ (**), $P<0.05$ (*), $P<0.1$ (+).

					***P* level**		
	CO_2	**Low N**	**Mid N**	**High N**	**CO_2**	**N**	**C*N**
C inc. from sowing	Con	314 ± 11	408 ± 18	821 ± 35	**	***	ns
	Enr	390 ± 27	532 ± 26	992 ± 46			
	$R_{e/c}$	*1.24*	*1.30*	*1.21*			
LAI	Con	0.31 ± 0.01	0.57 ± 0.01	1.52 ± 0.18	*	**	ns
	Enr	0.23 ± 0.01	0.50 ± 0.05	1.21 ± 0.13			
	$R_{e/c}$	*0.74*	*0.88*	*0.80*			
Green leaf C	Con	17.4 ± 1.2	47.1 ± 2.0	147.1 ± 3.8	ns	***	ns
	Enr	18.8 ± 0.4	45.8 ± 1.6	140.3 ± 6.0			
	$R_{e/c}$	*1.08*	*0.97*	*0.95*			
Senesced leaf C	Con	95.4 ± 1.5	122.5 ± 2.2	219.5 ± 4.6	***	***	***
	Enr	108.3 ± 1.9	147.6 ± 1.3	289.5 ± 4.3			
	$R_{e/c}$	*1.13*	*1.21*	*1.32*			
Surface litter C	Con	53 ± 9	58 ± 6	185 ± 31	ns	**	ns
	Enr	82 ± 8	109 ± 25	180 ± 27			
	$R_{e/c}$	*1.54*	*1.88*	*0.97*			
Root C	Con	87 ± 2	106 ± 4	172 ± 9	***	***	*
	Enr	92 ± 3	126 ± 5	247 ± 9			
	$R_{e/c}$	*1.06*	*1.19*	*1.43*			
Soil C	Con	363 ± 21	373 ± 12	399 ± 22	ns	ns	ns
	Enr	392 ± 21	409 ± 17	436 ± 21			
	$R_{e/c}$	*1.08*	*1.10*	*1.09*			

Appendix 6 Summary of nitrogen data from microcosm experiment two after 493 days of growth. All values are g m^{-2} unless otherwise stated, and are followed by one standard error. CO_2: Con ~350 μL L^{-1}, Enr ~700 μL L^{-1}. *P* levels (ANOVA) are *P*<0.001 (***), *P*<0.01 (**), *P*<0.05 (*), *P*<0.1 (+).

					***P* level**		
	CO_2	**Low N**	**Mid N**	**High N**	**CO_2**	**N**	**C*N**
N inc. from sowing	Con	7.6 ± 0.4	10.2 ± 1.2	28.7 ± 1.6	ns	***	ns
	Enr	7.1 ± 0.9	12.2 ± 0.7	25.5 ± 1.0			
	$R_{e/c}$	*0.93*	*1.19*	*0.89*			
N balance	Con	4.46 ± 0.43	1.42 ± 1.16	2.66 ± 1.61	ns	P< 0.12	ns
	Enr	3.96 ± 0.89	3.41 ± 0.70	-0.52 ± 0.97			
	Re/c	-	-	-			
Green leaf N	Con	0.42 ± 0.02	1.40 ± 0.10	5.23 ± 0.09	**	***	ns
	Enr	0.37 ± 0.01	0.98 ± 0.02	3.49 ± 0.29			
	$R_{e/c}$	*0.89*	*0.70*	*0.67*			
Green leaf N conc. (mg N g^{-1} C)	Con	24.1 ± 0.7	29.7 ± 2.0	35.7 ± 1.5	***	***	*
	Enr	19.7 ± 0.2	21.4 ± 0.9	24.7 ± 1.2			
	$R_{e/c}$	*0.82*	*0.72*	*0.69*			
Senesced leaf N	Con	1.30 ± 0.04	1.86 ± 0.04	4.18 ± 0.10	***	***	**
	Enr	0.86 ± 0.04	1.39 ± 0.01	3.12 ± 0.06			
	$R_{e/c}$	*0.66*	*0.75*	*0.75*			
Surface litter N	Con	2.1 ± 0.4	2.5 ± 0.1	8.8 ± 1.3	ns	**	ns
	Enr	2.3 ± 0.2	3.3 ± 0.7	5.7 ± 0.6			
	$R_{e/c}$	*1.07*	*1.32*	*0.65*			
Root N	Con	2.20 ± 0.10	3.00 ± 0.09	5.78 ± 0.24	ns	***	*
	Enr	1.93 ± 0.05	2.93 ± 0.12	6.28 ± 0.14			
	$R_{e/c}$	*0.87*	*0.98*	*1.09*			
Root N conc. (mg N g^{-1} C)	Con	25.4 ± 1.2	28.3 ± 0.5	33.6 ± 0.5	***	***	*
	Enr	20.9 ± 0.5	23.2 ± 0.5	25.5 ± 0.4			
	$R_{e/c}$	*0.82*	*0.82*	*0.76*			
Soil N	Con	45.9 ± 1.2	45.7 ± 1.3	49.7 ± 1.3	+	*	ns
	Enr	46.3 ± 0.5	49.0 ± 0.7	52.0 ± 0.8			
	$R_{e/c}$	*1.01*	*1.07*	*1.05*			

Appendix 7 Summary of tissue quality and soil parameters for microcosm experiment two after 493 days of growth. All values are followed by one standard error. CO_2: Con ~350 $\mu L\ L^{-1}$, Enr ~700 $\mu L\ L^{-1}$. P levels (ANOVA) are $P<0.001$ (∗∗∗), $P<0.01$ (∗∗), $P<0.05$ (∗), $P<0.1$ (+).

					P level		
	CO_2	**Low N**	**Mid N**	**High N**	**CO_2**	**N**	**C∗N**
Total system C:N	Con	11.9 ± 0.1	13.0 ± 0.1	15.3 ± 0.2	∗∗∗	∗∗∗	ns
	Enr	13.4 ± 0.3	14.6 ± 0.3	18.4 ± 0.5			
	$R_{e/c}$	*1.13*	*1.12*	*1.20*			
Senesced leaf C:N	Con	74 ± 3	66 ± 2	53 ± 2	∗∗∗	∗∗∗	ns
	Enr	127 ± 6	106 ± 1	93 ± 1			
	$R_{e/c}$	*1.73*	*1.61*	*1.77*			
Surface litter C:N	Con	25 ± 2	23 ± 1	21 ± 1	∗∗∗	∗	ns
	Enr	36 ± 2	32 ± 1	31 ± 2			
	$R_{e/c}$	*1.43*	*1.41*	*1.50*			
Soil C:N	Con	7.9 ± 0.3	8.2 ± 0.1	8.0 ± 0.3	ns	ns	ns
	Enr	8.5 ± 0.4	8.4 ± 0.3	8.4 ± 0.4			
	$R_{e/c}$	*1.07*	*1.02*	*1.04*			
AM infection (%)	Con	63 ± 2	66 ± 4	69 ± 4	ns	ns	ns
	Enr	55 ± 4	66 ± 5	70 ± 3			
	$R_{e/c}$	*0.87*	*1.00*	*1.01*			
MBM C ($\mu g\ C\ g^{-1}$ soil DW)	Con	228 ± 52	332 ± 15	449 ± 66	+	∗	ns
	Enr	288 ± 25	370 ± 24	503 ± 25			
	$R_{e/c}$	*1.26*	*1.11*	*1.12*			
MBM C ($mg\ C\ g^{-1}$ soil C)	Con	117 ± 27	151 ± 6	173 ± 40	ns	ns	ns
	Enr	125 ± 6	149 ± 16	157 ± 23			
	$R_{e/c}$	*1.06*	*0.99*	*0.91*			
Pot. mineralisable N ($\mu g\ N\ g^{-1}$ soil DW)	Con	16 ± 2	23 ± 2	26 ± 2	∗	∗	ns
	Enr	22 ± 3	29 ± 1	33 ± 2			
	$R_{e/c}$	*1.36*	*1.24*	*1.27*			
Bulk density ($g\ mL^{-1}$)	Con	1.35 ± 0.02	1.37 ± 0.01	1.37 ± 0.02	ns	ns	+
	Enr	1.37 ± 0.03	1.37 ± 0.02	1.34 ± 0.02			
	$R_{e/c}$	*1.01*	*1.00*	*0.98*			
Pore Space (%)	Con	48.9 ± 0.9	48.4 ± 0.4	48.1 ± 0.6	+	ns	∗
	Enr	48.2 ± 1.0	48.4 ± 0.8	49.4 ± 0.8			
	$R_{e/c}$	*0.99*	*1.00*	*1.03*			
pH	Con	5.92 ± 0.06	5.91 ± 0.06	5.19 ± 0.77	ns	ns	ns
	Enr	5.86 ± 0.06	5.81 ± 0.17	5.60 ± 0.54			
	$R_{e/c}$	*0.99*	*0.98*	*1.08*