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Influence of elevated atmospheric CO_2 concentration on symbiotic N_2 fixation and availability of nitrogen in grassland ecosystems

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Before I came here I was confused about this subject.
...I am still confused. But on a higher level.

Enrico Fermi

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List of abbreviations

^{15}N atom %-excess	atom%^{15}N in excess of natural abundance
c	Gross NH_4^+ consumption
DM	dry matter
FACE	Free Air CO_2 enrichment
f_N	fraction of fertiliser N in the soil
i	N immobilisation
m	Gross N mineralisation
N*fert*	N derived from fertiliser
N*fix*	N derived from symbiotic N_2 fixation
Nfix_d	N derived from symbiotic N_2 fixation that was fixed and directly harvested
Nfix_t	N derived from symbiotic N_2 fixation that was fixed and directly harvested plus the fixed N, which was recovered in subsequent yields
N*soil*	N derived from fertiliser N plus mineralisation from soil N
N*som*	N derived from mineralisation of unlabelled soil organic matter
pCO_2	partial pressure of atmospheric CO_2
SOM	soil organic matter
Qc	specific microbial activity of gross NH_4^+ consumption
Qm	specific microbial activity of gross N mineralisation

Summary

The objective of this thesis was to investigate processes determining the response of grassland ecosystems to elevated atmospheric partial pressure (pCO_2). During the past two centuries, the atmospheric CO_2 partial pressure (pCO_2) has increased by 31 %, mainly due to the burning of fossil fuel and changes in land use. Due to the stimulatory effect of increased carbon (C) assimilation on plant growth, terrestrial ecosystems may potentially act as a sink for C and, thus, partly counterbalance the increase in pCO_2. Grasslands are of special interest as they cover large areas and store large amounts of organic matter in the soil. However, the plant-mediated input of C into the ecosystem may be limited by other growth resources, most often by nitrogen (N) supply. Mineralisation, immobilisation, losses of N as well as symbiotic N_2 fixation are the most important processes determining N availability for plant growth and subsequently the pCO_2 response of the ecosystem. Therefore, the specific objectives of this work were to investigate: i) gross N mineralisation and immobilisation rates after seven years of CO_2 fumigation in pure grass and legume swards, ii) the response of N yield and symbiotic N_2 fixation during ten years of elevated pCO_2, iii) the ten-year N budget of a grassland ecosystem in order to estimate the effect of elevated pCO_2 on losses and sequestration of N.

Lolium perenne and *Trifolium repens* were grown for ten years in monocultures and bi-species mixtures in a fertile soil. Elevated pCO_2 (60 Pa) was achieved by means of *F*ree *A*ir *C*arbon Dioxide *E*nrichment (FACE) technology. The swards were exposed to two levels of N fertilisation (14 and 56 $g\,m^{-1}a^{-1}$) and harvested frequently.

In the eighth year, gross N transformation rates in the soil were measured in intact soil cores, taken from the low-N fertilised *L. perenne* and *T. repens* swards during two days of incubation by means of the ^{15}N pool dilution method. Rates of gross N mineralisation and immobilisation and the amount of microbial N in the soil did not change significantly under elevated pCO_2. This suggests that the changes in soil organic matter or in microbial activity, which may have occurred during the seven-year exposure

to elevated pCO_2, had no major effect on gross N transformation in these soils.

The effects of 10 years of elevated pCO_2 on the N yield and the symbiotic N_2 fixation were studied in the *T. repens* and the *T. repens/L. perenne* mixtures by the ^{15}N-isotopic dilution method. At high N fertilisation the pCO_2 response of the symbiotically fixed N_2 decreased during the 10 years of the experiment from $5\,g\,N\,m^{-2}$ in 1993 to $-1\,g\,N\,m^{-2}$ in 2002. The additional N yield under elevated pCO_2 was exclusively derived from the N_2 fixation in the first three years only. Thereafter, the change in the N yield under elevated pCO_2 was a result of both N derived from soil and fixation. This was attributed to an insufficient availability of soil N to meet the greater N demand of the plant under elevated pCO_2 after the step increase of pCO_2. Due to the increased N_2 fixation in the first years of the experiment, more N may have been temporarily immobilised under elevated pCO_2 leading to an apparent input of N into the system. With the increased availability of soil N, due to the re-mineralisation of this pool in combination with the high fertiliser N input, the plant may has tended to acquire mineral N, which requires less energy. The low N treatments were continuously N-limited, leading to a higher (16 %) portion of symbiotically fixed N in the plant material under elevated pCO_2.

The calculation of the N budgets of the ecosystems suggests that there was no net sequestration of N at low N supply, while at high N supply a net N sequestration of N may have occurred. There were no significant differences in the N balance of the CO_2 treatments. Isotopic analysis of harvested plant material and soil N revealed that losses of fertiliser N from the systems were highest in the high-N clover swards (up to 58 % of N applied) with no significant differences between the pCO_2 treatments. A larger N input by symbiotic N_2 fixation in these swards counterbalanced the greater loss of fertiliser N.

Our data showed that the long-term response of fertile grassland ecosystems to elevated pCO_2 is governed by the availability of N. In grasslands receiving high amounts of N, the initially observed limitation of N derived from the soil is lessened with time and the system approaches equilibrium again after several years. This may be due to a consistent net input of N into the system. Under less favourable conditions, it may take much more longer to reach steady-state, because the net input of N into the system and, thus, the availability of N remain low.

Zusammenfassung

In der vorliegenden Arbeit wurden Prozesse untersucht, welche die Reaktion von Graslandökosystemen auf eine erhöhte atmosphärische CO_2-Konzentration (pCO_2) bestimmen. Der Verbrauch fossiler Brennstoffe sowie Veränderungen in der Landnutzung haben in den letzten 200 Jahren zu einem Anstieg von pCO_2 in der Atmosphäre um 31 % geführt. Durch den stimulierenden Effekt auf die Kohlenstoffassimilation und das Pflanzenwachstum könnten terrestrische Ökosysteme möglicherweise als Kohlenstoffsenken fungieren und so dem weiteren Anstieg der atmosphärischen CO_2-Konzentration entgegenwirken. Graslandökosysteme sind hier von besonderem Interesse, da sie große Gebiete bedecken und bedeutende Mengen an organischer Substanz im Boden speichern können. Der Eintrag von C durch die Vegetation kann jedoch durch die Limitierung anderer für das Pflanzenwachstum essentieller Ressourcen, wie zum Beispiel Stickstoff (N), begrenzt sein. Mineralisierung, Immobilisierung, symbiotische N_2 Fixierung sowie N-Verluste sind die wichtigsten Prozesse, welche die Verfügbarkeit von N für das Pflanzenwachstum und folglich die CO_2-Antwort eines Ökosystems bestimmen. In der vorliegenden Arbeit wurden deshalb folgende Aspekte eingehender untersucht: 1) die Raten der Brutto-N-Mineralisierung und Immobilisierung in reinen Gras- und Leguminosenbeständen nach sieben Jahren CO_2 Begasung, 2) der Einfluss von erhöhter atmosphärischer CO_2 Konzentration auf den N-Ertrag und die symbiotische N_2 Fixierung im Grasland während 10 Jahren, 3) der Einfluss von 10 Jahren erhöhter CO_2 Konzentration auf den N-Haushalt, N-Verluste sowie die mögliche Einlagerung von Stickstoff in den Boden.

Auf einem nährstoffreichen Boden wurden Rein- und Mischbestände von *Lolium perenne* und *Trifolium repens* angelegt und unter Verwendung der FACE-Technik (*F*ree *A*ir *C*arbon Dioxide *E*nrichment) mit CO_2 angereicherter Luft begast. Die Bestände wurden mit zwei unterschiedlichen Mengen an mineralischem N-Dünger (14 und 56 $g\,m^{-1}a^{-1}$) gedüngt und regelmäßig geschnitten.

Die Messung der Bruttoraten der N-Flüsse im Boden erfolgte im achten

Jahr des Versuches unter Verwendung der ^{15}N-Pool-Verdünnungsmethode in intakten Bodenproben von den niedrig gedüngten *L. perenne*- und *T. repens*-Beständen. Die Bruttoraten der N-Mineralisierung und -Immobilisierung sowie die Menge des mikrobiellen N zeigten keine signifikanten Veränderungen unter erhöhtem pCO_2. Dies deutet darauf hin, dass die Veränderungen der bodenorganischen Substanz und der Aktivität der Mikroorganismen, welche durch die siebenjährige CO_2 Begasung möglicherweise stattgefunden haben, keinen wesentlichen Einfluss auf die Umsetzung des N im Boden hatte.

Der Effekt von zehnjähriger CO_2-Begasung auf den N-Ertrag und die symbiotische N_2-Fixierung wurde in den *T. repens*- und den *T. repens*/-*L. perenne*-Beständen unter Verwendung der ^{15}N-Isotopen-Verdünnungsmethode untersucht. Bei hoher N-Düngung verminderte sich die CO_2 Antwort der symbiotischem N_2-Fixierung während der 10 Jahre des Experiments von $5\,g\,N\,m^{-2}$ in 1993 zu $-1\,g\,N\,m^{-2}$ im Jahr 2002. Der zusätzliche N-Ertrag unter erhöhtem pCO_2 kam nur in den ersten drei Jahren ausschließlich von der symbiotischen N_2 Fixierung. Danach war der veränderte N-Ertrag unter erhöhtem pCO_2 das Ergebnis der Assimilation von N aus dem Boden und der symbiotischen N_2-Fixierung. Dies wurde einer unzureichenden Verfügbarkeit von N aus dem Boden zugeschrieben, welche den größeren N-Bedarf der Pflanze nach der Erhöhung von pCO_2 nicht decken konnte. In den ersten Jahren kam es durch die gesteigerte N_2-Fixierung unter erhöhtem pCO_2 wahrscheinlich zu einer vermehrten Immobilisation von N, was zu einem scheinbaren Netto-Eintrag von N in das System führte. Durch die spätere Re-Mineralisierung dieses Pools in Kombination mit hoher N-Düngung erhöhte sich die Verfügbarkeit von N im Boden. Dadurch favorisierte die Pflanze die weniger energieintensive Aufnahme von mineralischem N und der Anteil an symbiotisch fixiertem N im Pflanzenmaterial sank ab. Bei niedriger N-Düngung blieb das System in Bezug auf mineralischem N stärker limitiert, was in einer fortgesetzt hohen symbiotischen N_2-Fixierung (+16 %) resultierte.

Die N-Bilanzen der Ökosysteme lassen vermuten, dass es bei niedriger N-Düngung zu keiner Netto-Festlegung von N in das System kam, während eine solche bei hoher N-Düngung möglicherweise stattfand. Zwischen den beiden CO_2-Verfahren wurden keine signifikanten Unterschiede der N-Bilanzen festgestellt. Die Analyse mittels stabiler Isotope des geernteten Pflanzenmaterials sowie des Boden-N zeigten, dass die Verluste an Dünger-N in den hoch gedüngten *T. repens*-Beständen mit bis zu bis zu 58 % der applizierten

Menge am höchsten ausfielen. Signifikante Unterschiede zwischen den CO_2-Verfahren wurden nicht festgestellt. Ein grösserer N-Input durch die symbiotische N_2-Fixierung in diesen Beständen glich die höheren Verluste an Dünger-N aus.

Unsere Daten zeigten, dass die CO_2-Antwort in nährstoffreichen bewirtschafteten Graslandsystemen maßgeblich von der Verfügbarkeit von N für das Pflanzenwachstum bestimmt wird. In Systemen, welche hohe Gaben an Dünger-N erhalten, geht die anfängliche Limitierung bezüglich Boden-N zurück und das System erreicht nach einigen Jahren ein neues Gleichgewicht. Dies wurde auf steten Netto-Input von N in das System zurückgeführt. Unter weniger produktiven Bedingungen könnte die Angleichung an ein neues Gleichgewicht viel länger dauern, da der Netto-Input an N sowie die N-Verfügbarkeit niedrig bleibt.

1 General introduction

1.1 Increasing atmospheric CO_2 concentration and its implications for global carbon cycling

Global changes in the environment have attracted the attention of society, politicians and scientists in recent years. An increasing population combined with changes in human activities have led to enormous changes in land use, a worldwide decline in biodiversity and a dramatic increase in the emissions of greenhouse gases. The most reliable aspect of global climate change is the current increase in the partial pressure of atmospheric CO_2 (pCO_2). Since the beginning of the 19th century atmospheric pCO_2 has increased by 31 % from post-glacial values to the present level of 36.7 Pa (IPCC, 2001). The actual annual increase in pCO_2 is 0.15 Pa, and it is anticipated that pCO_2 will have doubled by the mid of this century (IPCC, 2001). This increase is undoubtedly of anthropogenic origin because changes in the ratios of ^{13}C:^{12}C and ^{14}C:^{12}C, i. e. the isotopic composition of the atmospheric CO_2, demonstrates that the origin of the increase is the combustion of fossil fuels. Moreover, atmospheric O_2 is declining at a rate comparable to the increase in these fossil fuel emissions of CO_2.

The steady increase in pCO_2 has lead to an imbalance in the global C cycle, i. e. more CO_2 is released into the atmosphere than is absorbed. However, from the anthropogenic release of about 5.5 Gt C a^{-1} by fossil fuels and 1.6 Gt C a^{-1} by changes in land-use only about half is found in the atmosphere. About 2.0 Gt C a^{-1} are estimated to be deposited in the ocean and the remaining 1.4 Gt C cannot be attributed to one of the known sinks (Gifford, 1994). It has been suggested that the increased storage of C in the terrestrial biosphere, i. e. in the vegetation and soil organic matter (SOM), may account for this 'missing C sink' and may help to decelerate the increase in atmospheric CO_2. The potential importance of terrestrial ecosystems as C sinks is evident when the amounts of C stored globally in living biomass (650 Gt C), in SOM (2000 Gt C) and the approximate CO_2 exchange through

photosynthesis and respiration (60 Gt C a^{-1}) (IPCC, 2001) are compared with the 'missing' C sink. Thus, relatively small changes in gross primary production and/or respiration may significantly regulate the trend of the steady increase in atmospheric pCO_2. Results of long-term studies indicate that the potential increase in C sequestration may be much lower than originally predicted (Arnone and Körner, 1995). There is little information about net balance of C in terrestrial ecosystems, and hardly any knowledge is available about the numerous processes that govern the sequestration of C. Thus, an understanding of the potential effect of environmental changes on the activity of C sink as well as of the interactions of elevated pCO_2 with other growth factors on terrestrial ecosystems is necessary.

1.2 Grassland ecosystems

Natural and managed grasslands cover 24 million km^2 worldwide, which is nearly one fifth of the world's land surface. However, the role of grasslands in the global carbon cycle was not sufficiently recognised, even though grassland soils represent a significant C pool (Scurlock and Hall, 1998). In humid temperate regions this C pool can be even larger than that of forest soils (Goudriaan, 1992). The great potential for C storage in grassland arises from the most common management regimes, i. e. vegetation covers the soil for the whole year and the soil is usually not disturbed. Considerable proportions of biomass are transferred to below-ground plant parts and decay rates of SOM are relatively slow, leading to a potential accumulation of SOM. The functioning and productivity of grassland ecosystems and thus, their potential capacity to store C depend on changes in the environment, such as changes in temperature, precipitation, radiation and nutrient supply. Consequently, the change in the atmospheric CO_2 concentration will probably lead to changes in the functioning of plants and the ecosystem, because CO_2 is an essential resource for photosynthesis.

With a few exceptions, such as in alpine areas, the grasslands in central Europe are not ecosystems in the natural climax stadium but are the result of agricultural use; e. g. in Switzerland grasslands account for 80 % of the agricultural used area (BFS, 2001). Grasslands are generally multi-species systems with a considerable variation in age and botanical composition. Graminaceous species are most abundant but usually include other functional groups such as legumes and non-leguminous dicots. This variety

in biodiversity is of ecological and economical significance; the presence of legumes may improve the nutritional value of the forage because of their high digestibility and protein content. Due to their ability to fix atmospheric N_2 symbiotically, the legumes are less dependent on the supply of mineral N and may contribute to a sustainable management of grassland ecosystems.

Previous investigations of the effect of pCO_2 on grasslands were focused mainly on natural grassland communities such as tallgras prairie (Owensby *et al.*, 1999), California annual grassland (Higgins *et al.*, 2002) and nutrient-poor calcareous grassland (Leadley *et al.*, 1999). However, due to the significant area covered by agricultural grasslands in temperate regions, studies of the effect of elevated pCO_2 in such ecosystems are also required.

1.3 Importance of nitrogen

Up to the CO_2 saturation of the photosynthesis reaction, atmospheric pCO_2 is certainly a main parameter determining the amount of C imported into the ecosystem. However, the rate of photosynthetic fixation of C by the vegetation also depends on other environmental parameters, including climatic conditions, light and the availability of nutrients. One of the most significant parameters affecting C assimilation and, consequently, the effect of pCO_2 on the ecosystem is the availability of nitrogen (N) since N is a key element for plant growth. Consequently, the cycles of C and N in the ecosystem are generally coupled (Rastetter *et al.*, 1997).

Nitrogen that is potentially available to the biosphere includes the huge reservoir of gaseous N_2 in the atmosphere, the soil pools of organic and inorganic N and the N in the microbial, plant and animal biomass. Due to the high mobility of N, there is an intensive exchange between these pools, which includes diverse chemical transformations. Processes, which lead to the loss of N from the ecosystem, include leaching, runoff and gaseous emissions through denitrification/nitrification and the volatilisation of ammonia. Nitrogen is introduced into the ecosystem by wet and dry deposition and biological N_2 fixation. Managed grassland systems may be subjected to additional input (fertilisation) and outputs (removal of above-ground biomass) of N.

Plants and most microorganisms are mainly restricted to the uptake of inorganic N, i. e. nitrate (NO_3^-) and ammonium (NH_4^+). However, inorganic

N is the smallest N pool in the soil; the large organic N pool can be utilised only after mineralisation. Therefore, in terrestrial ecosystems, the amount of biomass produced by living organisms is often limited by the availability of N (Larcher, 1995). It is evident that potential responses to changes in environmental parameters, such as elevated pCO_2, would also be limited by plant-available N in the system. Hence, the effects of elevated pCO_2 on the plant and the ecosystem can not be explained mechanistically without a knowledge of the effect of elevated pCO_2 on processes governing N flow and N availability in the ecosystem.

1.4 Effect of elevated pCO_2 on the ecosystem with respect to N: state of research

1.4.1 Plant response to elevated pCO_2

In the majority of studies, the rates of photosynthesis (Drake *et al.*, 1997) and production of plant biomass (Kimball, 1983) was increased. The key reaction of C assimilation is the carboxylation of ribulose-1,5-bisphosphate (RubP), catalysed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is not saturated at current atmospheric pCO_2. Moreover, the oxygenase reaction (photorespiration), which decreases the net efficiency of photosynthesis, is inhibited by CO_2. Both lead to a higher net uptake of C per quantum light under elevated pCO_2 and, consequently, to a higher net rate of photosynthesis. Further responses to elevated pCO_2 are a decrease in stomatal conductance and transpiration, which may improve water use efficiency. Key enzymes of photosynthesis were reduced after acclimation to higher pCO_2, which increased nitrogen use efficiency, i. e. the rate of carbon assimilation per unit of N. As well as a greater concentration of carbohydrates in plant tissue, this decrease in rubisco and in the photosynthetic capacity causes the commonly observed change in the chemical composition of the plant biomass, such as an increase in the C/N ratio.

Due to the increased C input as a result of the enhanced photosynthesis an increase in the production of plant biomass is expected. In the vast majority of studies a generally positive response of plant production to elevated pCO_2 was observed. An average increase in yield of about 33 % was calculated by Kimball (1983) who compared more than 430 studies, conducted under controlled conditions, of horticultural and field crops, grasses

and trees. However, the effects of elevated pCO_2 on spaced plants under controlled conditions are of limited value when such results are extrapolated to the actual situation in a complex ecosystem. There are numerous interactions among plants in the field, including competition for nutrients, light, and space and interactions between the plant and the soil. In a recent comparison of many different field studies Kimball *et al.* (2002) showed, that the magnitude of these responses varied with the functional plant type and with soil nitrogen; the effects of pCO_2 on dry matter and nutrient yield was often found to be weaker than expected from the increase in photosynthesis. Such inter-species and resource-dependent responses to elevated pCO_2 were also found in the first years of the Swiss FACE (Free Air Carbon Dioxide Enrichment) experiment. In pure stands of *Lolium perenne* there was a significant increase in root biomass with no or a very slight (7 %) increase in the shoot fraction (at low and high N fertilization, respectively), while both the root and shoot biomass of *Trifolium repens* increased remarkably (Hebeisen *et al.*, 1997; Jongen *et al.*, 1995; Lüscher *et al.*, 1998). These interspecific differences in the pCO_2 response were attributed to the limited availability of N in the grass swards and to the unlimited access to the atmospheric N pool through symbiotic N_2 fixation by the clover. This interpretation was confirmed by an indication that the growth of *Lolium perenne* under elevated pCO_2 was limited by N (Zanetti *et al.*, 1997; Fischer *et al.*, 1997) and by the fact that the proportion of N assimilated from the soil and fertiliser N in a *L. perenne/T. repens* mixture decreased (Zanetti *et al.*, 1997). Daepp *et al.* (2000) found that the response of the annual dry matter (DM) and N yield of *L. perenne* monocultures to elevated pCO_2 increased over six years in the high-N system; this was attributed to a steady increase in the availability of mineral N in the soil. These facts clearly show that the different processes in the ecosystem are not affected simultaneously to elevated pCO_2, i. e. the plant may respond quickly, while processes in the soil may change much more slowly. In natural ecosystems, most of the N absorbed by plants becomes available through the decomposition of organic matter, i. e. N mineralisation, and in the case of legumes through symbiotic N_2 fixation. It is, therefore, evident that potential pCO_2-induced changes in both N mineralisation and symbiotic N_2 fixation may fundamentally change the regulation of processes in the ecosystem.

1.4.2 Response of N cycling in the soil to elevated pCO_2

More than 99 % of soil N is bound to decayed organic matter derived from plants, animals and microbes. Only after mineralisation does this N becomes available for plant growth. Mineralisation is the conversion of carbon and nutrients from organic to inorganic forms due to the breakdown of litter and soil organic matter, which results almost entirely from microbial activity. In contrast, immobilisation is the removal of inorganic nutrients from the available pool by microbial uptake and chemical fixation. As well as gaseous and aqueous losses of N, the mineralisation/immobilisation turnover determines the availability of N in the soil for plant growth and, subsequently, its response to pCO_2. The concentration of CO_2 in the soil is 10 to 50 times higher than that in the atmosphere (Richter and Markewitz, 1995), a direct effect of elevated atmospheric pCO_2 on the soil microbes and, thus, on N cycling in the soil is negligible. However, the microbial communities may be influenced indirectly by a plant-mediated change in the cycling of C and N, changes in biomass allocation, rhizodeposition and quality of forage and litter. There is experimental evidence that significant changes occurred in the few soil organisms studied thus far with respect to elevated pCO_2. A distinct increase in the *Pseudomonas* population in the rhizosphere of white clover versus perennial ryegrass (Marilley *et al.*, 1999) was detected. The size of the *Rhizobium leguminosarum* population, which is the N_2-fixing symbiont of white clover, increased in the rhizosphere of the latter (Schortemeyer *et al.*, 1996). Elevated atmospheric CO_2 increased the competitive ability of root nodule symbionts (Montealegre *et al.*, 2000). However, the extent and type of influence of elevated atmospheric pCO_2 on processes of N cycling is a controversy topic (Zak *et al.*, 2000), because the rates of N mineralisation in soils exposed to elevated pCO_2 increased (Hungate *et al.*, 1997), decreased or remained constant (Gloser *et al.*, 2000). Stimulation of N mineralisation in grassland soils was observed when ample fertiliser N was applied, whereas N mineralisation and microbial immobilisation did not change or even decreased in poorly fertilised soils (van Ginkel *et al.*, 1997; Gloser *et al.*, 2000; Loiseau and Soussana, 2000). This phenomenon is known as the 'priming effect' or 'additive nitrogen interaction' (Jenkinson *et al.*, 1985; Kuzyakov *et al.*, 2000). An increased release of labile C and N through rhizodeposition (van Veen *et al.*, 1991) may stimulate the activity of the microbial community. On the one hand, this could lead to a greater availability of soil N by increased mineralisation (Zak *et al.*, 1993)

or to a lower availability of N due to an increase in microbial immobilisation (Díaz *et al.*, 1993) on the other. Moreover, the poorer quality (increased C/N ratio) of the plant material at elevated pCO_2 (Cotrufo *et al.*, 1998; Hartwig *et al.*, 2002) may affect the digestibility and kinetics of decomposition (Gorissen *et al.*, 1995) and reduce the amount of inorganic N released into the soil. The greater allocation of biomass below ground (Jongen *et al.*, 1995; Hebeisen *et al.*, 1997) may result in a transient accumulation of soil organic matter followed by an increase in mineralisation. It becomes even more complicated when the time factor is considered, i. e. when the size of the pools and the components of N cycling in the soil change with time. Plant and soil processes may adapt to elevated pCO_2, and the system may reach a new C/N equilibrium. Given the complexity of soil organisms, their interaction with each other and with abiotic factors, which may change over time, an understanding of N cycling in a world of increasing pCO_2 can only be obtained in long-term field studies.

1.4.3 Response of symbiotic N_2 fixation to elevated pCO_2

Symbiotic N_2 fixation is based on the mutual interaction of soil-born *Rhizobium* bacteria and legumes. Symbiotic N_2 fixation reduces the dependency of the legume on other N sources. As a result, the associated N_2 fixing bacteria will be supplied with assimilates needed for the energy demanding reduction of N_2.

Symbiotic N_2 fixation in the ecosystem is considered to be the major process for introducing N into grassland. In managed grasslands, the presence of red or white clover may result in an input of symbiotically fixed N up to $300\,kg\,ha^{-1}a^{-1}$ (Boller and Nösberger, 1987, 1988; Seresinhe *et al.*, 1994; Zanetti *et al.*, 1996, 1997). It is assumed that the sequestration of C and N into an ecosystem is closely linked (Granhall, 1981; Gifford, 1992; van Groeningen *et al.*, 2002), an increase in C availability would ultimately lead to a greater N requirement of the plant and the ecosystem. Symbiotic N_2 fixation may counterbalance a lack of available soil N and may maintain the C/N balance of the ecosystem (Zanetti *et al.*, 1997; Hartwig *et al.*, 2000; Lüscher *et al.*, 2000).

Recent results of laboratory experiments indicate that, under elevated pCO_2, symbiotic N_2 fixation increases parallel to the assimilation of mineral N in order to meet the greater demand for N as a result of a higher

growth rate (Zanetti *et al.*, 1998; Almeida *et al.*, 2000). This strongly indicates that elevated pCO_2 *per se* did not influence the percentage of N derived from symbiosis in *Alnus glutinosa* grown in soil (Vogel *et al.*, 1997) and *Acacia melanoxylon* (Schortemeyer *et al.*, 1999) and *T. repens* (Zanetti *et al.*, 1998) grown hydroponically in nutrient solution. In contrast, under field conditions in the Swiss FACE, the response of symbiotic N_2 fixation to elevated pCO_2 differed from that in the controlled environment. The percentage of N from symbiosis in white clover increased in such a way that all the additionally assimilated N came from symbiotic N_2 fixation and none from the soil or the fertiliser (Zanetti *et al.*, 1996; Zanetti and Hartwig, 1997). Similar results were obtained with lucerne grown under similar conditions (Lüscher *et al.*, 2000) and *T. repens* grown in soil monoliths in the greenhouse (Soussana and Hartwig, 1996).

Plants unable to fix atmospheric N_2 can benefit from the presence of a legume as a result of the subsequent transfer of N derived from the atmosphere (Boller and Nösberger, 1987; Giller *et al.*, 1991). This flow occurs mainly through the decomposition of legume debris and the uptake of remineralised N by the receiver plant. However, also other pathways, e. g. via root exudates (Paynel *et al.*, 2001) and through mycorrhizal fungi interconnecting the root systems (Johansen *et al.*, 1993; Simard *et al.*, 1997) have been reported. The presence of the legume increased the supply of available N in the root zone, also through its lower demand for mineral N. The apparent N transfer contributed up to 60 % of the total N assimilated by *L. perenne* grown in a mixture with *T. repens* (Høgh-Jensen and Schjoerring, 1997). Under elevated pCO_2, the N concentration in *L. perenne* clearly increased when grown in association with *T. repens* compared to the monoculture (Zanetti *et al.*, 1997; Hartwig *et al.*, 2000). In the mixture, the apparent CO_2-induced increase in the limiting effect of N in *L. perenne* swards was lower than in the monoculture (Soussana and Hartwig, 1996; Fischer *et al.*, 1997; Zanetti *et al.*, 1997). More N may have been available due to the apparent transfer of N and because the grass with its very dense rooting system can easily explore the soil for nutrients, leaving the legume to meet its N demand mainly (up to 90 %) by symbiotic N_2 fixation (Zanetti *et al.*, 1996). The positive interaction of the legume and the non-legume may ensure a more efficient acquisition of atmospheric and soil-derived N. This demonstrates that interactions and feedback mechanisms in the plant soil system play a key role in the response of grassland ecosystems to elevated pCO_2.

1.5 Unanswered questions and objectives of the study

The effect of elevated atmospheric pCO_2 on the primary assimilation of C and N by single plants was mainly investigated so far (§ 1.4.1). If the results are scaled-up to a field situation, they suggest that the increase in atmospheric pCO_2 changes the C budget of the grassland ecosystem. The N budget is also affected, and more complicated, this feeds back again to the plant. There is a lack of experimental data on the dynamics of N cycling under elevated pCO_2 for longer time periods. There is experimental evidence that the initial N limitation of the growth of non-leguminous plants may be reduced over time (§ 1.4.1; Daepp *et al.*, 2000). However, there is less information about the underlying processes, which determine the availability of N for plant growth, i. e. mineralisation, immobilisation and loss of N from the soil. Likewise, the potential effect of such changes over time on biological N_2 fixation, the most important parameter of N-import in natural grassland ecosystems, is a matter of speculation. CO_2-induced changes in the input of N by means of N_2 fixation ultimately affect the translocation of N in the ecosystem as well as the potential sequestration of N into the soil. In turn, such changes may have significant economic (loss of fertiliser N) and ecological (leaching of nitrate into the ground-water, emissions of the greenhouse gas N_2O) implications for management. For these reasons, it is necessary to study the influence of elevated pCO_2 on processes, which affect availability of N for plant growth, i. e. N mineralisation, N immobilisation and symbiotic N_2 fixation. Moreover, to understand the significance of the N flow in the ecosystem as an important determinant for the ecosystem C flow and C/N interaction, an ecosystem N budget is needed. The results of such studies will provide a basic understanding of the processes involved in the regulation of components of the global C cycle, in particular under elevated atmospheric pCO_2. Furthermore, the data may be important for improving the modelling of the ecosystem (Thornley and Cannell, 1997, 2000), i. e. integrating processes related to the plant and to the cycling of N in the ecosystem (Rastetter *et al.*, 1997; Cannell and Thornley, 1998) under increasing atmospheric pCO_2.

The objective of this work was to study processes determining N availability for plant growth as well sequestration into the soil in managed grassland ecosystems as affected by elevated atmospheric pCO_2. The following issues were investigated in detail:

1. gross N mineralisation, NH_4^+ consumption and immobilisation rates after seven years of CO_2 fumigation in pure grass and legume swards
2. the effect of ten years elevated pCO_2 on the N yield and symbiotic N_2 fixation in a pure legume and grass/legume mixed swards
3. to establish ten-years N budgets of grassland ecosystems in order to estimate the effect of elevated pCO_2 on N economy, i. e. losses and sequestration of N

1.6 Experimental approach

Long-term experiments in the field, where the availability of growth resources varies and the plants interact with each other and with the soil, are necessary for predicting the effect of elevated pCO_2. Therefore, the study was conducted at the Swiss FACE experimental site, which provides unique set of data about the functioning of the ecosystem in temperate managed grassland and its response to elevated atmospheric pCO_2. The FACE technique (Lewin *et al.*, 1994) enabled the direct investigation in the field with no changes in the microclimate, which might occur in enclosures. The investigations focused on two representative plant species, *Lolium perenne* L. and *Trifolium repens* L., which are abundant in managed grasslands in temperate climates. *L. perenne* requires large amounts of mineral N, while *T. repens* has access to atmospheric N_2 due to its symbiotic association with *Rhizobium leguminosarum* bv. *trifolii*. Two levels of N fertilisation enabled the study of the combined effects of elevated pCO_2 and the availability of mineral N. The use of ^{15}N-enriched fertiliser N enabled the quantification of the different N sources for plant growth and in the soil, i. e. symbiotic N_2 fixation, soil organic matter and fertiliser. Rates of gross N transformations were measured in short-term incubation studies by means of the ^{15}N pool dilution technique; the activity of soil microbes was estimated by measuring respiration and N_2O emissions. Net N mineralisation was measured for a whole growth season by means of the sequential soil coring method. Gaseous losses from the system were assessed using closed flux chambers.

2 Gross fluxes of N in grassland soil exposed to elevated atmospheric pCO_2 for seven years

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Abstract Gross rates of nitrogen (N) mineralisation were examined in grassland soils exposed to ambient (36 Pa) and elevated (60 Pa) atmospheric CO_2 partial pressure (pCO_2) for seven years in the Swiss Free Air Carbon Dioxide Enrichment (FACE) experiment. It was hypothesized that the increased translocation of photoassimilates below-ground at elevated pCO_2 would lead to a temporary increase in immobilisation of N due to an excess supply of energy to the roots. Intact soil cores were sampled from *Lolium perenne* and *Trifolium repens* swards in May and September, 2000. The rates of gross N mineralisation (m) and NH_4^+ consumption (c) were determined using ^{15}N isotopic dilution during a 51-hour period of incubation. The rate of N immobilisation was estimated either as the difference between m and the net rate of N mineralisation after 51 hours or as the amount of ^{15}N released from the microbial biomass after chloroform fumigation. Soil samples from both swards showed that the rates of gross N mineralisation and NH_4^+ consumption and the portion of microbial N and ^{15}N released by the chloroform fumigation did not change significantly under elevated pCO_2. Gross rates of N mineralisation, NH_4^+ consumption and microbial ^{15}N did not differ significantly between the two swards. A considerably larger amount of NO_3^- was recovered at the end of incubation in the soils taken from the *T. repens* swards compared to soils taken from the *L. perenne* swards. Ten percent of the added ^{15}N were recovered in the roots in the cores sampled under *L perenne*, while only 5 % were recovered in the cores sampled under *T. repens*. These results show that the calculation of N immobilisation from gross and net rates of mineralisation in soils with a high root biomass does not reflect the actual immobilisation of N in the microbial biomass. The results of this study suggest that the mineralisation and immobilisation of N, measured in grassland soils in short-term experiments, are not affected by long-term exposure to elevated pCO_2.

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2.1 Introduction

The atmospheric CO_2 partial pressure has increased continuously over the past decades, and it is expected to double by the end of the 21st century compared to pre-industrial values (IPCC, 2001). The enhanced pCO_2 stimulates net photosynthesis and, as a consequence, plant biomass production. In terrestrial ecosystems, the extent of this pCO_2-induced response is strongly dependent on nutrient supply, particularly with regard to nitrogen (Daepp *et al.*, 2000). In addition to symbiotic N_2 fixation, mineral fertilisers, wet and dry N deposition, and the mineralisation of soil organic matter are the main sources of plant-available nitrogen in managed grassland. While knowledge of the responses of plants to elevated pCO_2 has grown in recent years, the effects of pCO_2 on soil N cycling are still a matter of controversy (Zak *et al.*, 2000), because the rates of N mineralisation in soils exposed to elevated pCO_2 were shown to increase (Hungate *et al.*, 1997), decrease or to remain constant (Gloser *et al.*, 2000). In a recent review, Zak *et al.* (2000) explained these contradictory results by the fact that these studies were conducted in different ecosystems (forests vs grasslands), using different methods (net mineralisation vs gross mineralisation) and focussing on different processes (mineralisation, immobilisation, soil respiration, etc.). Zak *et al.* (1993) observed that soils exposed to elevated pCO_2 in open-top chambers show a higher net N mineralisation and explained this result by an increased release of labile C into the rhizosphere due a stronger photosynthetic response. On the other hand, Díaz *et al.* (1993) observed an increased immobilisation in the soil microbial biomass under elevated pCO_2 which they explained by an enhancement of C-rich substrate in the rhizosphere. The transformation of nitrogen in grassland soils was stimulated under elevated pCO_2 when ample fertiliser N was applied, whereas N transformations did not change or even decreased in poorly fertilised soils (van Ginkel *et al.*, 1997; Loiseau and Soussana, 2000; Gloser *et al.*, 2000).

In the Swiss Free Air Carbon Dioxide Enrichment (Swiss FACE) experiment, the root biomass of *Lolium perenne* and *Trifolium repens* increased when both plants were exposed to elevated pCO_2 (Hebeisen *et al.*, 1997; Hartwig *et al.*, 2000), while symbiotic N_2 fixation increased (Zanetti *et al.*, 1996) and changes in the microbial community were observed in the rhizosphere of both plants (Schortemeyer *et al.*, 1996; Sowerby *et al.*, 2000). Enhanced rhizodeposition, associated with a larger root biomass, may have the potential to higher microbial activity in the rhizosphere and to a tem-

porary increase in the immobilisation of mineral N. Gloser *et al.* (2000), however, did not detect pCO_2-induced changes in the content of mineral N in the soil or in net N mineralisation. However, studies of net mineralisation provide information about changes in the overall mineral N in the soil but can not quantify the components of this change, such as gross mineralisation, immobilisation and N losses. The assessment of gross N fluxes (gross N mineralisation and gross N consumption) as proposed by Kirkham and Bartholomew (1954), is a more direct estimation of the transformations of N in soils exposed to elevated pCO_2.

The present study was undertaken to test the hypothesis that increased below-ground allocation of C under elevated pCO_2 enhances gross rates of N transformation. Gross N mineralisation, NH_4^+ consumption and immobilisation rates were evaluated using the ^{15}N dilution technique in intact soil cores taken from *Lolium perenne* and *Trifolium repens* swards, grown for seven years under ambient and elevated atmospheric pCO_2. The activity of the soil microbial biomass was assessed by measuring soil respiration and denitrification.

2.2 Materials and methods

2.2.1 Experimental site and management conditions

The experimental site is located in Eschikon, Switzerland (20 km east of Zurich, 47°27′N and 8°41′E at 550 m above sea level). The soil was a clay loam, eutric cambisol, with pH values (0 to 20 cm topsoil) between 6.5 and 7.6. Since May 1993, monocultures of *Trifolium repens* L. and *Lolium perenne* L. have been grown in plots (2.8 × 1.9 m) at two pCO_2 levels (ambient = 36 Pa and elevated = 60 Pa) in the Swiss FACE experiment (Zanetti *et al.*, 1996; Hebeisen *et al.*, 1997). CO_2 fumigation using FACE technology (Lewin *et al.*, 1994) was carried out in three circular areas (diameter 18 m) for each pCO_2 treatment. The plots were fertilised with 14 g N m^{-2}, 5.5 g P m^{-2} and 24 g K m^{-2} as described in Zanetti *et al.* (1996). From 1996, the swards were cut five times a year, 5 cm above ground level. The design of the experimental site is described in detail in Zanetti *et al.* (1996) and Hebeisen *et al.* (1997).

2.2.2 Soil sampling, ^{15}N application, incubation procedure and trace gas sampling

Gross rates of N mineralisation and NH_4^+ consumption were measured using a technique based on changes in the ^{15}N-labelled soil mineral N pool due to biotic and abiotic processes (Kirkham and Bartholomew, 1954). Pairs of intact soil cores (5.5 cm in diameter, 7 cm deep) were sampled from the *Lolium perenne* and *Trifolium repens* plots on 2 May and 11 September in 2000, and the remaining shoot tissue was cut immediately. The samples were then processed within 24 hours: ^{15}N was injected with a 1-ml syringe as $(^{15}NH_4)_2SO_4$ in solution (5 atom % ^{15}N enriched) at a rate of 5 μg N g^{-1} dry soil. The solution was released through four lateral holes in the needle. Nine injections were carried out on each side of the cores to a depth of 3.5 cm, slowly withdrawing the needle as each injection progressed; 5.4 ml of solution were supplied to each core (0.3 ml per injection). Each pair of cores was incubated at 18°C, one core for 3 and one for 51 hours. After three hours, one core of each pair was removed for quantification of the initial mineral N (M_0) and mineral ^{15}N content (H_0, see below). The other core was put into a 1500-ml jar (Fisher Scientific, UK), fitted with a septum, and left to incubate for the remaining 48 hours at a constant temperature of 18°C. Thereafter, post-incubation mineral N and ^{15}N content (M_1 and H_1) were quantified from the second core.

Carbon dioxide and nitrous oxide emissions were measured after 45 hours of incubation. Samples of 12 ml were placed in evacuated gas vials (Labco Ltd., UK, Cat. No. 139B) and stored at 4°C prior to analysis using an Pye Unicam gas chromatograph equipped with an electron capture detector (N_2O) and an infra red gas analyser (CO_2, ADC-225-MK3, Analytical Development Co. Ltd, UK).

2.2.3 Analyses of mineral N pool and ^{15}N enrichment

The top 3.5 cm and the bottom 3.5 cm of soil were separated after 3 and 51 hours respectively. A 5-g sub-sample of each soil fraction was dried (105°C for 24 hours) for moisture determination; 40 g of each fraction (fresh weight) were shaken for 1 h in 150 ml of a 1 M KCl solution, and the solution was then filtered through a Whatman No. 1 filter paper. Concentrations of NH_4^+ and NO_3^- in the extract were determined colorimetrically using a Burkhard SF-A2 continuous-flow analyser. A modified diffusion procedure (Brooks

et al., 1989) was used for ^{15}N analysis of the KCl extracts. Aliquots of each KCl extract, containing about 100 μg N, were put into 500-ml jars (Fisher Scientific, UK). Potassium chloride was added so that the final concentration was about 2 M. The solution turned alkaline after adding 0.7 g MgO to generate NH_3. A 5-mm glass fibre disc (Whatman GF/D paper), acidified with 10 μl of 2.5 M $KHSO_4$, was placed onto a hook attached to the underside of the lid. The lid was sealed and the sample incubated for seven days at 28°C. Then the disc was removed, placed into a tin capsule and dried for 2 h at 55°C prior to analyses for ^{15}N enrichment of NH_4^+ using a 20-20 PDZ mass spectrometer (Europa Scientific, UK) coupled to an automated C/N analyser. A fresh disc containing 10 μl of 2.5 M $KHSO_4$ was then put onto each lid. Devarda's alloy, which reduces NO_3^- to NH_4^+, followed by another 0.7 g MgO were added to each jar, and another diffusion procedure was performed as described above. The discs were treated and analysed for ^{15}N enrichment of NO_3^- as above.

2.2.4 Determination of chloroform extractable N and ^{15}N

Microbial N was estimated by the chloroform fumigation extraction method (Brookes *et al.*, 1985). Forty grams of each fraction of the soil sampled after 51 hours of incubation were placed into a dessicator containing 50 ml of distilled chloroform in the well. The dessicator was lined with wet tissue to keep the atmosphere moist. The dessicator was evacuated prior to incubation. After 48-hour incubation, the samples were extracted with KCl as described above. Forty millilitres of the chloroform-incubated extract (fumigated) and 40 ml of the non-fumigated extract (initial) were digested according to the Kjeldahl procedure. A sub-sample of each sample, containing about 100 μg N, was then subjected to diffusion for the ^{15}N isotope analysis as described above. The N and ^{15}N recovered in the non-fumigated samples were subtracted from the N and ^{15}N recovered in the fumigated samples to yield the microbial N and ^{15}N. A correction factor (k_N) was not used to determine microbial N, because the main focus of this study was to compare treatments and not to obtain absolute values.

2.2.5 Calculations

Isotope dilution calculations were carried out according to Kirkham and Bartholomew (1954).

$$m = \frac{M_0 - M_1}{t} \times \frac{\log(H_0 M_1 / H_1 M_0)}{\log(M_0 / M_1)} \tag{2.1}$$

$$c = \frac{M_0 - M_1}{t} \times \frac{\log(H_0 / H_1)}{\log(M_0 / M_1)} \tag{2.2}$$

M_0 NH_4^+ content [μg N g^{-1} soil] after 3 h of incubation

M_1 NH_4^+ content [μg N g^{-1} soil] after 51 h incubation

H_0 $^{15}NH_4^+$ content [μg N g^{-1} soil] in excess of natural abundance after 3 h of incubation

H_1 $^{15}NH_4^+$ content [μg N g^{-1} soil] in excess of natural abundance after 51 h incubation

m gross mineralisation rate [μg N g^{-1} soil d^{-1}]

c gross NH_4^+ consumption rate [μg N g^{-1} soil d^{-1}]

t time (2 days) between initial and post-incubation harvest [days]

Nitrogen immobilisation (i) was quantified in two ways: i) the amount of ^{15}N in the chloroform-labile microbial biomass (see above) was measured, and ii) according to equation 2.3 (Wang *et al.*, 2001):

$$i = m - N_{min} \tag{2.3}$$

m gross N mineralisation rate [μg N g^{-1} soil d^{-1}]

N_{min} net N mineralisation, calculated from the difference in the mineral (NH_4^+ and NO_3^-) N content after 3 and 51 hours of incubation.

The mineralisation quotient (Qm) and the NH_4^+ consumption quotient (Qc) were used to express the rate of gross N mineralisation or NH_4^+ consumption per unit of chloroform-labile microbial N.

Several key elements and potential errors must be considered when the ^{15}N pool dilution technique is applied to assess gross N fluxes in soils (Kirkham and Bartholomew, 1954; Davidson *et al.*, 1991). Davidson *et al.* (1991) reported rapid (within minutes) abiotic consumption (clay fixation) of applied NH_4^+ in a silt loam mineral grassland soil, leading to an overestimation of nitrification and, thus, NH_4^+ consumption. This potential error

was overcome by an initial sampling three hours after $^{15}NH_4^+$ application. The uneven distribution of applied ^{15}N can lead to potential errors as well (Davidson *et al.*, 1991; Monaghan, 1995; Murphy *et al.*, 1999). These errors can be significant with a non-random spatial distribution of microbial processes in undisturbed, intact soil cores (Davidson *et al.*, 1991). The ^{15}N was, therefore, injected evenly from the top and the bottom of the core to minimize systematic biases of the label within the core. Re-mineralisation of immobilised ^{15}N can lead to a considerable underestimation of the gross mineralisation rate (Bjarnason, 1988; Davidson *et al.*, 1991); however, due to the short incubation period (2 days) in the present study, this error should be insignificant.

2.2.6 Determination of total soil N/^{15}N

After the above measurements, the roots were separated manually from the remaining soil, placed onto a 250-μm sieve, washed gently, dried at 45°C and analysed for N and ^{15}N on a 20-20 PDZ mass spectrometer (Europa Scientific, UK) linked to an automated C/N analyser. Soil samples used for total N/^{15}N analysis were oven-dried (48 h at 45°C) and finely ground before N/^{15}N analysis.

2.2.7 Statistical analyses

The soil cores were replicated three times for each treatment and sampling date (2 pCO_2 levels, 2 sward types and 2 sampling dates). The design of the experiment was a split-split-plot with pCO_2 and sampling time as the main plot factors and sward type and soil fraction as sub plot factors. Effects of pCO_2, sward types and sampling time were analysed by ANOVA using the values of the whole soil core. To estimate the effects between the soil layers, a separate ANOVA procedure was carried out using the values of the two soil layers (0–3.5 cm and 3.5–7 cm). To assess the significance of the interrelationships between the variables measured, Pearson correlation coefficients were determined. To estimate the correlation coefficients, values of each core were used. All the statistical analyses were performed using the SAS statistical analysis package (SAS Institute, Cary, NC, USA).

Table 2.1: Initial (after three hours incubation) and post-incubation (51 hours) NH_4^+ and NO_3^- pool sizes [μg N g soil^{-1}] and ^{15}N atom %-excess (^{15}N %), averaged for the two sampling dates.

	Initial (3 hrs)				Post-incubation (51 hrs)			
pCO_2	NH_4^+	^{15}N %	NO_3^-	^{15}N %	NH_4^+	^{15}N %	NO_3^-	^{15}N %
				Lolium				
36 Pa	6.59	2.06	4.18	1.34	3.21	0.257	8.90	1.56
60 Pa	8.84	1.87	2.88	1.27	4.13	0.163	7.90	1.36
				Trifolium				
36 Pa	5.51	2.22	6.07	1.28	1.88	0.035	17.45	1.34
60 Pa	5.99	1.85	6.86	1.19	2.26	0.296	15.79	1.32
SE	*0.48*	*0.15*	*0.89*	*0.12*	*0.28*	*0.08*	*1.69*	*0.14*
ANOVA								
pCO_2	NS	NS	NS	NS	(+)	NS	NS	NS
Sward	(+)	NS	(+)	NS	**	NS	**	NS
pCO_2 × Sward	NS	NS	NS	NS	NS	NS	NS	NS

Significance: (**) $p < 0.01$; (+) $p < 0.1$; NS not significant.
SE: standard error of the means (n = 6)

2.3 Results

2.3.1 Daily rates of gross N mineralisation, NH_4^+ consumption and N immobilisation

There were no significant differences in the initial and post incubation mineral content between pCO_2 treatments (Table 2.1). While differences in the initial content of mineral N between the swards types were only significant at the 10 % level, significantly more nitrate had accumulated after 51 hours in the *Trifolium* treatment, which was associated with a lower NH_4^+ content. The ^{15}N atom %-excess of the mineral N did not differ between the CO_2 treatment and the sward type, and there were no significant interactions with pCO_2.

Gross mineralisation (m) as well as NH_4^+ consumption (c) varied considerably; hence, the changes in the rates were not statistically significant under elevated pCO_2 in the *Lolium* and *Trifolium* treatments (Figure 2.1). No significant differences were found between the two sward types. Gross

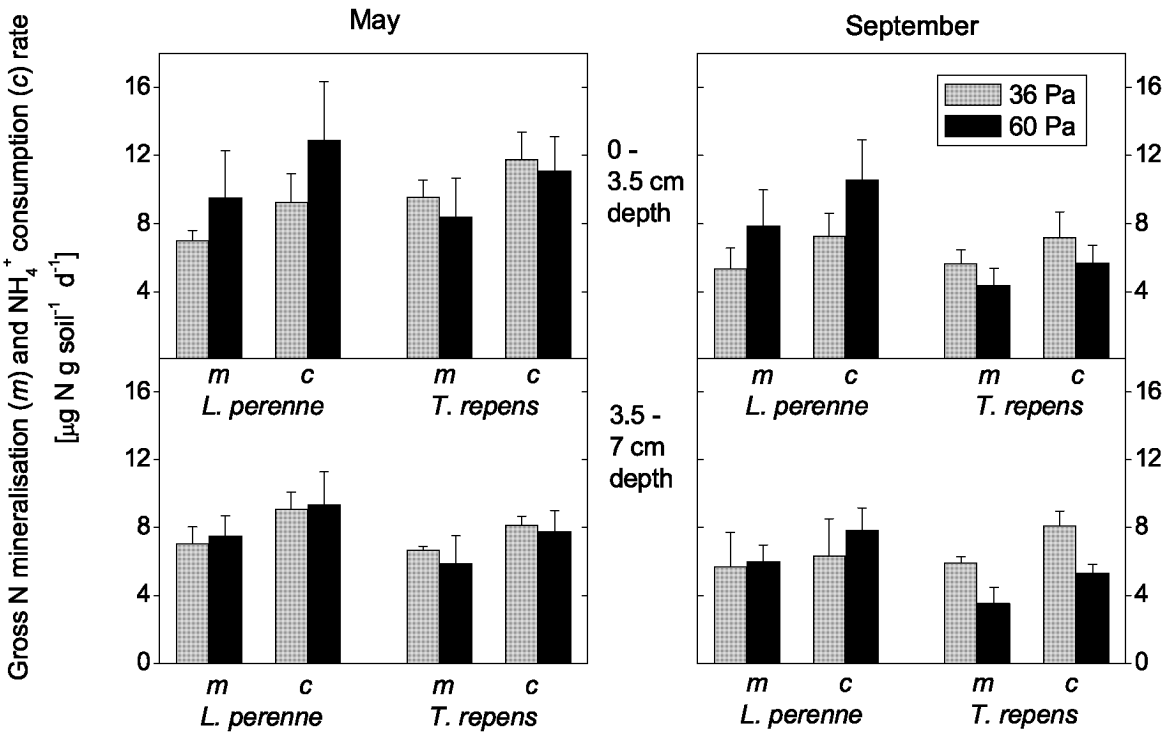

Figure 2.1: Rates of gross N mineralisation (m) and NH_4^+ consumption (c) in intact soil cores taken from *L. perenne* and *T. repens* swards in the field in May and September 2000. Swards were grown at two pCO_2 levels. Vertical bars are standard errors of the mean (n = 3).

rates of m and c were significantly ($p < 0.05$) higher in the soil layer 0 to 3.5 cm than from 3.5 to 7 cm; this difference was most evident from the differences in the May sampling. Comparing the two samplings (May and September), gross N rates of m and c were significantly ($p < 0.05$) lower for cores sampled in September.

2.3.2 Chloroform-labile microbial N and ^{15}N

The amount of chloroform-labile microbial N in the core was greater in the *Lolium* (Table 2.2) than in *Trifolium* soils on both sampling dates. The amount of chloroform-labile microbial N decreased in soils sampled in September compared to soils sampled in May 2000 ($p < 0.05$). Elevated pCO_2 did not have a significant effect on the amount of chloroform-labile microbial N. Elevated pCO_2 had no effect on the ^{15}N atom %-excess in the chloroform-labile microbial N, and no differences were found between the sward types and sampling dates. The ^{15}N atom %-excess of chloroform-labile

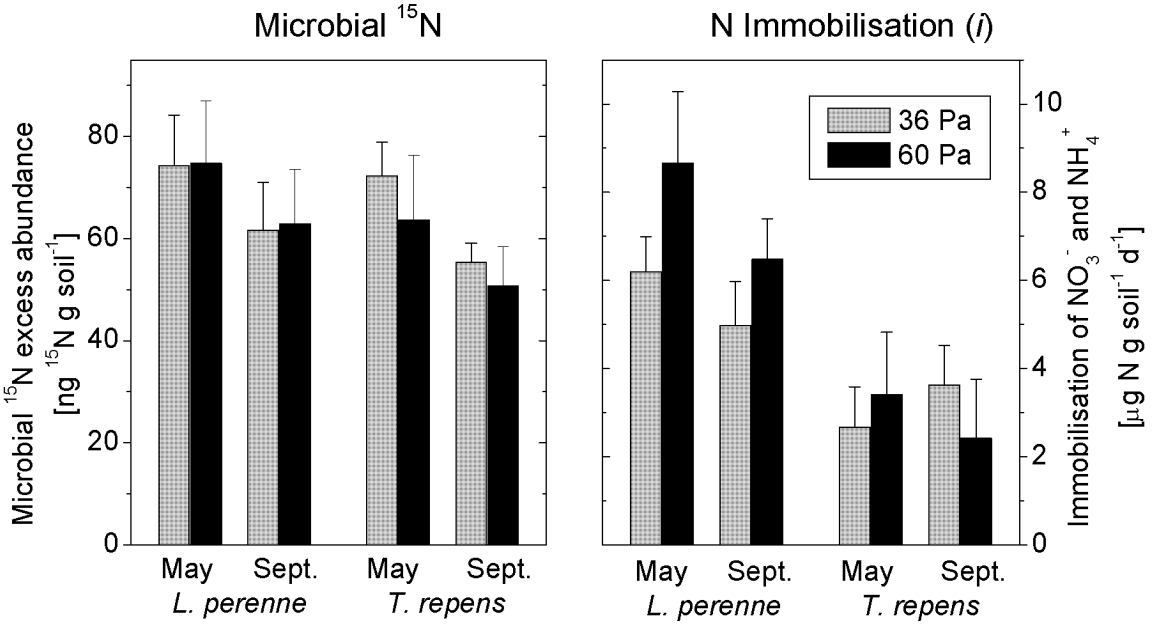

Figure 2.2: Amount of ^{15}N immobilised in the microbial N pool 48 h after application of $^{15}NH_4^+$ solution and daily rate of $NH_4^+ + NO_3^-$ immobilisation in intact soil cores taken from *L. perenne* and *T. repens* swards in the field in May and September 2000. Swards were grown at two pCO_2 levels. Vertical bars are standard errors of the mean (n = 3).

microbial N in the soil was significantly greater from 3.5 to 7 cm than in the upper layer of the soil when analysed for both sward types, pCO_2 treatments and sampling dates. The increase of the *Qm* and *Qc* values in the 3.5–7 cm soil layer (Table 2.2) indicated the proportionally greater efficiency of microbes to mineralise and consume N in this soil layer. This was confirmed by the higher ^{15}N atom %-excess of chloroform-labile microbial N in the lower fraction, i. e. increased immobilisation of the applied mineral ^{15}N per unit biomass N.

Immobilisation (*i*) (Figure 2.2) of mineral N (both NH_4^+ and NO_3^-) was not affected significantly by elevated pCO_2, soil depth and sampling date. However, *i* increased significantly ($p < 0.05$, averaged over pCO_2 treatments, sampling dates and soil layers) in the *L. perenne* swards compared to the *T. repens* treatments (Figure 2.2). In contrast, the amount of ^{15}N immobilised in the chloroform-labile microbial N pool was similar in both swards (Figure 2.2). Furthermore, no significant differences in the amount of microbial ^{15}N were found between the two pCO_2 levels and sampling dates. CO_2 emissions from soil cores, i. e. respiration of soil microbes measured

Table 2.2: Amount of microbial N, proportion of ^{15}N in microbial N and specific activity of gross mineralisation (Qm) and gross NH_4^+ consumption (Qc) in intact soil cores of *Lolium perenne* and *Trifolium repens* swards grown under two pCO_2 levels. Soil layer a: top 3.5 cm; soil layer b: 3.5–7 cm. *SE*: Standard error of the means (n = 3).

Sampling	Sward	pCO_2	Soil depth	Microbial N and ^{15}N		Specific microb. activity	
				N	$^{15}N(\text{microb. N})^{-1}$	Qm	Qc
		[Pa]		$[\mu g\,(g\,soil)^{-1}]$	$[\mu g\,g^{-1}\times10^{-4}]$	$[\mu g\,N(g\,microb\,N)^{-1}\times10^{-2}]$	
May	*Lolium*	36	a	145	5.9	4.82	6.3
			b	84	8.08	8.3	10.77
		60	a	140	5.51	6.6	9.05
			b	94	9.41	8.39	10.66
	Trifolium	36	a	123	6.7	7.96	9.82
			b	84	7.45	8.26	10.14
		60	a	118	5.81	6.86	9.54
			b	83	6.66	6.83	9.87
September	*Lolium*	36	a	86	8.65	6.14	8.44
			b	64	8.16	8.35	9.09
		60	a	139	5.15	6.13	8.56
			b	91	5.92	6.73	8.83
	Trifolium	36	a	84	6.74	7.1	9.17
			b	66	8.24	9.13	12.57
		60	a	92	5.52	5.07	7.11
			b	63	10.2	6.11	11.31
SE				*10.6*	*1.6*	*0.73*	*1.28*

Table 2.2: continued: Source of variation (ANOVA)

ANOVA	micob. N	microb. ^{15}N	Qm	Qc
pCO_2	NS	NS	NS	NS
Sward	*	NS	NS	NS
Sampling	*	NS	NS	NS
Soil depth	*	*	***	**
pCO_2 × Sward	(+)	NS	NS	NS
pCO_2 × Sampling	NS	NS	NS	NS
pCO_2 × soildepth	NS	NS	NS	NS
Sward × Sampling	NS	NS	NS	NS
Sward × Soildepth	NS	NS	NS	NS

Significance: (***) $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$; (+) $p < 0.1$; NS not significant.

after 45 h incubation (Table 2.3) were significantly greater from the *Lolium* soils than from *Trifolium* swards on both sampling dates. In both sward types, CO_2 emissions were lower in samples taken in September. No significant effects of elevated pCO_2 were detected for sward treatment or sampling date. There were no significant differences in N_2O emissions between sward types and CO_2 treatments (Table 2.3). N_2O emissions were generally stronger from the samples taken in May but only at $p < 0.1$.

2.3.3 Relationship between gross N fluxes, microbial N and CO_2 emissions from the soil

There was a significant positive correlation between gross mineralisation and chloroform-labile microbial N in both sward types (Table 2.4). The NH_4^+ consumption correlated well with chloroform-labile microbial N in *Lolium* soils, whereas the correlation was lower and insignificant in the *Trifolium* soils. Significant correlations were found for microbial ^{15}N-content (a measure of the immobilisation of applied NH_4^+) with the immobilisation rate calculated according to equation 2.3 and for NH_4^+ consumption in the *Trifolium* cores. However, in the case of *Lolium perenne*, no significant correlations between these variables were observed, indicating different contributions of the soil microbes to N immobilisation on the one hand and/or sinks other than chloroform labile biomass for the applied ^{15}N. Total mineral N immobilisation was strongly related to NH_4^+ consumption in the *Lolium* cores, but this was not the case for *T. repens*. In comparing chloroform-labile microbial N and ^{15}N with CO_2 emissions from the soil cores, significant correlations were detected only in the *Trifolium* soil. The same was found when gross mineralisation and NH_4^+ consumption were correlated with CO_2 emissions. The correlation coefficients were generally lower and insignificant in the individual treatments (pCO_2, sampling time), mainly due to the small number of observations (n = 6 or fewer).

2.3.4 Distribution of applied ^{15}N in the various N pools after incubation

Forty micrograms of ^{15}N were applied to each core, resulting in approximately 0.3 $\mu g\,^{15}N\ g^{-1}$ oven-dried soil. Almost all the applied $^{15}NH_4^+$ was transformed in both soils of the sward types and pCO_2 treatments (Table 2.5). A maximum of 5 % of applied ^{15}N was still present as NH_4^+ af-

Table 2.3: CO_2 and N_2O emissions during 45 h in intact soil cores of *Lolium perenne* and *Trifolium repens* swards grown under ambient (36 Pa) and elevated (60 Pa) pCO_2.

Sampling	Sward	pCO_2	CO_2	N_2O
		[Pa]	$[\mu g(g\,soil)^{-1}]$	$[\mu g\,(g\,soil)^{-1} \times 10^{-6}]$
May	*Lolium*	36	177	20.4
		60	174	28.8
	Trifolium	36	128	10.9
		60	142	19.3
September	*Lolium*	36	80	8.5
		60	130	18.7
	Trifolium	36	71	6.9
		60	64	13.2
SE			*21.2*	*5.82*
ANOVA				
pCO_2			NS	NS
Sward			*	NS
Sampling			**	(+)
pCO_2 × Sward			NS	NS
pCO_2 × Sampling			NS	NS
Sward × Sampling			NS	NS

Significance: (**) $p < 0.01$; (*) $p < 0.05$; (+) $p < 0.1$; NS not significant.
SE: Standard error of the means (n = 3).

ter 51 h of incubation. 17 to 24 % of applied ^{15}N was immobilised in the chloroform-labile microbial biomass, with no significant differences between sward types and pCO_2 levels. The amount of $^{15}NO_3$ was significantly higher in the *Trifolium* cores (up to 75 % of applied ^{15}N) than the *Lolium* cores (at the most 52 %). However, no significant changes were detected under elevated pCO_2 or between the sampling dates. The increased amount of $^{15}NO_3^-$ in the *Trifolium* cores resulted in a significantly higher total ^{15}N recovery compared to the *Lolium* cores. Whereas 90 to 99 % of the applied ^{15}N was recovered in the *Trifolium* cores, only 53 to 78 % of ^{15}N was found as mineral and chloroform-labile microbial N in the *Lolium* cores after incubation. The amount of ^{15}N lost through N_2O emission was negligible; total $^{14+15}N_2O$ values were 7 to 29 × 10^{-6} μg N g^{-1} soil (Table 2.3). Recovery of total ^{15}N from the soil (Table 2.6) was 99 to 101 % three hours after isotope application in both sward treatments.

Table 2.4: Correlation coefficients (r-values) between gross transformation rates, microbial N and CO_2 emissions over both pCO_2 treatments and sampling times, determined separately for *L. perenne* and *T. repens* (n = 12).

T. repens	Immobilisation	Microb. N	Microb. ^{15}N	CO_2 emission
Microbial N	0.647 *			
Microbial ^{15}N	0.673 *	0.911 ***		
CO_2 emissions	0.163^{NS}	0.669 *	0.650 *	
Gross mineralisation	0.657 *	0.699 *	0.797 **	0.673 **
Gross NH_4^+ consum.	0.526^{NS}	0.502^{NS}	0.623 *	0.657 *
L. perenne	Immobilisation	Microb. N	Microb. ^{15}N	CO_2 emission
Microbial N	0.646*			
Microbial ^{15}N	-0.110^{NS}	0.395^{NS}		
CO_2 emissions	0.311^{NS}	0.292^{NS}	0.083^{NS}	
Gross mineralisation	0.858 ***	0.796 **	0.249^{NS}	0.183^{NS}
Gross NH_4^+ consum.	0.918 **	0.765 **	0.126^{NS}	0.292^{NS}

Significance: (***) $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$; (NS) not significant.

While recovery of total soil ^{15}N remained high in the *Trifolium* soil after incubation, isotope recovery was much lower in *Lolium* soil, consistent with the lower recovery of ^{15}N in the mineral and microbial N pools. About twice as much ^{15}N was recovered in the coarse roots of the *L. perenne* cores compared to the *T. repens* cores (Table 2.6).

2.4 Discussion

2.4.1 Does long-term exposure to elevated pCO_2 affect N fluxes in grasslands?

Daily rates of gross mineralisation (Figure 2.1) were consistent with rates of gross mineralisation reported for fertile grassland soils (Davidson *et al.*, 1991; Ledgard *et al.*, 1998; Watson *et al.*, 2000). Although rates of gross N mineralisation and NH_4^+ consumption tended to increase in the *Lolium* soils but to decrease in *Trifolium* soils under elevated pCO_2, the changes were not statistically significant (Figure 2.1). The absence of a significant pCO_2 effect on gross N mineralisation is in accordance with the results of Gloser *et al.* (2000), who found no difference in *in situ* net mineralisation in

Table 2.5: Percentage of applied ^{15}N found after 48 h incubation in the mineral (as $^{15}NH_4^+$ and $^{15}NO_3^-$) and microbial N pool and in both pools combined.

			^{15}N recovery as:			
Sampling	Sward	pCO_2	$^{15}NH_4^+$	$^{15}NO_3^-$	Microbial ^{15}N	All fractions
May	*Lolium*	36 Pa	1.62	42.3	24.1	68.1
		60 Pa	3.75	24.9	24.4	53.1
	Trifolium	36 Pa	0.37	75.2	23.4	98.9
		60 Pa	3.73	68.5	21.3	93.5
September	*Lolium*	36 Pa	5.07	52.2	21.1	78.4
		60 Pa	1.04	43.1	18.7	62.9
	Trifolium	36 Pa	0.11	70.8	18.6	89.6
		60 Pa	1.34	74.7	17.4	93.4
SE			*1.78*	*8.01*	*3.43*	*9.61*
ANOVA						
pCO_2			NS	NS	NS	NS
Sward			NS	**	NS	***
$pCO_2 \times$ Sward			NS	NS	NS	(+)

Significance: (***) $p < 0.001$; (**) $p < 0.01$; (+) $p < 0.1$; NS not significant.
SE: Standard error of the means (n = 6)

the *L. perenne* swards in 1997. The lack of a significant response of gross N mineralisation corresponds to the lack of a response of CO_2 respiration to elevated pCO_2 and a similar soil microbial N content (Table 2.2 and 2.3). Thus, the initial hypothesis - increased below-ground allocation of photosynthates under elevated pCO_2, observed by Hebeisen *et al.* (1997) and confirmed in our study (Table 2.6), enhances gross rates of N transformation - was not true in the long run.

Plant-driven effects, such as root dynamics as well as biological and physical soil conditions, are factors which determine the rates of N transformation and, consequently, the pCO_2 response. Fitter *et al.* (1997) found that root biomass and turnover increased in two contrasting grassland systems under elevated atmospheric pCO_2, which could potentially lead to an increase in N transformation. Changes in plant root exudates, which can

Table 2.6: Total N and percentage of applied ^{15}N found in the soil and root samples of the May sampling after 3 h and 51 h incubation. Values in brackets are standard errors of the mean.

		soil			roots	
Sward	pCO_2	N [mg g^{-1}]	^{15}N (3 h) [% of applied]	^{15}N (51 h)	DM [mg g^{-1}]	^{15}N (51 h) % of appl.
Lolium	36 Pa	3.67 (0.1)	101 (5.9)	57 (-)	14.4	12.0 (2.9)
	60 Pa	3.72 (0.1)	98 (4.5)	-	16.8	9.6 (3.2)
Trifolium	36 Pa	3.44 (0.1)	87 (5.4)	99 (12.5)	7.7	6.3 (1.2)
	60 Pa	3.81 (0.1)	100 (6.6)	86 (2.5)	9.1	5.02 (2.2)

constitute up to 52 % of the net photosynthesis (Marschner *et al.*, 1996; Hodge *et al.*, 1998) may influence the flow of nutrients through the microbial biomass. However, no estimates are available and, although there are indications that changes occurred in the microbial communities under elevated pCO_2 (Schortemeyer *et al.*, 1996; Marilley *et al.*, 1999; Montealegre *et al.*, 2000), the effects of such changes were probably too small to have had a significant influence on our results. On the other hand, Ross *et al.* (1995) found no effect of elevated atmospheric pCO_2 on the decomposition of roots or on net N mineralisation in a ryegrass/white clover sward. Moreover, Blum *et al.* (1997) observed that the C/N ratio of dead tillers, leaves (necromass) and root biomass did not respond to pCO_2. Both findings may explain why N transformation did not change significantly. Physical soil factors like soil moisture also influence soil N transformation. Hungate *et al.* (1997) found that increased soil moisture under elevated pCO_2 stimulated gross N mineralisation. Gross N mineralisation was correlated with soil moisture ($r = 0.60$, $p = 0.002$) in our study; nevertheless, the moisture of the soil cores was not influenced by CO_2 fumigation. Furthermore, due to the application of the ^{15}N solution, soil moisture was increased by only 2 % (from 31 to 33 %, averaged over all treatments), which is assumed to be of no significance for N transformation processes. Denitrification too, measured as N_2O emissions in the same plots (Baggs *et al.*, 2003b) was also not affected by elevated pCO_2. This indicates that there were no alterations of availability of mineral N (NO_3^-) and/or a greater demand of the plants for N under elevated pCO_2.

The fact that neither microbial biomass N nor gross N mineralisation

was affected significantly by pCO_2 may also be due to the fact that below-ground transformations of N were not significant during the short period of incubation. For example, apart from seasonal differences, the time of the measurements in relation to the time of harvesting may also be important, because a greater amount of C may be released after such events. Further work should concentrate on seasonal variations and changes that occur in the below-ground allocation, which may influence N dynamics after periods of sward re-growth.

The ^{15}N dilution technique proved to be very useful, however short incubation times are necessary to avoid re-mineralisation of ^{15}N. Therefore, this technique has the disadvantage that it provides only short-term insight into N fluxes.

2.4.2 Effect of season and soil layer on microbial biomass

Rates of gross N mineralisation and NH_4^+ consumption were higher in the upper 3.5 cm of the soil than in the lower soil layers (Figure 2.1, $p < 0.05$), consistent with the higher content of microbial N in the top layer (Table 2.2). Differences were not as large as reported elsewhere (Murphy *et al.*, 1998) but indicated a higher microbial activity in the upper layer, possibly due to a greater availability of plant residues and a higher oxygen concentration. There were no significant differences in the soil moisture and the incubation temperature between the layers. In contrast, the specific mineralisation (Qm) (Table 2.2) was significantly higher in the 3.5 to 7 cm layer. Consequently, the microbes in the lower soil layer mineralised the organic N more efficiently, and/or a greater fraction of microbial biomass was involved in N mineralisation, contrary to the findings of Murphy *et al.* (1998). These findings suggest a vertical variation in the quantity and/or the composition of the microbial community as well as uneven contribution of the different components of the microbial biomass to N mineralisation (Puri and Ashman, 1998). The vertical stratification of the specific microbial activity, as discussed for gross mineralisation, was also evident in the case of gross NH_4^+ consumption. Furthermore, the total amount of ^{15}N immobilised in the microbial biomass was greater in the upper soil layer, whereas the fraction of ^{15}N in the microbial N was significantly higher in the lower layer (Table 2.2). This illustrates a greater immobilisation of N per unit microbial N or a lower N turnover in this soil layer.

2.4.3 Effect of sward type on N fluxes

Rates of N immobilisation were considerably higher in the soils taken from the *Lolium* swards (Figure 2.2). In contrast, microbial ^{15}N, a direct measure of N immobilisation, was almost the same in both sward types (Figure 2.2), indicating a similar rate of microbial N immobilisation. As shown in Table 2.4, there was a significant correlation between microbial ^{15}N and N immobilisation in the soil of *Trifolium* swards but not in that of *Lolium* swards. This indicates that the amount of microbial ^{15}N may be an adequate measure of microbial N immobilisation in the *Trifolium* soils but not in the *Lolium* soils. Since rates of N immobilisation were calculated from gross mineralisation (which was similar in both sward types) and net mineralisation, the discrepancy was due to differences in net mineralisation. As shown in Table 2.1, significantly more NO_3^- accumulated during incubation in the *T. repens* soils. Considering the fact that the ^{15}N enrichment of the post-incubation NO_3^- pool was the same in both soils (Table 2.1), an additional source of NO_3^-, other than nitrification from the labelled NH_4^+ pool, can be excluded in the *T. repens* cores. On the other hand, NO_3^- was not leached from the soil cores, and denitrification was probably unimportant during the two-day incubation period, as indicated by the low emissions of nitrous oxide (Table 2.3). A possible explanation may be the NO_3^- uptake by roots in the *Lolium* soil cores although the plant tissue had been removed at ground level to eliminate the demand of the plant for N. Nitrogen that disappears into roots can hardly be detected by chloroform fumigation or by KCl extraction. Root biomass was reported to be up to seven times greater in the *Lolium* swards than in *Trifolium repens* swards (Hartwig *et al.*, 2000) and was found to be as twice as high in the *Lolium* soils in the present study (Table 2.6). A greater root biomass could absorb more mineral N. This would also explain the lower total recovery of applied ^{15}N (Table 2.5) as chloroform-labile microbial N and mineral N in the grass soils (65 to 70 % of applied ^{15}N) after incubation compared to the clover soils (90 to 100 %). We suggest the nitrate uptake by roots in the *Lolium* soil cores by the following facts: a) Soil respiration was significantly higher in the *Lolium* soil cores (Table 2.3), although the amount of chloroform-labile microbial N was similar in both sward types. The CO_2 emissions correlated well with the size of the total microbial N pool in the *Trifolium* soils but not in the *Lolium* soils (Table 2.4). This indicates that there was a source of CO_2 other than the microbial biomass in the soils from the *Lolium* swards, e. g. enhanced

root respiration. b) Rates of gross mineralisation and NH_4^+ consumption correlate well with microbial N in both sward types, illustrating an equal contribution of soil microbes to the gross N transformation. However, soil respiration correlates with rates of m and c only in the *Trifolium* soils, thus supporting our hypothesis that there is a source of CO_2 emissions in the *Lolium* soils other than microbial respiration. c) In a total N/^{15}N determination of the remaining samples from May 2000, nearly all the ^{15}N was recovered in both sward types three hours after ^{15}N application (Table 2.6). In contrast, after 51 hours of incubation in the *Lolium* soil, only 57 % of the applied ^{15}N was recovered in the soil, while 92 % was recovered in the *Trifolium* soil. d) A greater amount of ^{15}N was found in the *Lolium* roots (11.4 % of the applied ^{15}N) than in the *Trifolium* roots (5.4 %) in the remaining soil in May (Table 2.6); this was related to the larger amount of root biomass in the *Lolium* soils. These values may not be strictly quantitative due to the fact that the fine roots in particular not sampled from the soil. However, the data support the hypothesis that N/^{15}N was taken up by roots during incubation. This potential error source, leading to a considerable overestimation of microbial N immobilisation, should be considered when the ^{15}N pool dilution technique is applied to intact soil cores containing large amounts of root remains. Hence, the amount of immobilised ^{15}N (Figure 2.2) may be a better value for comparing microbial N immobilisation in different treatments.

2.4.4 Conclusions

After seven years of exposure to elevated pCO_2, gross mineralisation, NH_4^+ consumption and N immobilisation in both the *Lolium perenne* and the *Trifolium repens* soils did not show significant responses to CO_2 on the two sampling dates. The size of the microbial N pool and immobilisation of applied mineral ^{15}N were also not affected by elevated pCO_2. This suggests that the changes in soil organic matter or in microbial activity, which may have occurred during the seven-year exposure to elevated pCO_2, had no significant effect on gross N transformations in the soils measured on a two-days incubation.

The immobilisation of ^{15}N in the microbial biomass was similar in soils taken from the *L. perenne* and *T. repens* swards, whereas nitrogen immobilisation was significantly lower in the *Trifolium* swards. N immobilisation in the microbial biomass, determined from gross and net mineralisation, was

overestimated in the *Lolium* cores due to a high rate of immobilisation of mineral N in the roots. This may indicate that measurements of microbial N immobilisation may be significantly overestimated, when a potential N-uptake by roots stacked in the soil cores is not taken into consideration.

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3 Symbiotic N_2 fixation in grassland swards during ten years of free air CO_2 enrichment

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Abstract Most information concerning the dynamics of grassland ecosystems and the response to N availability after a step increase in the partial pressure of atmospheric CO_2 (pCO_2) has been obtained in short-term studies. However, there is little information about the long-term effects of elevated pCO_2 on such ecosystems. The influence of free-air carbon dioxide enrichment (FACE) on symbiotic N_2 fixation in *Trifolium repens* (white clover) and bi-species mixtures of clover with *Lolium perenne* (rye grass) was studied for 10 years under field conditions. Plants were grown under ambient (36 Pa) and elevated (60 Pa) pCO_2 in combination with two N fertilisation treatments (14 and 56 g N $m^{-2}a^{-1}$). The N derived from N_2 fixation was measured by means of the ^{15}N dilution technique. At high N fertilisation, the response of the symbiotic fixed N_2 to pCO_2 decreased from 5 g N m^{-2} in 1993 to -1 g N m^{-2} in 2002. The higher N yield under elevated pCO_2 was solely derived from N_2 fixation in the first three years. This was attributed to an insufficient availability of soil N to meet the greater demand of the plant for N under elevated pCO_2. Thereafter, changes in the N yield under elevated pCO_2 were the result of soil-derived N and from N_2 fixation. Our results suggested that, as a result of the increased N_2 fixation in the first three years, more N was temporarily immobilised under elevated pCO_2, leading to a transient net input of N into the system. With the increasing availability of soil N, due to the re-mineralisation of this pool and large inputs of fertiliser N, the plant may have acquired more of its N from the soil, a process which requires less energy. In contrast, the plants remained limited in N in the low-N treatment, leading to a high N_2 fixation under elevated pCO_2. Our results indicate that legume-grass systems, exposed to a step increase in elevated pCO_2, may act temporarily as an N sink until a new equilibrium in the plant-soil system has been established.

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3.1 Introduction

Considerable attention is being paid to the potential impact of elevated pCO_2 on various ecosystems as the concentration of atmospheric CO_2 will undoubtedly continue to rise, it is predicted to double by the end of this century (IPCC, 2001). The potential positive effect of elevated pCO_2 on the primary production as a result of the additional input of C may be limited by other environmental factors such as water supply, temperature, irradiation and the availability of nutrients. In temperate terrestrial ecosystems, nitrogen (N) availability is one of the key factors that may limit the positive response of plant growth. It is assumed that sequestration of C and N into an ecosystem are closely related (Granhall, 1981; Gifford, 1992; van Groeningen *et al.*, 2002). Thus, an increase in the availability of C would ultimately lead to an increased demand for N by the plants and the ecosystem as a whole. Symbiotic N_2 fixation, one of the main processes by which N is introduced into terrestrial ecosystems, may counterbalance a lack of available N in the soil and may maintain the C/N balance in a given ecosystem (Zanetti *et al.*, 1997; Lüscher *et al.*, 2000; Hartwig *et al.*, 2000). In various ecosystems, symbiotic N_2 fixation has been shown to depend strongly on the supply of soil N and on fertiliser N (reviewed by Hartwig, 1998; Singh *et al.*, 2002). However, the influence of elevated pCO_2 on the availability of soil N for plant growth is still under debate. On the one hand, Díaz *et al.* (1993) suggested a feedback mechanism, in which an increased release of substrate under elevated CO_2 causes an increase in the microbial immobilisation of N as a result of a greater number of microflora. This would lead to a decline in the N availability in the soil. In contrast, Zak *et al.* (1993) suggested a positive feedback on soil N and C dynamics due to greater inputs of C below ground, causing an increase in the microbial biomass in the soil and higher rates of organic matter turnover and N availability.

Results from the first three years of the Swiss FACE experiment indicate that the uptake of N from the soil by plants in managed grassland systems is limited after a step increase in pCO_2. Net mineralisation did not change under elevated pCO_2 (Gloser *et al.*, 2000). The harvested above-ground dry matter (DM) and N yield increased only in swards with white clover (Zanetti *et al.*, 1996; Hebeisen *et al.*, 1997), and the increase in the N yield was solely derived from the symbiotic N_2 fixation (Zanetti *et al.*, 1996, 1997). In contrast, a ryegrass sward monoculture showed a negative, or no, response to elevated pCO_2 (Hebeisen *et al.*, 1997; Daepp *et al.*, 2000; Suter *et al.*,

2002). There was a strong increase in root biomass in the ryegrass swards (Hebeisen *et al.*, 1997; Suter *et al.*, 2002) and, to a lesser extent, in the root biomass in the clover swards (Zanetti *et al.*, 1996; Hebeisen *et al.*, 1997). These data were obtained after two to three years of enhanced pCO_2. In a recent study, Daepp *et al.* (2000) found elevated pCO_2 resulted in an increase in dry matter yield and N over six years in *L. perenne* monocultures treated with high N input. This was attributed to a steady increase in the availability of soil N. Thus, the different processes in the ecosystem do not respond simultaneously to elevated pCO_2. The plant may respond very quickly, e. g. with changes in the rate of photosynthesis or alterations in the leaf area and allocation of biomass, while changes in soil processes, such as mineralisation of soil organic matter, may occur much more slowly, as the ecosystem adapts to the new conditions. Consequently, the short-term response of an ecosystem to a step increase in pCO_2 may not represent an appropriate base for an accurate prediction of the ecosystem response to a gradual slow increase in pCO_2. For that purpose, long-term experiments are a necessary to adequately determine and model how terrestrial ecosystems will respond to elevated pCO_2 in the long term.

The objective of this study was to examine the influence of long-term (10 years) elevated pCO_2 (60 Pa) on N yield, symbiotic N_2 fixation and N availability in the soils planted with white clover and mixed swards of white clover/perennial ryegrass. To assess the N availability the annual rates of the net mineralisation of N were measured during the eighth year of CO_2 fumigation. Two rates of N fertilisation enabled the determination of combined effects of elevated pCO_2 and N availability.

3.2 Materials and Methods

3.2.1 Experimental site and plant material

The FACE technology (Lewin *et al.*, 1994) was used to investigate the long-term influence of elevated pCO_2 on grassland ecosystems in the field. The Swiss FACE experimental site, located in Eschikon near Zurich, Switzerland (47°27′N and 8°41′E at 550 m above sea level), consisted of three blocks (Zanetti *et al.*, 1996; Hebeisen *et al.*, 1997). Each block consisted of two circular areas (18 m diameter), one CO_2-fumigated with CO_2 ($pCO_2 = 60$ Pa) and a control ($pCO_2 = 35$ Pa) area. The fumigation began on May 31, 1993

and took place during the daytime from 1993 to 2001 throughout the growing season from March to November, provided that the air temperature was above 5°C.

The soil was a eutric cambisol with pH-values of 6.5 to 7.5 (20 cm topsoil). Phosphorus, K and Mg were applied as fertiliser at the beginning of each growing season to remove potential nutrient deficiencies and to adjust for differences in nutrient availability between the several blocks (Zanetti *et al.*, 1996; Hebeisen *et al.*, 1997).

In mid-August 1992, *Lolium perenne* and *Trifolium repens* were sown in monoculture and two-species plots (2.8 m × 1.9 m). Swards were cut in mid-April and mid-May, before the beginning of CO_2 fumigation on May 31, 1993. In each year, the plots were treated to prevent *Fusarium* and clover rot as described by Zanetti *et al.* (1996) and Hebeisen *et al.* (1997).

3.2.2 Treatments

Two defoliation regimes were applied in the first three years of the experiment: infrequent cutting (5 cuts per year) and frequent cutting (7 cuts in 1993 and 8 cuts in 1994 and 1995). From 1996 to 2001 all the swards were harvested five times a year. The cutting height was 5 cm above ground level.

Nitrogen fertiliser was applied at two levels, 10 and 14 $g\,m^{-2}$ (1993 and 1994–2001, respectively) on the low-N plots and 42 and 56 $g\,m^{-2}$ (1993 and 1994–2001, respectively) on the high-N plots. The N fertiliser was supplied as NH_4NO_3 in solution; the amounts were adjusted in proportion to the expected biomass production in the year (Zanetti *et al.*, 1996). The sampling area of each plot (1 m^{-2}) was treated with ^{15}N-enriched fertiliser. Nitrate and ammonia were each labelled with a ^{15}N atom %-excess of 0.4 (1993–1994) and 0.3 (1995–2001) in the low-N treatment and 1.6 (1993–1994) and 1.3 (1995–2001) in the high-N treatment.

The levels of atmospheric pCO_2 were ambient (36 Pa) and elevated (60 Pa). This resulted in a total of 16 treatments in the years 1993 to 1995 with two sward types (*T. repens* and *L. perenne*/*T. repens* mixture), two N treatments (low N, high N) two levels of pCO_2 (ambient and elevated) and two defoliation frequencies (frequent, infrequent). There were 12 treatments from 1996 to 2001 without varying the frequency of defoliation.

3.2.3 Sampling of plant material

The harvested plant material from the inner part (0.25 m^2) of the sampling area was separated into *L. perenne*, *T. repens* and unsown species, dried at 65°C for 48 h and ground to a fine powder according the procedure described in Zanetti *et al.* (1996). Analyses for $N/^{15}N$ concentration were done using a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK) at the University of California, Davis, USA.

3.2.4 Calculation of N parameters

The amount of N in the harvested above-ground plant material (N*yield*) was derived from symbiotic N_2 fixation (N*fix*) and from the soil (N*soil*):

$$\mathrm{N}yield = \mathrm{N}fix + \mathrm{N}soil \tag{3.1}$$

The amount of N in the plant material of *T. repens* derived from symbiotic N_2 fixation (N*fix*) was calculated using the standard ^{15}N isotope dilution protocol according to McAuliffe *et al.* (1958):

$$\mathrm{N}fix = \left(1 - \frac{^{15}\mathrm{N\ atom\ \%\ excess_{clover}}}{^{15}\mathrm{N\ atom\ \%\ excess_{reference\ plant}}}\right) \times \mathrm{N}yield \tag{3.2}$$

where *L. perenne* grown in the mixed plots of the corresponding treatment served as the reference crop (Boller and Nösberger, 1988).

N*soil* included the N derived from the fertiliser (N*fert*) and from mineralisation of soil organic matter (N*som*).

$$\mathrm{N}soil = \mathrm{N}fert + \mathrm{N}som \tag{3.3}$$

N*som* includes the N that was in the soil before the experiment started as well as the N that was introduced by symbiotic N_2 fixation, sequestered into the SOM and remineralised, i. e. unlabelled N. However, it does not include the N derived from re-mineralisation of the fraction of fertiliser added since 1993 and that which was immobilised. The amount of N in the plant material derived from the unlabelled soil N (N*som*) was calculated as follows:

$$\mathrm{N}som = \mathrm{N}yield - \mathrm{N}fert - \mathrm{N}fix \tag{3.4}$$

$$\mathrm{N}som = \mathrm{N}yield - \left(\frac{^{15}\mathrm{N\ atom\ \%\ excess_{fertiliser}}}{^{15}\mathrm{N\ atom\ \%\ excess_{plant\ material}}} \times \mathrm{N}yield\right) - \mathrm{N}fix \tag{3.5}$$

All these parameters were calculated separately for each re-growth period and then added to obtain the annual values.

3.2.5 Content of fertiliser-N in the soil and annual net N mineralisation

Total soil N and the ^{15}N content were determined at end of 2001. Three soil cores (2 cm in diameter and 25 cm depth) were sampled from each plot and pooled together. Fresh samples were sieved through a 2-mm sieve and dried at 50°C for 3 days. Dry samples were ground to a fine powder (ball mill MM2, Retsch, Switzerland) and analysed for total N and ^{15}N using a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK) at the University of California, Davis, USA.

The fraction of fertiliser N in the soil (f_N) was calculated as follows:

$$f_N = \left(\frac{^{15}\text{N atom \% excess}_{\text{soil}}}{^{15}\text{N atom \% excess}_{\text{fertiliser}}} \right) \times 100 \qquad (3.6)$$

Due to the changed ^{15}N-enrichment of the fertiliser after 2 years, a weighted average of ^{15}N atom %-excess of the fertiliser was used.

Rates of net N mineralisation in the soil were assessed during the whole growing season 2000 (7 April to 20 October) for all the sward types and treatments. The experiment was conducted twice per re-growth period (10 measurements in total) using the sequential soil coring method (Raison *et al.*, 1987; Berendse *et al.*, 1994). Three sets of two soil cores were taken from each plot at the beginning of the incubation period. The first core of each pair was extracted immediately after sampling, and the second core was taken with a PVC tube (15 cm × 1.6 cm) so that the core consisted of an undisturbed 10-cm column of soil. The tube was closed at both ends to prevent run-off of the soil solution, and the tube was driven into the soil and incubated for 10 to 29 days. Aeration was ensured by drilling small holes in the side walls of the tubes, which extended above the soul surface. Samples from each plot were pooled and the rates of net mineralisation were calculated by subtracting the mineral N content (NH_4^+ and NO_3^-) of the initial sample from the mineral N content of the incubated soil sample. For the determination of NH_4^+ and NO_3^-, soil samples (30 g fresh weight) were shaken for 1 h with 150 ml 2 M KCl solution, and the extract was filtered through ash-less filter paper (No. 589², Schleicher & Schuell, Germany). The concentration of NH_4^+ and NO_3^- ions in extracts was determined colorimetrically using a segment flow analyser (Alliance Evolution II, Alliance Instruments SA, France).

3.2.6 Statistical analysis

The design of the experiment was a split-plot design with pCO_2 as the main plot factor and N fertilisation as the sub-plot factor. The data were analysed using the general linear model (GLM) procedure of the SAS statistical analysis package (SAS Institute, Cary, NC, USA). Because there were no significant differences in the treatment effects between the two cutting frequencies from 1993 to 1995, only the means of the cutting frequencies are presented in the figures. The first re-growth in 1993 was not included in the analysis of data as it was not fumigated with elevated pCO_2.

3.3 Results

3.3.1 Influence of elevated pCO_2 and N fertilisation on N yield and contribution of N sources

Elevated pCO_2 increased the annual above-ground yield of N (N*yield*) by 4 to 6 $g\,m^{-2}$ in both sward types and for both N treatments ($p < 0.1$, averaged over all treatments and years) except towards the end of the experiment (Figures 3.1a and 3.2a). Annual dry mass yield followed the same pattern as N yield. However, due to the slightly lower N concentration of the herbage under elevated pCO_2 the increase in dry matter yield under elevated pCO_2 was greater (on average 18 %, data not shown) than that of N yield (14 %). With respect to the sources of N used by the plants, the response to elevated pCO_2 in the ten years depended strongly on N fertilisation. At low N fertilisation, the percentage of symbiotic N_2 fixation (%N*fix*) contributing to N yield was significantly greater ($p < 0.05$, averaged over all growing seasons and both sward types) during the whole experimental period when the plants were grown under elevated pCO_2 (Figures 3.1b and 3.2b).

At the same time, the proportion of N derived from the mineralisation of unlabelled SOM (%N*som*) was consistently lower by 4 % ($p < 0.1$) under elevated pCO_2. At high N, however, there was a statistically significant positive response of %N*fix* to elevated pCO_2 ($p < 0.1$ in both years, averaged over the sward types) only in 1993 and 1994 (Figures 3.1b and 3.2b). The proportion of N*som* did not change significantly under elevated pCO_2 in these years. In the following seasons, the positive effect of elevated pCO_2 on %N*fix* declined in both sward types, while %N*som* increased under ele-

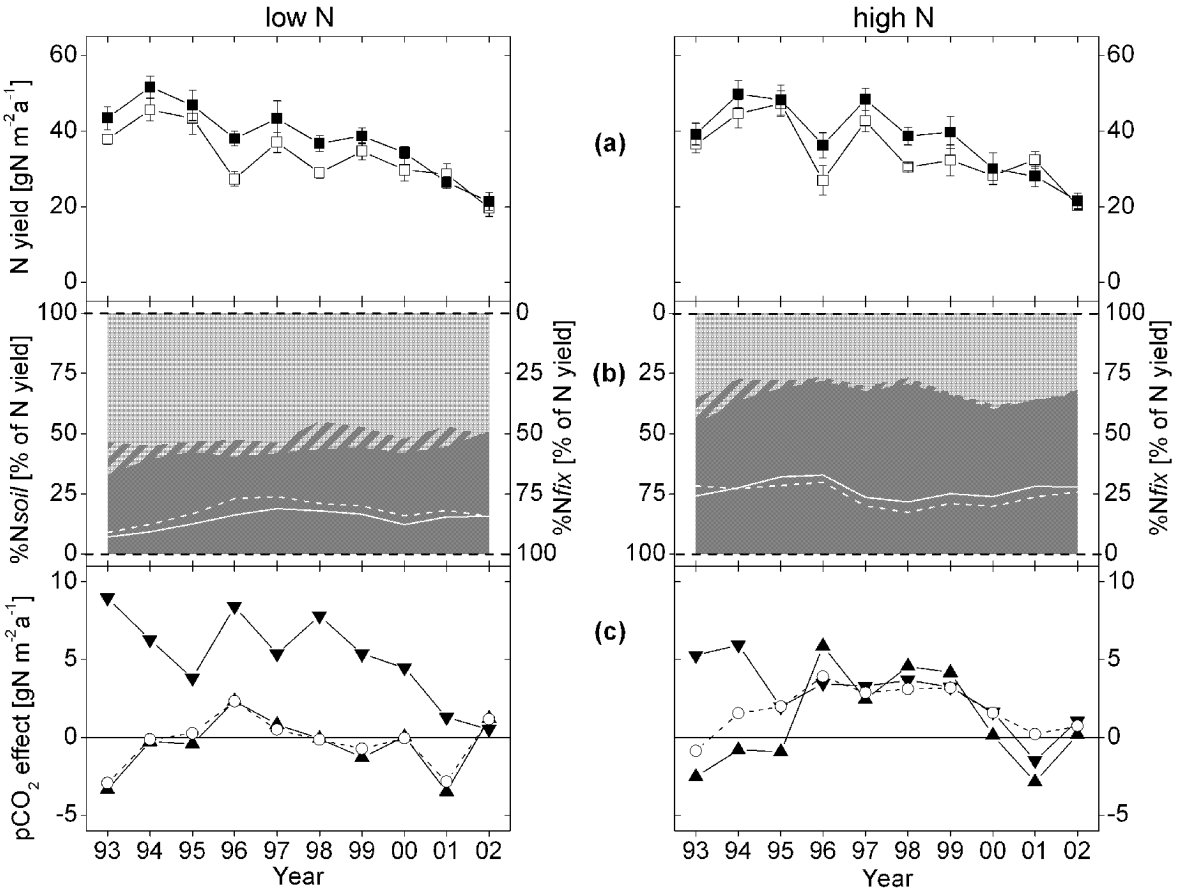

Figure 3.1: Annual harvested above ground N (N yield) (a), percentage of N sources (%N*fix*, %N*soil*, %N*som*) (b), and absolute pCO_2 response separated by the N sources (c) of *T. repens* monoculture swards at ambient and elevated pCO_2 in combination with two nitrogen fertilisation levels from 1993 to 2002. (a) CO_2 treatments: □ = 36 Pa; ■ = 60 Pa. Error bars are standard error of the means, n = 6. (b): light grey = fixed N at 36 Pa, with hatching at 60 Pa; dark grey = soil derived N (%N*soil*) at 60 Pa, with hatching at 36 Pa; white lines represent soil derived N without fertiliser (%N*som*), dotted = 36 Pa; straight = 60 Pa. (c) N sources: ▼ = N*fix*; ▲ = N*soil*; ○ = N*som*.

vated pCO_2 in both sward types ($p < 0.1$) by an average of 5 % from 1996 (Figures 3.1b and 3.2b). As a consequence thereof, the pCO_2-induced increase in the N yield in the low N treatments of *Trifolium* was solely derived from symbiotic N_2 fixation ($p < 0.05$) during the whole experimental period (Figure 3.1c). The trend in the mixture was similar, but there was a tendency to a greater contribution of soil-derived N (N*soil*) after the third year (Figures 3.1 and 3.2). In the high-N treatments, the higher N yield under elevated pCO_2 was solely due to symbiotic N_2 fixation only in the first two years. Thereafter, the change in the N yield under elevated pCO_2

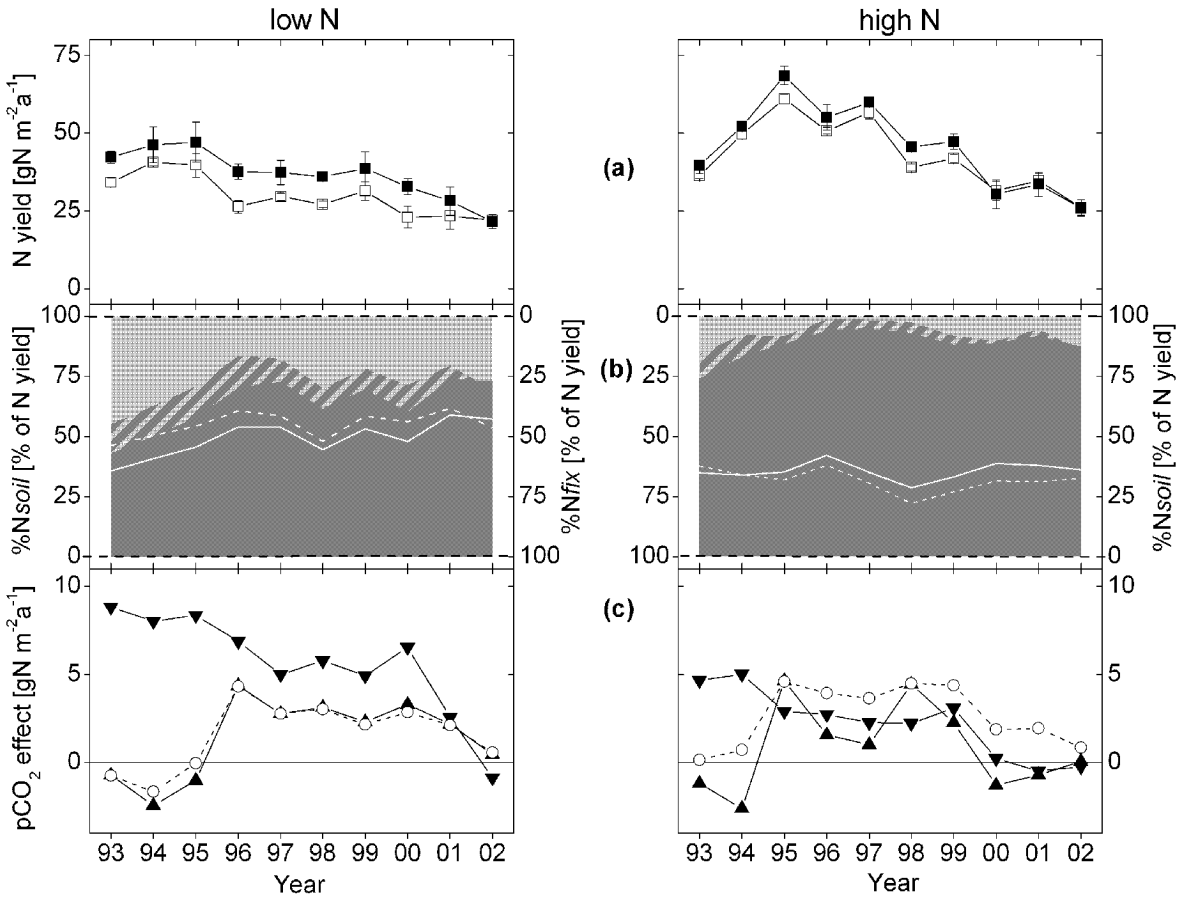

Figure 3.2: Annual harvested above ground N yield (N yield) (a), percentage of N sources (%N*fix*, %N*soil*, %N*som*) (b), and absolute pCO_2 response separated by the N sources (c) of *T. repens/L. perenne* mixed swards at ambient and elevated pCO_2 in combination with two nitrogen fertilisation levels from 1993 to 2002. For details see Figure 3.1.

was a result of changes in N derived from symbiosis and from the soil (N*soil*, Figures 3.1c and 3.2c).

High N fertilisation induced a 26 % increase of the N yield ($p < 0.0001$) in the mixed swards, whereas in the *Trifolium* monocultures no changes were detected. In each growing season high N fertilisation caused a significantly lower %N*fix* in both sward types ($p < 0.0001$). This decrease was about 40 % in the *T. repens* monoculture and about 70 % in the mixed swards, averaged over all the growing seasons and pCO_2 treatments. High N fertilisation resulted in a significant increase from 25 % at low N to 36 % at high N ($p < 0.0001$, averaged over all years and both pCO_2 treatments) in the annual proportion of N in harvested above-ground plant yield derived from the mineralisation of unlabelled SOM (%N*som*) in the *Trifolium* swards

Table 3.1: Total contents of soil N, amount of fertiliser N and fraction of fertiliser N (f_N) in the top 25 cm of soil sampled in December 2001 from *T. repens* and *T. repens*/*L. perenne* swards grown under ambient and elevated pCO_2 in combination with two N fertilisation treatments. Indication of significance are given.

Sward	N fertilisat.	pCO_2 [Pa]	Total soil N [g m^{-2}]	Fertiliser N [g m^{-2}]	f_N [% of soil N]
Trifolium	low	36	713	18.1	2.54
		60	817	21.0	2.57
	high	36	679	47.3	6.97
		60	732	53.3	7.28
Lp/*Tr*-Mix	low	36	658	20.8	3.16
		60	756	19.5	2.58
	high	36	717	55.1	7.69
		60	738	53.2	7.21
SE			*59.7*	*5.14*	*0.59*
Source of variance					
CO_2			0.204	0.634	0.658
N			0.484	< 0.001	< 0.001
Sward			0.426	0.526	0.409
$CO_2 \times$ N			0.777	0.681	0.937
$CO_2 \times$ Sward			0.211	0.442	0.576
N $\times$ Sward			0.335	0.626	0.783
$CO_2 \times$ N $\times$ Sward			0.864	0.733	0.955

SE: Standard error of the means (n = 3).

(Figure 3.1b). In the mixed swards, %N*som* significantly ($p < 0.0001$) decreased from 52 % to 33 % (Figure 3.2b).

3.3.2 Fertiliser and total soil N and annual net N mineralisation

At the end of the 2001 growing season, the total N content in the top 25 cm of the soil did not differ significantly for the sward types and the N and pCO_2 treatments (Table 3.1). The fraction of fertiliser N (f_N) in the soil was not influenced by elevated pCO_2 and sward type. However, at high N fertilisation, f_N increased by a factor of 2.5 compared to the low-N treatments ($p < 0.0001$).

Net N mineralisation in the eighth year of fumigation (2000) ranged from 19 to 33 g N m^{-2} (Figure 3.3). Differences between the treatments were not significant due to the considerable variation during the growing seasons, as well as variation among replicates. However, while there was a strong tendency for the annual net mineralisation of N to be greater under elevated pCO_2 at high N fertilisation (35 % in the *T. repens* swards and and 26 % in the *T. repens/L. perenne* mixtures), there was not a clear tendency in the changes in net N mineralisation under elevated pCO_2 in the low-N treatment. This differences in the pCO_2 response between the N treatments ($pCO_2 \times$ N interaction) was statistically significant at $p < 0.1$.

3.4 Discussion

3.4.1 The pCO_2 response of symbiotic N_2 fixation declined due to increased availability of soil N

The effect of elevated pCO_2 on symbiotic N_2 fixation (N*fix*) decreased significantly in the high N treatment in both sward types and in the low N treatment mixed swards during the experimental period (Figures 3.1b and 3.2b). The higher yield of N under elevated pCO_2 was derived solely from symbiotic N_2 fixation in the first two to three years only. Thereafter, the changes in the N yield under elevated pCO_2 was a result of changes in N derived from symbiosis and from the soil (N*soil*) (Figures 3.1c and 3.2c). The increased symbiotic N_2 fixation under elevated pCO_2 observed in the first three years of the experiment (Zanetti *et al.*, 1996, 1997) as well as in other field studies (Lüscher *et al.*, 2000; Hartwig *et al.*, 2002) was generally interpreted as a result of an greater demand of the plant for N caused by the increase in the production of plant biomass. Under conditions of a continuous supply of mineral N, the proportion of symbiotic N_2 fixation to N yield did not change, and thus N_2 fixation and the assimilation of mineral N contributed equally to the increase in N yield under elevated pCO_2 (Zanetti *et al.*, 1998; Schortemeyer *et al.*, 1999; Almeida *et al.*, 2000). In N-limited ecosystems, however, the C/N balance under elevated pCO_2 would be maintained by an increase in N_2 fixation according to the concept of the N demand-driven regulation of symbiotic N_2 fixation (Hartwig, 1998). The dependence of symbiotic N_2 fixation on the supply of soil N was confirmed in the present study: 1) The annual proportion of symbiotically derived N

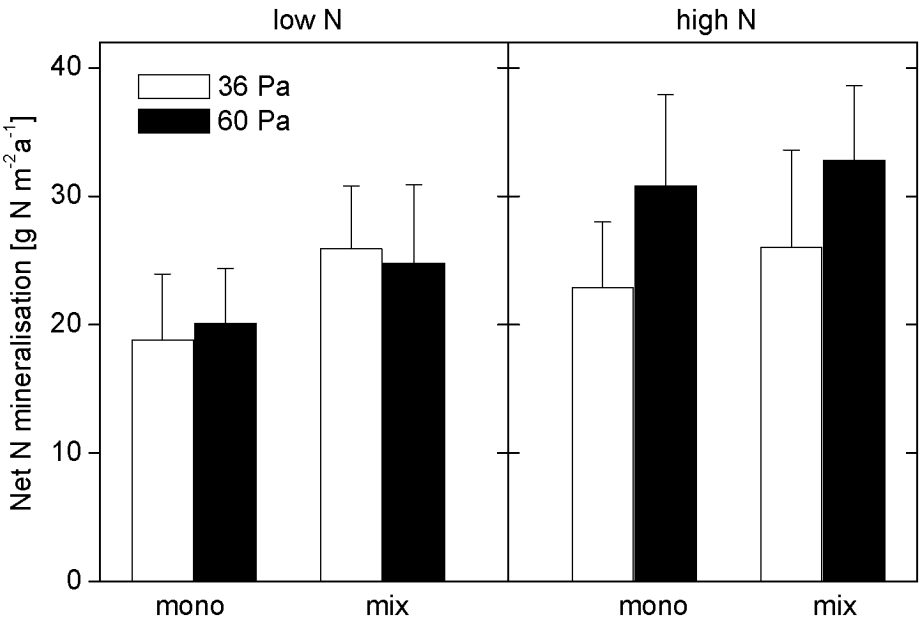

Figure 3.3: Annual net N mineralisation (0–10 cm topsoil) in *T. repens* monocultures and *T. repens*/*L. perenne* mixed swards in the season 2000. Swards were grown at two pCO_2 and two N fertilisation levels. Vertical bars are the standard errors of the mean (n = 3).

in the N yield (%N*fix*) was significantly higher ($p < 0.0001$) in the low N treatment of both sward types (Figures 3.1b and 3.2b) and 2) there was significantly higher ($p < 0.0001$) annual %N*fix* in the harvested above-ground biomass of *T. repens* in the mixture (72 % in the low-N and 40 % in the high-N treatment) compared to *T. repens* grown in monoculture (54 % in the low-N and 33 % in the high-N treatment), showing limited N in the soil due to a non-fixing crop competing for mineral N.

The decreasing effect of elevated pCO_2 on %N*fix* and the concomitant increasing pCO_2 effect on the proportion of N derived from SOM (%N*som*) (Figures 3.1b and Figure 3.2b) over the ten years indicates a decreasing limitation of mineral N in the high-N treatment under elevated pCO_2. This may be due to the less vigorous growth of these swards during the last three years, which is indicated by lower N yield (Figures 3.1a and 3.2a). Moreover, more soil N may have been available under elevated pCO_2. This is indicated by the stronger positive pCO_2 effect on the proportion of N in the yield that was not derived from the fertiliser (%N*som*) (Figures 3.1b and 3.2b). Although the increase in the annual net mineralisation of N under elevated pCO_2 at high N was not statistically significant in the year 2000, the extent of this increase (30 % averaged over both swards) as well as the

low probability value ($p = 0.12$) may imply an increased availability of N from mineralisation.

It has been suggested that the plants tended to acquire mineral N to meet the greater demand for N under elevated pCO_2. The cost of N_2 fixation in units of g C per g N is theoretically 2.5 (Warembourg and Roumet, 1989), but measured values ranged up to 7.5 (Heichel, 1985). In contrast, the energy required to convert NO_3^- to NH_4^+ is 1.75 unit of C per unit of N (Marschner, 1995). The mechanisms by which pCO_2 induces long-term alterations in the %N*fix* in the high-N treatments were caused, remains unclear. The doubled population of *R. leguninosarum* bv. *trifolii* (the symbiont of *T. repens*) after one year of CO_2 fumigation (Schortemeyer *et al.*, 1996), may have become smaller under elevated pCO_2 or the genetic differences and the superior competitive ability of the *Rhizobium* strains, as a result of elevated pCO_2 (Montealegre *et al.*, 2000) may have decreased or become weaker, respectively. Therefore, more experiments are needed to provide greater insight into the long-term dynamics of the microbial community under elevated pCO_2, especially with respect to the rhizosphere of plants, which fix N_2.

The consistent strong effect of elevated pCO_2 on %N*fix* in the low-N treatments, however, strongly indicates that the availability of soil N to plant plays the strongest role in the long-term trend. It is well established that a large supply of nitrate decreases nodule initiation and growth, and nitrogenase activity (Kaiser *et al.*, 1997; Blumenthal *et al.*, 1997; Fujikake *et al.*, 2002). Nitrate inhibition and regulation by N feedback mechanisms (Parsons *et al.*, 1993) have been proposed to explain the dependence of N_2 fixation on N availability and demand. While the permeability of the nodule to oxygen is probably the direct regulator of these processes (Vessey and Waterer, 1992; Kaiser *et al.*, 1997), the molecular mechanisms are still not understood. Bacanamwo and Harper (1997) found that the nitrogenase activity was inhibited after treatment with NO_3^-. This was associated with an increase in the concentrations of asparagine and aspartate/glutamate in the shoot and nodules, respectively. They suggested a feedback control of nodule activity by sensing asparagine and products of its metabolism in the nodule, which was first proposed by Parsons *et al.* (1993). There is further experimental evidence, which proves that high concentrations of reduced N (amino acids, amides) in the phloem sap may hinder the growth and activity of nodules. Increased concentrations of ureide (Serraj *et al.*, 2001) under drought stress and asparagine, glutamate and aspartate (Hartwig

and Trommler, 2001) after defoliation were observed. Drought stress as well as defoliation are known to inhibit symbiotic N_2 fixation. As the NO_3^- inhibition of N_2 fixation, the N feedback mechanism is probably triggered by the availability of O_2 in the nodule. However, the molecular mechanisms are still unclear and need further investigation.

3.4.2 What caused the changes in N dynamics under elevated pCO_2 in the high-N input system?

After the initial two to three years, greater amounts and a higher proportion of N*som* were found under elevated pCO_2 at high N fertilisation (Figures 3.1 and 3.2), while at low N such changes were not observed. N*som* does not represent the total amount of N derived from SOM, because it does not include the fertiliser N, which was incorporated into the SOM and then re-mineralised and taken up by the plant later on. Thus, the amount of N derived from SOM may be underestimated by the proportion of fertiliser N in the SOM (f_N) which was 2.5 to 3.2 % at low N and 6.9 to 7.7 % high N (Table 3.1). However, there were no significant differences between the pCO_2 treatments. Therefore, this potential underestimation is probably irrelevant for the observed long-term trend of the pCO_2-response of N*som*. Sources of unlabelled N, other than through the mineralisation of SOM-N and symbiotic N_2 fixation (lateral influx of unlabelled fertiliser, N from the surrounding plot, dry/wet N-deposition), did not depend on the treatment and were, thus, insignificant for the long-term trends. Therefore, the increasing proportion of %N*som* under elevated pCO_2 at high N may be the result of: 1) increasing N sinks for fertiliser N other than the harvested biomass, i. e. sequestration into the SOM and gaseous and aqueous losses, and 2) the increasing availability of N derived from the mineralisation of SOM. The unchanged fraction of fertiliser N (f_N) in the soil under elevated pCO_2 after the season in 2001 (Table 3.1) indicates that there was no substantial change in the sequestration of fertiliser N in the soil. Considerable amounts of nitrate from fertiliser may have been leached from the swards; the annual precipitation at the experimental site is relatively high ($1100\,mm\,year^{-1}$). (Jarvis, 2000) reported up to a $35\,g\,a^{-1}$ loss of N from temperate grassland, which was usually highest in legume and legume-grass swards. However, leaching from grasslands occurs most frequently during the winter when the demand for N is low or non-existent (Whitehead, 1995a). Leaching is associated also with heavy rainfall following the fertiliser application in spring

(Barraclough *et al.*, 1983), as was the case in the years 2000 and 2001 in the present experiment. However, these events, which occurred sporadically, are assumed to be of little significance in the long run when comparing the treatments. Gaseous losses of N often also play an important role in the N budget of ecosystems (Sheehy, 1987). Ineson *et al.* (1998) and Baggs *et al.* (2003b) found that annual N losses through N_2O emissions increased under elevated pCO_2 in the high-N mixed swards, and this was most evident right after fertilisation. However, no pCO_2-induced changes were found in the *Trifolium* swards. Furthermore, even if the total amount of emitted N_2O-N is assumed to be fertiliser N, it would account for at most 1.5 % of the applied fertiliser N. On the other hand, total denitrification (N_2O and N_2, measured in the *Lolium* swards) accounted for 5 and 17 % of the fertiliser ^{15}N applied in the ambient and elevated pCO_2 treatment, respectively (Baggs *et al.*, 2003a). However, these values were obtained right after fertilisation, which was followed by heavy rainfall; conditions, which favour denitrification. Variations in environmental factors such as temperature and rainfall right after fertilisation may have led to a lower net input of fertiliser N and, consequently, to a decreased availability of fertiliser N to the plants. However, in the present study, similar amounts of fertiliser N were recovered in the harvested biomass in both pCO_2 treatments (data not shown), which implies that there was no change in the availability of fertiliser N to the plants. Even if there were slightly greater losses of fertiliser N under elevated pCO_2, they may explain just in part the graeter fraction of N derived from SOM (%N*som*) in the plant yield but do not explain the weaker pCO_2 response of symbiotic N_2 fixation in the last six years.

Increased translocation of C and N to the roots, stubble and stolons occurred under elevated pCO_2 (Pregitzer *et al.*, 1995; Hebeisen *et al.*, 1997; Fitter *et al.*, 1997; Suter *et al.*, 2002). This was also the case with legumes but to a lesser extent than in pure grass swards (Zanetti *et al.*, 1996; Hebeisen *et al.*, 1997). A greater root biomass together with increased root branching (Zimmermann, pers. com.) may explore a greater soil volume and, consequently, acquire more N derived from mineralisation.

The increase in the residual biomass under elevated pCO_2 represents a greater net input of N into the system as a result of symbiotic N_2 fixation and to a lesser extent by fertiliser N. However, the size of these 'residual biomass' pools is apparently approaching a new equilibrium. The effect of elevated pCO_2 on the root biomass was observed to reach a maximum already after the second growing season under elevated pCO_2 (Hebeisen *et al.*,

1997). A continuous pCO_2-induced stimulation of the N input may result in the increased net sequestration of N into stable SOM pools or a greater amount of N being available for plant growth due to the accelerated turnover of labile SOM pools. Van Kessel *et al.* (2000) using a $\delta^{13}C$ tracer technique found that the sequestration of new C (and N) into the stable SOM pool in the same swards was very low and that the input and output of C in and from the labile SOM pool reached steady-state after the first three years. It is, therefore, suggested that, as a consequence of the increasing supply of N from the soil under elevated pCO_2, the N input through symbiotic N_2 fixation decreased, as discussed above. Hence the whole system approached equilibrium again. The increase in the N yield under elevated pCO_2, derived from the soil was mainly derived from unlabelled sources (Figures 3.1c and 3.2c). It is assumed that a considerable amount of this N was introduced previously by N_2 fixation. However, our approach did not enable us to separate the previously fixed N from N that was in the SOM pool before the experiment began. The tendency for an increase in the net N mineralisation under elevated pCO_2 at high N (Figure 3.3) may indicate a greater availability of mineral N under pCO_2, though with a low statistical power.

At low N, the proportion of symbiotically derived N in the plant yield was higher under elevated pCO_2 over the ten years. Moreover, the contribution of N derived from soil to the increase in the N yield was much smaller. This indicates that these systems had a limited supply of mineral N, leading to a consistently high demand for symbiotically fixed N_2. A possible explanation may be the competition between soil microbes and plants for available N. There is evidence that the microbial biomass increases in the rhizosphere under elevated pCO_2 (Zak *et al.*, 1993; Sowerby *et al.*, 2000). This was attributed to an enhanced rhizodeposition and exudation of labile C and N as a substrate for microbial growth. According to the feedback mechanism proposed by Díaz *et al.* (1993) this change in the microbial population may increase its competition for mineral N (Hodge *et al.*, 2000), resulting in a decrease in the availability of soil N. High rates of mineral N fertilisation may compensate for this effect, which could explain the greater availability of mineral soil N (%N*som*) for plant growth in the high-N treatments in our experiment as well as the consistent limitation at low N. At low N supply, the annual rate of net N mineralisation did not differ between the pCO_2 treatments (Figure 3.3), which may be due to the proportionally greater microbial immobilisation of N. The contribution of soil-derived N to the greater N yield under elevated pCO_2 tended to increase, particularly

in the mixed swards. However, due to the smaller input of fertiliser N it may take much longer to reach equilibrium in the respective N pools than it does at high N. Further studies should therefore focus on the long term effects of elevated pCO_2 in low-N input systems.

3.4.3 Conclusions

The response of symbiotic N_2 fixation to a step-increase in atmospheric pCO_2 changed markedly over the years in the system with high inputs of fertiliser N. The transient increase in the N yield under elevated pCO_2 resulted from an increase in symbiotic N_2 fixation in the first two to three years of CO_2 enrichment; in the following years the increase in the N yield was derived from both N_2 fixation and the mineralisation of SOM-N. We suggest that a temporary immobilisation of N in the first years under elevated pCO_2 led to an apparent input of N into the system. With the greater availability of soil N, caused by the re-mineralisation of this pool and the high input of fertiliser N, the plant may have tended to acquire mineral N, which uses less energy. In contrast, at low N supply, the system was continuously N-limited and N_2 fixation remained markedly higher under elevated pCO_2.

4 Ten years nitrogen budget in grassland under elevated pCO_2

Abstract Ecosystem N budgets were established for ten years under ambient and 60 Pa pCO_2 at two levels of N fertilisation (140 and 560 kg N $ha^{-1}a^{-1}$) on *Trifolium repens*, *Lolium perenne* and bi-species mixed swards in the Swiss FACE (Free Air Carbon Dioxide Enrichment) experiment. Elevated pCO_2 decreased the annual N yield of *L. perenne* (−16 %) at low N while at high N the N yield did not change significantly. In the *T. repens* and mixed swards elevated pCO_2 induced an increase in the N yield from 8 % (low N mixed swards) to 23 % (high N mixed swards). At low N all the additionally assimilated N under elevated pCO_2 in the plant was the result of symbiotic N_2 fixation. At high N this was the case only when previously fixed N, which was recovered from the soil, was found in the subsequent yields. This fraction has to be taken into account to avoid underestimating the input of N into the ecosystem when assessing the N budget. The higher input of N by symbiotic N_2 fixation (97 to 217 kg N $ha^{-1}a^{-1}$) into the ecosystems with legumes was partly counterbalanced by the greater losses of fertiliser N from these systems (32 to 58 % of applied) compared to the loss of fertiliser N (14 to 26 %) and the higher yield of the pure grass stands. Despite the uncertainty with regard to the quantification of the gaseous and aqueous loss of N, our data indicate that there was no net input of N into the low-N systems; a potential temporary net input into the high-N systems is feasible. Elevated pCO_2 did not significantly change the input/output balance of N in the ecosystem, which implies that there were no pCO_2-induced changes in the total loss of N or net N sequestration.

Abbreviations

Partial pressure of atmospheric CO_2 (pCO_2), Soil organic matter (SOM), Free Air CO_2 Enrichment (FACE), dry matter (DM), N concentration in the plant biomass ([N]), fixed N which was harvested directly in the above-ground N ($Nfix_d$), $Nfix_d$ plus the fixed N that was transferred to the soil and recovered in subsequent yields ($Nfix_t$), N from mineralisation of unlabelled soil organic matter ($Nsom$) and from fertiliser N ($Nfert$)

4.1 Introduction

It is predicted that the continuous increase in the atmospheric CO_2 partial pressure (pCO_2) will stimulate primary production and lead to greater input of carbon (C) into the soil (van Veen *et al.*, 1991; Drake *et al.*, 1997). The potentially positive plant response under elevated pCO_2 would lead to a greater demand for N. Low N availability in particular is the limiting factor in many terrestrial ecosystems (Vitousek and Howarth, 1991) and, consequently, if the availability of N does not increase, the plant response to elevated pCO_2 will remain limited. Previous studies under field conditions showed species-specific responses of the production and allocation of biomass to elevated pCO_2, which were clearly controlled the interactions of the availability of and demand for N. These interactions resulted in a greater allocation of C and N to the roots (Hebeisen *et al.*, 1997; Suter *et al.*, 2002; Hartwig *et al.*, 2002) with a negative or unchanged response of aboveground biomass. In systems with legumes, a substantial increase in the proportion of N derived from symbiotic N_2 fixation was detected; this increase was consistent with a simultaneous increase in the above ground biomass of clover (Soussana and Hartwig, 1996; Zanetti *et al.*, 1996; Hartwig *et al.*, 2002) and lucerne (Lüscher *et al.*, 2000). These alterations in the allocation of C and N, in the C/N ratio of the plant tissue and in the litter may affect the composition and size of the soil microbial community (Schortemeyer *et al.*, 1996; Marilley *et al.*, 1999; Montealegre *et al.*, 2000) and, thus, the immobilisation/decomposition of SOM. These changes may have the potential to alter sink/source relations of the ecosystem and, consequently, the sequestration of C and N into the soil. In theory, the calculation of N import and export parameters of the N budget of the ecosystem enables the quantification of potential net N sequestration or N loss from the system. However, it is difficult to measure some of these parameters, especially, gaseous losses and leaching. Consequently, the calculated balance is often a rough estimate.

After the first six years of the Swiss FACE experiment, Daepp *et al.* (2000) reported that the N balance was in equilibrium in the *Lolium* swards under low N fertilisation, while it was slightly positive at high N supply. They assumed that a net input of N into the ecosystem at high N provided the N to overcome the initial N-limited response of yield to elevated pCO_2. However, atmospheric inputs and losses of N from the system were not discussed. Hartwig *et al.* (2002) found that N was retained by the soil (without pCO_2 induced changes) as a result of the fertiliser N in *L. perenne*

and symbiotic N_2 fixation in *T. repens* swards. However, their experiment was conducted for four years in model ecosystems; plants were grown in soil that had been re-packed in boxes. The associated confounding variable by soil disturbance and inside-edge effects may have led to conditions, which did not reflect the actual situation in the field. Therefore, studies in less disturbed soils, which may have reached equilibrium of the soil organic matter, are necessary.

This study was conducted to investigate the effect of elevated pCO_2 for ten years on white clover, perennial ryegrass and bi-species mixed swards in the Swiss FACE experiment. By use of ^{15}N tracer technique, the significance of the different N sources (mineral fertiliser, mineralisation of soil organic matter and symbiotic N_2 fixation) for plant growth were quantified. Likewise, the pathway of applied ^{15}N-labelled fertiliser N was monitored; the fertiliser N in the harvested plant tissue, sequestration of N into the soil and the total loss of fertiliser N were determined. The input and output of N in the ten years of the Swiss FACE experiment were quantified and the N budgets of the ecosystems were estimated. The parameters of N output which were not measured (nitrate leaching, ammonia volatilisation) or were quantified for limited periods only (gaseous losses through nitrification/denitrification), will be discussed with regard to their significance for the N budget.

4.2 Material and Methods

4.2.1 Experimental site and plant material

The FACE technology (Lewin *et al.*, 1994) was used to investigate the long-term effect of elevated pCO_2 on grassland ecosystems in the field. The Swiss FACE experimental site was established at Eschikon, near Zurich, Switzerland (47°27′N and 8°41′E at 550 m above sea level) and consists of three blocks. Each block has two circular areas (18 m diameter); one fumigated with CO_2 (pCO_2 = 60 Pa) and one with ambient (pCO_2 = 36 Pa). The fumigation started on May 31, 1993 and took place in the daytime during the growing season from March to November (1993 to 2002) provided that the air temperature was above 5°C.

The soil was an eutric cambisol (28 % clay, 33 % silt, 36 % sand, 2.9 to 5.1 % organic matter content at the beginning of the experiment in 1993).

The pH was 6.5 to 7.5. Phosphorus, K and Mg were applied at the beginning and around the middle of June in each growing season to similarly maintain the availability of nutrients in the blocks (Zanetti *et al.*, 1996; Hebeisen *et al.*, 1997). In mid-August 1992, *Lolium perenne* and *Trifolium repens* were sown in monoculture and bi-species plots (2.8 × 1.9 m). Swards were cut (5 cm above the soil surface) in mid-April and mid-May before CO_2 fumigation on 31 May 1993. Each year the plots were protected from *Fusarium* and clover rot as described detailed in Zanetti *et al.* (1996) and Hebeisen *et al.* (1997).

4.2.2 Treatments

In the first three years of the experiment there were two defoliation regimes: infrequent (4 cuts per year) and frequent (7 cuts in 1993 and 8 cuts in 1994 and 1995). From 1996 to 2001 all the swards were harvested five times a year. The cutting height was always ≈ 5 cm above the soil surface.

Nitrogen fertiliser was applied as follow: 10 g m^{-2} (1993) and 14 g m^{-2} (1994–2001) in the low-N plots and 42 g m^{-2} (1993) and 56 g m^{-2} (1994–2001) in the high-N plots. The N was in an NH_4NO_3 solution and the amounts applied were adjusted to the expected biomass yield in the respective re-growth (Zanetti *et al.*, 1996). The sampling area of each plot (1 m^{-2}) was treated with ^{15}N-enriched fertiliser in the same way. Nitrate and ammonia were each labelled with a ^{15}N atom %-excess of 0.4 % (1993–1994) and 0.3 % (1995–2002) in the low N treatment and 1.6 % (1993–1994) and 1.3 % (1995–2002) in the high N treatment.

The atmospheric pCO_2 was ambient (36 Pa) or elevated (60 Pa). This results in a total of 24 treatments from 1993 to 1995 with three sward types (*L. perenne*, *T. repens*, *L perenne*/*T. repens* mixture), two N treatments (low N, high N) two pCO_2 levels (ambient and elevated) and two defoliation frequencies (frequent, infrequent) and 12 treatments from 1996 to 2001 (defoliation was not varied).

4.2.3 Sampling of plant material

The harvested plant material from the inner part (0.25 m^2) of the sampling area was separated into *L. perenne*, *T. repens* and unsown species and dried at 65°C for 48 h and then ground to a fine powder according the procedure as described in Zanetti *et al.* (1996). Analyses to determine N and

^{15}N concentrations were done using a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK) at Stable Isotope Facility (University of California, Davis, USA).

4.2.4 Determination of N parameters

The amount of N in the harvested above-ground plant material (N*yield*) was derived from symbiotic N_2 fixation (Nfix_d), fertiliser (N*fert*) and mineralisation of the unlabelled soil organic matter (N*som*).

$$\mathrm{N}yield = \mathrm{N}fix_\mathrm{d} + \mathrm{N}fert + \mathrm{N}som \tag{4.1}$$

These parameters were calculated for each re-growth period and then added together.

The amount of symbiotically fixed N in N*yield* of *T. repens* (Nfix_d) was calculated according to the standard ^{15}N isotopic dilution protocol (McAuliffe *et al.*, 1958):

$$\mathrm{N}fix_\mathrm{d} = \left(1 - \frac{^{15}\mathrm{N\,atom\,\%\,excess_{clover}}}{^{15}\mathrm{N\,atom\,\%\,excess_{reference\,plant}}}\right) \times \mathrm{N}yield \tag{4.2}$$

L. perenne grown in the mixture of the appropriate treatment served as the reference plant for calculating the N, which was fixed and directly harvested in the above-ground N in (Nfix_d) the plants (Boller and Nösberger, 1988). To calculate the total amount of symbiotically fixed N, including the fixed N that was transferred below ground and recovered in subsequent yields (Nfix_t), *L. perenne* in monocultures was the reference. In the mixed swards, Nfix_t included the apparent transfer of N from the legumes to non legumes (N*trans*), i. e. the percentage of N in the N yield of the non-fixing grass originated from the N_2 fixation of the legume.

$$\mathrm{N}fix_\mathrm{t}\ (\mathrm{mix\,total}) = \mathrm{N}fix_\mathrm{t}\ (\mathrm{mix\,clover}) + \mathrm{N}trans \tag{4.3}$$

N*trans* was calculated according to Vallis *et al.* (1977):

$$\mathrm{N}trans = \left(1 - \frac{^{15}\mathrm{N\,atom\,\%\,excess_{grass\,mono}}}{^{15}\mathrm{N\,atom\,\%\,excess_{grass\,mix}}}\right) \times \mathrm{N}yield \tag{4.4}$$

N*yield* represents here the total N yield of *L. perenne* in the mixed plots.

The amount of N in the plant material derived from fertiliser (N*fert*) was calculated using the ^{15}Natom%-excess of the plant material and the fertiliser.

$$\mathrm{N}fert = \left(\frac{^{15}\mathrm{N\,atom\,\%\,excess_{plant}}}{^{15}\mathrm{N\,atom\,\%\,excess_{fertiliser}}}\right) \times \mathrm{N}yield \tag{4.5}$$

N*yield* stands for the total harvestable N yield of the respective sward.

The amount of N in the harvested N, that was yield derived from unlabelled soil N (N*som*), was calculated according to Equation 4.6.

$$\mathrm{N}som = \mathrm{N}yield - \mathrm{N}fert - \mathrm{N}fix_{\mathrm{d}}, \quad (\mathrm{N}fix_{\mathrm{d}} \text{ for } L.\,perenne = 0) \quad (4.6)$$

N*som* includes the fixed N that was transferred below ground and yielded later on minus the N that was fixed directly (N$fix_{\mathrm{t-d}}$).

Wet N deposition was assessed by means of rain collectors (Jacot *et al.*, 2000) installed in three of the six FACE rings. Rain water was collected every two weeks, 50 cm above the ground. Sub-samples were taken and analysed colorimetrically for NH_4^+ and NO_3^- with a segment flow analyser (Alliance Evolution II, Alliance Instruments SA, Nanterre, France).

Total contents of soil N and ^{15}N were determined at the end of 2001. Three soil cores (2 cm in diameter) were taken from 0 to 50 cm from each plot and combined according to depth (0–10, 10–25 and 25–50 cm). Fresh samples were sieved through a 2-mm sieve and dried at 50°C for three days. Dry samples were ground to a fine powder and analysed for total N and ^{15}N using a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK) at Stable Isotope Facility (University of California, Davis, USA).

The amount of fertiliser N in the soil (N$fert_{\mathrm{soil}}$) was calculated to according equation 4.7.

$$\mathrm{N}fert_{\mathrm{soil}} = \left(\frac{^{15}\mathrm{N\ atom\,\%\ excess_{soil}}}{^{15}\mathrm{N\ atom\,\%\ excess_{fertiliser}}} \right) \times \mathrm{N}soil \quad (4.7)$$

where N*soil* is the total amount of N in the soil. Due to the change in ^{15}N enrichment of the fertiliser after two years, a weighted average of ^{15}N atom %-excess of the fertiliser was used.

4.3 Results

4.3.1 Annual dry matter yield and N concentration

Elevated pCO_2 induced a 21 % increase in the average annual dry matter yield of the *T. repens* swards (Table 4.1), independent of N fertilisation ($p < 0.1$), whereas in the *L. perenne* swards a significant CO_2 response (18 % increase, $p < 0.01$) was observed only in the high-N treatment. The total

Table 4.1: Average annual above ground dry matter (DM) yield and N concentration [N] in *L. perenne*, *T. repens* and bi-species mixed swards grown at ambient and elevated (60 Pa) atmospheric pCO_2 and two levels of N fertilisation (140 and 560 kg N $ha^{-1}a^{-1}$) from 1993 to 2002.

Sward	N-fertilis.	pCO_2 [Pa]	*T. repens* [%]	DM yield [t ha^{-1}]	[N] [mg N g^{-1}]
Lolium	low	36	-	6.76	21.9
		60	-	6.63	18.7
	high	36	-	12.09	33.2
		60	-	14.23	28.1
Trifolium	low	36	-	8.02	43.1
		60	-	9.70	40.5
	high	36	-	8.00	44.3
		60	-	9.70	40.6
Lp./Tr-Mixture	low	36	27.3	10.24	30.1
		60	38.9	12.61	30.2
	high	36	12.2	12.88	34.3
		60	29.8	14.54	32.5
SE			*4.73*	*0.27*	*0.56*
Source of variance					
Sward			-	<0.001	<0.001
N			0.017	<0.001	<0.001
CO_2			0.161	0.018	0.014
Sward × N			-	<0.001	<0.001
Sward × CO_2			-	0.048	<0.001
N × CO_2			0.669	0.113	0.025
Sward × N × CO_2			-	0.003	0.447

Standard error of the mean (*SE*, n = 3) and indication of significance are given.

herbage of the mixed swards increased under elevated pCO_2 ($p < 0.05$) by 23 % (low N) and 13 % (high N). The harvested biomass of the *L. perenne* and mixed swards increased under high N ($p < 0.0001$) at both levels of pCO_2, while *T. repens* did not respond to N fertilisation.

[N] of the harvested biomass was lower under elevated pCO_2 in the *L. perenne* (−15 %, $p < 0.05$) and *T. repens* monoculture swards (−6 % and −8 % at low and high N, respectively, $p < 0.01$). In the mixture, [N] of

the total herbage was not significantly affected by elevated pCO_2. High N fertilisation resulted in a strong increase (factor 1.5) in the N concentration of *L. perenne* ($p < 0.0001$), while in the mixed swards the increase in [N] was 10 % ($p < 0.05$). [N] of the *T. repens* plant material showed no significant differences between the N treatments. In general, [N] of the *T. repens* material was higher by a factor of 1.5 and 2 at high and low N, respectively, than in *L. perenne* ($p < 0.0001$) averaged over all sward types.

4.3.2 Nitrogen yield and N sources of harvested plant material

The average annual N yield of the harvested above-ground biomass (N*yield*) of *L. perenne* was 16 % lower under elevated pCO_2 at low N supply ($p < 0.1$) but was not affected at high-N fertilisation (Table 4.2). In the *T. repens* and bi-species mixture swards the N yield was higher under elevated pCO_2; however, this was statistically significant only in the low-N treatment ($p < 0.1$). High N fertilisation strongly increased the average annual N yield of the *L. perenne* swards by a factor of 3 ($p < 0.0001$), while the N supply had no influence on the N yield of *T. repens*. In the mixed swards, high N fertilisation enhanced the annual N yield as well but only by a factor of 1.3 ($p < 0.0001$).

Elevated pCO_2 did not significantly affect the amounts of N derived from unlabelled soil (N*som*) in the above-ground yield of *L. perenne*; significantly less fertiliser N (N*fert*) at low N (-18 %) was found, however, in the N yield at elevated pCO_2 ($p < 0.1$). High N fertilisation doubled the fraction of the total N yield that originated from the fertiliser ($p < 0.0001$).

A considerable portion of the N yield of the *T. repens* and mixed swards originated from symbiotic N_2 fixation (Nfix_d), leading to lower contribution of soil and fertiliser N to the total annual N yield. The amount of symbiotically fixed N increased under elevated pCO_2 in both N treatments ($p < 0.1$). High N supply substantially lowered the amount of fixed N in the yield of the *T. repens* and mixed swards ($p < 0.0001$). The amount N derived from the soil did not change under elevated pCO_2 in the low-N treatment but was significantly greater in the high-N treatment ($p < 0.1$). This was due mainly to the positive pCO_2-response of the fraction of soil N, which originated from previous N_2 fixation. When this fraction was subtracted from the total N*yield* the amount of soil-derived N was unaffected by response to elevated pCO_2 at high N.

Table 4.2: Average annual N budget for harvested above-ground plant biomass, separated into N from fertiliser (N*fert*), N from soil (N*som*), N from N_2 fixation ($N fix_d$), N from fixation, which was immobilised and re-mineralised later on ($N fix_{t-d}$) and N yield (N*yield*) in *L. perenne*, *T. repens* and bi-species mixed swards. Swards were grown at ambient and elevated (60 Pa) atmospheric pCO_2 and two levels of N fertilisation (140 and 560 kg N $ha^{-1}a^{-1}$) from 1993 to 2002.

Sward	N fertilis.	pCO_2 [Pa]	N*fert*	N*som*	$N fix_{t-d}$	$N fix_d$	N yield
			[kg N ha^{-1} a^{-1}]				
Lolium	low	36	46	102	-	-	148
		60	38	86	-	-	124
	high	36	287	114	-	-	401
		60	271	129	-	-	400
Trifolium	low	36	39	136	46	171	346
		60	37	138	52	218	393
	high	36	161	87	17	106	354
		60	155	109	30	130	394
Lp./Tr	low	36	56	171	81	81	308
		60	55	186	92	139	379
	high	36	276	140	71	26	442
		60	255	169	102	49	473
SE			*3.85*	*4.88*	*6.15*	*13.6*	*11.3*
Source of variance							
Sward			<0.001	<0.001	<0.001	<0.001	<0.001
N			<0.001	0.111	0.012	<0.001	<0.001
CO_2			0.138	0.163	0.152	0.065	0.154
Sward × N			<0.001	<0.001	0.013	0.724	<0.001
Sward × CO_2			0.275	0.111	0.181	0.761	<0.001
N × CO_2			0.022	0.002	0.138	0.031	0.512
Sward × N × CO_2			0.455	0.447	0.490	0.642	0.281

Standard error of the mean (*SE*, n = 3) and indication of significance are given.

4.3.3 Fate of fertiliser N after nine years

Elevated pCO_2 decreased the export of fertiliser N with the harvested material (Table 4.4); this was significant, however, only for *L. perenne* at low N ($p < 0.05$). The total amounts of N, as well as the percentage of fertiliser loss were not affected by pCO_2. In both N treatments, the amount of fertiliser N retained by the soil was greatest in the *L. perenne* and lowest in the *T. repens* system. A substantial amount of fertiliser N was apparently lost during the nine years of the experiment. These losses were highest in both N treatments in the *T. repens* swards and lowest in the *L. perenne* swards. ($p < 0.0001$). The losses were significantly greater in the high-N than in the low-N treatments. ($p < 0.0001$).

4.3.4 Nine-year nitrogen budget and N balance

Symbiotic N_2 fixation accounted for 19 % (high N) and 61 % (low N) of the total N input into the swards containing *T. repens* (Table 4.3). Due to the increase in symbiotic N_2 fixation, the N input was greater under elevated pCO_2. The amount of N that was not accounted for, i. e. the apparent N balance, was significantly ($p < 0.0001$) greater at high N supply and was highest in the *T. repens* system with 332 kg N ha^{-1} a^{-1} (averaged over both pCO_2 treatments). The lowest apparent N balances were found in the mixed swards at low N (2 % of the applied N). Elevated pCO_2 did not have a significant effect on the apparent N balance.

4.4 Discussion

4.4.1 Harvested biomass: response to elevated pCO_2, N fertilisation and significance of symbiotic N_2 fixation for the N budget

The use of ^{15}N-enriched fertiliser enables the determination of the sources of the N in the harvested plant biomass: fertiliser N, N_2 fixation and mineralised SOM-N. Elevated pCO_2 and N fertilisation induced significant interspecific differences in plant growth. The *L. perenne* yield increased at high N fertilisation but showed no response to elevated pCO_2, while the *T. repens* yield was not affected by N fertilisation but increased under elevated pCO_2. These species-dependent responses to N fertilisation and elevated pCO_2 were

also reported by Hebeisen *et al.* (1997) after three years in the same experiment and by Lüscher *et al.* (1998) and Hartwig *et al.* (2002) in additional experiments conducted at the FACE site. The lower nitrogen concentration of the harvested plant tissue in the monocultures under elevated pCO_2 is also well documented (Hartwig *et al.*, 2002; Daepp *et al.*, 2000; Cotrufo *et al.*, 1998). In contrast, [N] of the mixed swards did not change signifi-

Table 4.3: Annual ecosystem N budget and apparent N balance [kg N $ha^{-1}a^{-1}$], averaged over 10 years (1993–2002) in *L. perenne*, *T. repens* and bi-species mixed swards grown at ambient and elevated (60 Pa) atmospheric pCO_2 and two levels of N fertilisation (140 and 560 kg N $ha^{-1}a^{-1}$). Inputs of N: fertiliser, wet deposition (wet dep.) and symbiotically fixed N ($Nfix_t$); outputs: N in the harvested above-ground biomass (N*yield*).

Sward	pCO_2	N input			N output	N balance
	[Pa]	Fertiliser	wet dep.	$Nfix_t$	N yield	
Lolium	36	136	18	-	148	6
	60		18	-	124	30
	36	546	18	-	401	163
	60		18	-	400	164
Trifolium	36	136	18	217	346	25
	60		18	270	393	31
	36	546	18	123	354	333
	60		18	160	394	330
Lp/Tr Mix	36	136	18	162	308	8
	60		18	231	379	6
	36	546	18	97	442	219
	60		18	151	473	242
SE			-	*13.6*	*11.3*	*6.51*
Source of variance						
Sward			-	0.006	<0.001	<0.001
N			-	<0.001	<0.001	<0.001
CO_2			-	0.111	0.154	0.611
Sward × N			-	0.155	<0.001	<0.001
Sward × CO_2			-	0.423	<0.001	0.082
N × CO_2			-	0.450	0.512	0.175
Sward × CO_2 × N			-	0.977	0.280	0.309

Standard error of the mean (*SE*, n = 3) and indication of significance are given.

Table 4.4: Recovery of fertiliser N (kg N ha^{-1}) in the harvested plant biomass (1993–2001) (N*fert*) and in soil and roots (Nov. 2001) (0–50 cm depth) in *L. perenne*, *T. repens* and bi-species mixed swards. Plants were grown at ambient and elevated (60 Pa) pCO_2 and two levels of N fertilisation (140 and 560 kg N $ha^{-1}a^{-1}$).

Sward	N applied	pCO_2	Fertilis. N recovered		Loss of fertiliser N	
	[kg ha^{-1}]	[Pa]	Biomass	Soil	[kg ha^{-1}]	% applied
Lolium	1220	36	429	479	310	26
		60	347	551	320	26
	4900	36	2730	1500	670	14
		60	2540	1230	1130	23
Trifolium	1220	36	311	314	600	49
		60	296	347	580	47
	4900	36	1300	776	2820	58
		60	1250	952	2700	55
Lp/Tr-Mix	1220	36	502	360	360	29
		60	501	327	390	32
	4900	36	2620	960	1320	27
		60	2430	899	1570	32
SE			*53.1*	*125*	*325*	*7.05*
Source of variance						
Sward			<0.001	0.079	<0.001	<0.001
N			<0.001	<0.001	<0.001	0.177
CO_2			0.065	0.923	0.391	0.453
Sward × N			<0.001	0.298	<0.001	0.026
Sward × C			0.299	0.623	0.383	0.478
N × CO_2			0.037	0.678	0.238	0.637
Sward × N × CO_2			0.352	0.534	0.506	0.824

Standard error of the mean (*SE*, n = 3) and indication of significance are given.

cantly at elevated pCO_2. This was attributed to the greater proportion of the N-rich *T. repens* in the mixtures (Table 4.1) and the greater symbiotic N_2 fixation (Table 4.2). The positive interaction between clover and ryegrass seems to ensure a more efficient utilisation of N derived from the soil and from N_2 fixation (Table 4.2), thus leading to the highest DM yields in all the treatments compared to the other sward types (Table 4.1). These

findings are in agreement with the observations reported by Soussana and Arregui (1995), Høgh-Jensen and Schjoerring (1997), Zanetti *et al.* (1997) and Hartwig *et al.* (2000).

In the *L. perenne* swards more soil-derived N was taken up by the plants at high fertilisation, especially at elevated pCO_2, a phenomenon which is referred to as 'added nitrogen interaction' or 'priming effect' (Jenkinson *et al.*, 1985; Kuzyakov *et al.*, 2000). On the one hand, this priming effect may be apparent when there is an exchange of added (fertiliser) ^{15}N with unlabelled soil-N pools, which show no change in the turnover rate. On the other hand, an accelerated mineralisation of soil organic matter may lead to an increase in the availability of mineral N, which is a real priming effect. The present study indicated an increased mobilisation of N from the soil under elevated pCO_2 and high N supply, because the increase in the proportion of N*som* in the harvested plant yield was correlated with an increase in DM and N yield over the ten years of the experiment. (Daepp *et al.*, 2000; Schneider, 2003). The amount of fertiliser-derived N in the harvested plant material was calculated according equation 4.5; it includes the fraction of fertiliser added since 1993, which was temporarily immobilised. Since the fraction of fertiliser N in the soil ranged from 2.5 % to 7.7 % (Table 3.1), the actual uptake of fertiliser N by the plant may be overestimated. Thus, N*som* is therefore underestimated. However, the present study focuses on the N economy, N budget and the fate of the fertiliser applied over the 10 years; thus the calculation of N*fert* and N*som* by means of equations 4.5 and 4.6 is a valid approach.

In the *T. repens* and mixed swards considerable amounts of the N yield were derived from the symbiotic N_2 fixation (Tables 4.2 and 4.3), being higher at low N and elevated pCO_2. An equal label of the soil under the legume and the reference plant (*L. perenne* grown in mixture), which is a prerequisite for quantifying symbiotically fixed N_2 (discussed by Boller and Nösberger, 1988), may not be met entirely in the *T. repens* monoculture. A greater input of symbiotically fixed N in these swards may lead to a greater dilution of the ^{15}N label in the soil. However, in the present experiment the ^{15}N label in the soil differed only marginally between the *T. repens* and the mixed swards (Table 3.1), thus minimising potential sources of error.

Previous field studies showed that the higher N yield under elevated pCO_2 was derived entirely from symbiotically fixed N (Soussana and Hartwig, 1996; Hartwig *et al.*, 2002; Zanetti *et al.*, 1996). In the present study this

was the case only when the total amount of fixed N, i. e. the N that was fixed during the re-growth (Nfix_{d}) plus previously fixed N which was transferred below ground and then found in the yield, was taken into account (Nfix_{t}) (Table 4.3). The directly fixed N (Nfix_{d}) was responsible for the entire N yield increase under elevated pCO_2 but only at low N (Table 4.5) (less evident in the mixed swards) and high N in the first three years of the experiment (Figure 3.1 and 3.2); this implies a change in the significance of the N sources for plant growth in the high-N treatment. As a consequence,

Table 4.5: Absolute pCO_2-response of total N yield (ΔN*yield*) compared to the pCO_2 response of total fixed N (ΔNfix_{t}) and directly fixed N (ΔNfix_{d}) of *T. repens* and *T.repens/L. perenne* mixed swards grown at ambient and elevated pCO_2 with two levels of N fertilisation (140 and 560 kg N $ha^{-1}a^{-1}$).

Sward	N fertiliser	ΔN*yield*	ΔNfix_{t}	Nfix_{d}
		[kg N ha^{-1} a^{-1}]		
Trifolium	low	47	53	47
	high	40	37	24
Lp./Tr Mix	low	71	69	58
	high	31	54	23

the symbiotically fixed N that was transferred below ground must be taken into consideration when calculating the N budget (Table 4.3) to avoid underestimating the N input by symbiotic N_2 fixation and thus misinterpreting the N input/output balance. However, the calculation of symbiotically fixed N by the grass grown in the monoculture is not without problems. In the legume plots, the dilution of the ^{15}N label in the soil was caused not only by an increase in the amount of unlabelled atmospheric N, but also by a greater loss of labelled fertiliser N (Table 4.4). Fixed N (equation 4.4) in *L. perenne* of the monoculture (reference crop) may have originated from mineralisation of SOM that was in the soil before the experiment began in 1993. However, our experimental approach did not enable us to distinguish between these sources of N.

4.4.2 N budget and apparent N balance in the ecosystem

The consistently positive N balance (Table 4.3), at least in the high N systems, implies an apparent net sequestration of N into the ecosystem and/or

N losses from the system. However, N was lost (nitrate leaching, NH_3 volatilisation), for which no data are available. Emissions of N_2O, N_2 and NO were monitored only for limited time intervals. As well as the identification of the N sources for plant growth, the use of ^{15}N-labelled fertiliser enabled an accurate quantification of the amount of fertiliser N lost from the system, a major advantage of the applied experimental approach. The high fractions of fertiliser N not recovered in the plant biomass or the soil (Table 4.4) indicate substantial losses of fertiliser N through the above mentioned processes. The loss of fertiliser N may be overestimated, because the N in the soil below 50 cm was not considered. However, the amount of fertiliser N declined with soil depth and was marginal at 50 cm (data not shown). The fraction of fertiliser N in the soil may also be underestimated due to the lateral influx of fertiliser and unlabelled soil N. Hartwig *et al.* (2002) reported similar losses (up to 71 % in a high-N *T. repens* system) of fertiliser N from model ecosystems in boxes where a lateral substitution of labelled and unlabelled N did not occur. When subtracting these losses of fertiliser N (converted into annual N losses) (Table 4.4) from the N balance (Table 4.3), the N balance is about 53 kg N $ha^{-1}a^{-1}$ at high N (averaged over all swards and the pCO_2 treatments) and is negative at low-N (-30 kg N $ha^{-1}a^{-1}$) with no significant differences between the pCO_2 treatments. This implies that, at least in the low N systems, net sequestration of N and C into the soil did not occur, which was confirmed by previous FACE studies (van Kessel *et al.*, 2000a,b; van Groeningen *et al.*, 2002). At high N, a temporary increase in the N sequestration and immobilisation may have occurred in the soil organic matter under elevated pCO_2. This is supported by studies of the long-term dynamics of N in *L. perenne* (Schneider, 2003) *T. repens* and mixed swards (§ 3). In both studies an increasing availability of N during the ten years of elevated pCO_2 was reported, which was attributed to an apparent net input of N into the system during the first years. However in the high N treatments too, total N losses may be higher than reported here; not only can fertiliser N be leached or given off as gas from the system, but also N derived from mineralisation of the soil organic matter may be lost. On the other hand, the input of N through symbiotic N_2 fixation may have been underestimated; only the fixed N in the harvested above-ground plant tissue was accounted for. The symbiotically fixed N sequestered into more stable SOM pools was, however, not quantifiable. The retention of symbiotically fixed N in the soil may partly counterbalance the greater losses of fertiliser N (Table 4.4) in the *T. repens* and mixed swards. In the pure grass stands,

fertiliser N seemed to play the main role in retention of N in the soil. This would confirm the results obtained in the four year-study of model ecosystems (Hartwig *et al.*, 2002). In theory, the net sequestration of N into the ecosystem or its loss from the ecosystem could be assessed by monitoring the change in total soil N. However, considering the large standing soil N, which equals approximately 98 % of the total N in grassland systems (Whitehead, 1995b), even comparably large changes in N in/output or changes in turnover rates would probably hardly lead to significant detectable changes in the total soil N content (Neff *et al.*, 2002). Long experimental periods and modelling are necessary before changes in the soil N can be predicted and assessed.

4.4.3 Loss of nitrogen

The above-mentioned N sinks, i. e. leaching of mineral N and gaseous emissions (NH_3 volatilisation, emissions of N_2O, N_2 and NO), may lead to substantial losses of N from the system; consequently, potential pCO_2-induced changes may alter the N budget of the ecosystem under elevated pCO_2. During the experiment gaseous emissions of N through denitrification and nitrification were estimated: i) in a short-term study in July 1995 (Ineson *et al.*, 1998), ii) in a one-year study in summer/autumn 2000 and spring 2001 (Baggs *et al.*, 2003b), and in a short-term study in June 2001 (Baggs *et al.*, 2003a). Ineson *et al.* (1998) measured very high N_2O emissions (averaged 0.83 kg N_2O-N $ha^{-1}d^{-1}$) in the high-N *L. perenne* swards with a 27 % increase under elevated pCO_2. Baggs *et al.* (2003b) measured annual N_2O emissions between 3.43 and 9.6 kg N_2O-N $ha^{-1}a^{-1}$; on average there was a 52 % increase in the high-N *L. perenne* and mixed swards under elevated pCO_2. Furthermore, N_2O emissions increased by approximately 40 % in the high-N *T. repens* swards, which may explain in part the high losses of fertiliser N in these swards (Table 4.4). Baggs *et al.* (2003a) reported that N losses through denitrification/nitrification accounted for up to 17 % of applied fertiliser and were N highest under elevated pCO_2, which may also explain the slow recovery of fertiliser N. However, it is not possible to generalise from short-term experiments; there may be large diurnal and seasonal variations as well as increasing differences with time between the treatments as the systems adapt to specific conditions. Furthermore, N_2O measurements do not include all the N lost through denitrification/nitrification; it is difficult to extrapolate, because the N_2/N_2O ratio of the emitted gas may

vary from about zero to 410 (Baggs *et al.*, 2003a). Volatilisation of ammonia has not been measured. Its significance as a potential N sink in the FACE experiment is, however, assumed to be low. In recent studies, cumulative NH_3 emissions (from 1–5 kg N $ha^{-1}a^{-1}$) have been found for different crops (Yamulki *et al.*, 1996; Schjoerring and Mattsson, 2001). Herrmann *et al.* (2001) found no emissions of NH_3 but a net deposition of about 1 kg $ha^{-1}a^{-1}$ in a fertilised grass/clover sward, similar to that in the present study.

As well as gaseous losses, significant amounts of nitrate and, thus, fertiliser N may be leached from the swards; losses of up to 590 kg $ha^{-1}a^{-1}$ from heavily fertilised and freely drained grass swards have been reported (Garwood and Ryden, 1986). Nitrate leaching tends to be higher in legume and legume-grass swards (Stockdale *et al.*, 2002), usually attributed to the smaller root system and the additional N input through symbiotic N_2 fixation leading to a decrease in the uptake of nitrate by the plants. This could explain the extraordinarily large losses of fertiliser N under *T. repens* in the present study. Leaching from grasslands has been reported to occur most frequently during the winter when the demand of the plant for N is low or non-existent (Whitehead, 1995a) or is associated with heavy rainfall following the fertiliser application in spring (Barraclough *et al.*, 1983), as was the case in 2000 and 2001 in this study. Another explanation for the high losses of fertiliser may be the fact that the fertiliser N was applied immediately after cutting when demand of the plant for N is low and the availability of mineral N is high; thus more fertiliser nitrate is lost through leaching and denitrification. In general, it is assumed that the apparent net N input (Table 4.3) was lost from the system and that some of the differences between the pCO_2 treatments were too small to have had a detectable effect on the total N budget.

4.4.4 Conclusions

Significant changes in the input/output balance of N in the ecosystem under elevated pCO_2 were not detected. Our data suggest that net sequestration did not occur at low N supply, while at high N net sequestration of N may occurred for a certain period of time. Symbiotic N_2 fixation played a major role in N input. It has been shown that previously fixed N, which was transferred below ground and found in subsequent harvests must be taken into account to avoid an underestimation of the input of N into the ecosystem. Significant amounts of fertiliser N (up to 58 % of applied) were

not recovered, which implies that substantial amounts of mineral N were lost from the system. In addition to a number of disadvantages, our ^{15}N studies proved to be a valuable tool for investigating temporal fluxes and fate of N in soil-crop systems.

5 General discussion and conclusions

The effect of elevated pCO_2 in combination with two levels of N fertilisation, on processes determining the sequestration of N and the N availability for plant growth in grassland ecosystems was investigated. It has been shown that gross mineralisation and immobilisation of N, measured on two days of incubation, in *Lolium perenne* and the *Trifolium repens* swards did not change significantly after seven years of CO_2 fumigation. Rates of net mineralisation during the eighth year of CO_2 enrichment also showed no significant pCO_2 response. However, there was a strong tendency for annual net mineralisation to increase under elevated pCO_2 at high N fertilisation (30 % on average), while there was no clear tendency for net N mineralisation to change under elevated pCO_2 in the low N treatment. The annual response of symbiotic N_2 fixation to elevated pCO_2 decreased over the ten years of fumigation in the high-N system. At low N supply, N_2 fixation was always higher and increased markedly under elevated pCO_2 throughout the experiment. The balance of input and output of N calculated for the ten years of the experiment showed no significant pCO_2-induced changes, which indicates that the sequestration of N into the soil organic matter did not change.

5.1 Effect of elevated pCO_2 on the availability of N for plant growth

It has been argued that the productivity of an ecosystem exposed to elevated pCO_2 may be limited by resources such as N (§ 1.3). The continuous excess input of C at elevated pCO_2 due to the increased photosynthesis (Drake *et al.*, 1997; Ainsworth *et al.*, 2003) alters the C/N ratio of the ecosystem. This might be counterbalanced by an additional N input (fertiliser, N_2 fixation) or by an increased availability of N from sources in the soil.

5.1.1 Availability of N from the soil

The amount of N available for plant growth is determined by the conversion of SOM-N into an inorganic plant available form and, in turn, on the removal of this inorganic N from the available pool by microbial immobilisation and gaseous or aqueous losses of mineral N. Consequently, potential changes in N availability for plant growth under elevated pCO_2 are dependent on pCO_2-induced changes in these processes.

In theory, the rate of *net N mineralisation* represents the amount of N that becomes potentially available for plant growth during a defined time period. However, the experimental approach of sequential soil coring utilised for the determination of net mineralisation (§ 3.2.5) leads to uncertainty, which may lead to a misinterpretation of the data. The soil coring technique allowed microbial immobilisation as well as gaseous losses to occur during the *in-situ* incubation. In contrast, nitrate leaching and the lateral efflux of mineral N is prevented due to the enclosure of the soil column in the PVC tube. Consequently, the rate of net N mineralisation may be overestimated by the amount of nitrate, which was leached outside of the incubation tube. In turn, the rate of net mineralisation may have been underestimated as there were no plants growing in the incubated tubes. Microbes inside the incubated soil core may immobilise a greater amount of mineralised N than would occur when plant roots compete for NH_4^+ and NO_3^- (Hodge *et al.*, 2000). Studies of net N mineralisation provide information about overall changes in mineral N in the soil but cannot decouple *gross mineralisation/immobilisation* turnover, which can only be studied using isotope techniques. The ^{15}N pool dilution technique, as proposed by Kirkham and Bartholomew (1954), provides a powerful technique to obtain insight into soil N cycling. However, the main assumptions, which have to be made, i. e. no discrimination between ^{14}N and ^{15}N, a constant rate of measured N fluxes during incubation and no re-mineralisation of applied ^{15}N, are not always quite true (Davidson *et al.*, 1991; Watson *et al.*, 2000). Short incubation periods are required to minimise the experimental error, especially due to re-mineralisation of ^{15}N. Therefore, the combination of both methods, i. e. long-term studies of net mineralisation by sequential soil coring and additional frequent short-term gross N flux measurements, may be suitable for assessing the availability of N in the soil for plant growth.

As outlined in § 1.4.2 and 2.1, elevated pCO_2 is considered to increase N availability through the stimulation of mineralisation of SOM (Zak *et al.*,

1993) or to decrease N supply due to a greater immobilisation by soil microbes (Díaz *et al.*, 1993). However, the microbial biomass in the soil cannot grow infinitely, and the size of the N pool that is sequestered into the microbial biomass is too small to explain an effect of the long-term availability of N.

The results of the long-term experiments in the Swiss FACE, i. e. an increasing yield response of the pure grass swards to elevated pCO_2 (Daepp *et al.*, 2000; Schneider, 2003) and the decreasing contribution of symbiotically fixed N to the yield increase in the legume plots (Figures 3.1 und 3.2), strongly indicates an increase in the availability of N in the soil for plant growth during the ten years. However, this contradicts the lack of a significant response of the annual net mineralisation rates to pCO_2 in 2000 (§ 3.3.2 and Figure 3.3). Moreover, Gloser *et al.* (2000) found no statistically significant changes in net N mineralisation in the *L. perenne*, swards measured for four weeks in 1997. In spite of the potential sources of error discussed above, it is noteworthy that the gross rates of N mineralisation are consistently higher at elevated pCO_2 under *L. perenne* (on average 27 %, Figure 2.1). Furthermore, the annual net rates of N mineralisation (Figure 3.3) were higher in all sward types with the exception of the mixed sward and *T. repens* plots at low N. The amounts of mineral N absorbed by ion exchange resins nearly doubled under elevated pCO_2 at high N supply (Gloser *et al.*, 2000). Although these differences were not significant, there is a general trend, which indicates a greater availability of mineral N in the soils at elevated pCO_2. Baggs *et al.* (2003a,b) measured grater emissions of N_2O and N_2 under elevated pCO_2 from these swards in 2001, which also indicates an increased availability of nitrate as a substrate for denitrification. The lack of statistical significance may be due to the strong spatial variability of the soils in general, as was obvious at the FACE site. Furthermore, due to the split-plot design of the Swiss FACE experiment (§ 3.2.6), the differences between the pCO_2 levels are hardly to be detected as statistically significant.

Factors which potentially determine the pCO_2 response of N mineralisation are discussed in detail in § 1.4.2 and 2.4.1. In brief, there were changes in the biophysical environment (e. g. soil moisture), in the quantity and quality of substrates and in the characteristics of the microbial community. An increased water use efficiency of plants under elevated pCO_2 (Conley *et al.*, 2001; Li *et al.*, 2003) may cause a decrease in the rate of transpiration of plants, leading to an increase in soil moisture. In particular

in dry periods this may result in an increase in mineralisation (Hungate *et al.*, 1997) due to the higher activity of soil microbes. The microbial community changed in size and structure under elevated pCO_2 (Schortemeyer *et al.*, 1996; Marilley *et al.*, 1999; Montealegre *et al.*, 2000). However, it is unknown how these changes affected the mineralisation/immobilisation turnover. Increased mineralisation may also occur as increased immobilisation, which would lead to a greater availability of mineral N on the one hand and a decrease in the mineral N content in the soil on the other. The N concentration of the *L. perenne* plant tissue decreased under elevated pCO_2 (Table 4.2; Zanetti *et al.*, 1997; van Kessel *et al.*, 2000a), which may lead to lower rates of litter decomposition and, subsequently, to the reduced release of mineral N into the soil (van Ginkel *et al.*, 1997, 2000). However, the quality of litter did not change under elevated pCO_2 in the Swiss FACE (Blum *et al.*, 1997) and there were minimal changes in other studies (reviewed by Norby *et al.*, 2001), which may be due to the resorption of nutrients by the plant. In swards containing legumes, elevated pCO_2 had little effect on the N concentration of plant tissue (Zanetti *et al.*, 1997; Hartwig *et al.*, 2000); consequently, no pCO_2-induced changes in the quality of litter should occur.

The data obtained in the present study do not give an ultimate answer to the question, if elevated pCO_2 led to a consistent increase of N availability from the soil for plant growth. However, the clear trend of an increased annual net N mineralisation as well as short-term gross mineralisation under elevated pCO_2 and the response of plant biomass production and N_2 fixation during the ten years of the experiment strongly support these findings. This may suggest that pCO_2-induced changes in the ecosystem functioning lead to a decrease in N limitation after the ecosystem has adapted to the new conditions.

5.1.2 Availability of N from symbiotic N_2 fixation

The amount of N in the harvested plant yield that was derived from the symbiotic N_2 fixation of *T. repens* generally increased under elevated pCO_2 (Figures 3.1 and 3.2; Table 4.2). The increase in the percentage of N from symbiosis was especially high in the first years of the experiment. With time this effect slowly became less strong at high N supply (Figures 3.1 and 3.2). This was attributed to an increasing availability of mineral N in the soil (discussed in § 3.4); according to the concept of the N demand-driven regulation of symbiotic N_2 fixation the proportion of N derived from

symbiosis responds quickly to the availability of soil N (Hartwig, 1998). There is indeed experimental evidence that plant-available N from sources in the soil did not increased proportional to the increased demand of the plants for a greater biomass production under elevated pCO_2. There was a very pronounced increase in the root/shoot ratio in the pure grass stands (Jongen *et al.*, 1995; Hebeisen *et al.*, 1997), a decrease in the N concentration of the plant tissue (Table 4.1; Zanetti *et al.*, 1997), a decrease in the N nutrition index (Soussana and Arregui, 1995; Zanetti *et al.*, 1997) and a increase in the proportion of clover in the mixed swards (Table 4.1). Any process in the ecosystem that leads to an increase in the availability of mineral N in the soil – at least in relation to plant growth – must therefore lead to a smaller percentage of N from N_2 fixation. In the experiments reported here it was assumed that the greater supply of mineral N was derived from the re-mineralisation of temporarily immobilised N. As shown in Table 4.2, considerable amounts of this soil-derived N was biologically fixed N, which was not harvested immediately but was sequestered into the SOM then re-mineralised and subsequently taken up by the plants. As the supply of mineral N for plant growth increased with time, the input of N by symbiotic N_2 fixation decreased and the ecosystem approached equilibrium among input through symbiosis, output and sequestration. At low N fertilisation, the availability of N system was more limited. N_2 fixation remained higher and was increased markedly under elevated pCO_2. However, contribution of soil-derived N tended to increase and that of symbiotic N_2 fixation tended to decrease under elevated pCO_2, particularly in the mixed swards. However, due to the smaller input of fertiliser N, it may take much longer to reach equilibrium in the respective N pools than at high N fertilisation.

5.2 What will be the final state of the N economy?

The FACE technique enabled the investigation of the impact of elevated pCO_2 on grassland ecosystems under actual field conditions. However, the experimental approach represented a simplified model system consisting of the two species representing two important functional groups, i. e. the C3 grass *L. perenne* and the legume *T. repens*. However, large areas of grassland, especially natural and extensively managed grasslands are usually multi-species communities with additional functional groups such as non-legume dicots and, in Mediterranean regions, C4 plants. These differences

must be considered in predicting the future response of grassland ecosystems to the continuous increase in atmospheric pCO_2, because the pCO_2-response of these functional types may vary. For instance, the growth of C4 plants responded less strongly than that of C3 plants (reviewed by Poorter and Navas, 2002), and the yield response of herbaceous species was weak in the first years, while thereafter it was stronger and comparable to that of legumes at elevated pCO_2 (Lüscher *et al.*, 1998). Consequently, the complexity of the effects of elevated pCO_2 increases with the number of plant species; the composition of multi-species mixtures may change due to competitive effects rather than to change in their biomass yield (Poorter and Navas, 2002).

The reduced water consumption of plants under elevated pCO_2 (Conley *et al.*, 2001; Li *et al.*, 2003) poses a further difficulty in predicting the pCO_2 response of grassland ecosystems. As discussed in § 5.1.1 and 2.4.1 this may lead to a change in the rate of N mineralisation and, thus, lead to substantial uncertainty. Nutrients other than N may also limit plant growth. There is experimental evidence that an insufficient supply of P prevents a positive response of symbiotic N_2 fixation and leguminous plant growth under elevated pCO_2 (Almeida *et al.*, 1999, 2000; Stöcklin and Körner, 1999). This may explain the different responses of legumes to elevated pCO_2 in a nutrient-poor calcareous grassland (Leadley and Stöcklin, 1996; Stöcklin *et al.*, 1998) as compared to the fertile conditions in the Swiss FACE experiment.

However, despite these uncertainties, our data indicate that the initially observed strong limitation of N derived from the soil lessens with time. This is evident from the gradually increasing response of perennial ryegrass to elevated pCO_2 at high N supply (Daepp *et al.*, 2000; Schneider, 2003). Likewise, the pCO_2 response of symbiotic N_2 fixation becomes weaker with time (Figures 3.1 and 3.2). These facts may indicate that, in a productive grassland ecosystem on fertile soil under favourable climatic conditions, a high rate of N fertilisation results in a new steady-state situation of N economy after some years of adjustment. Under less favourable conditions, it may take much longer to reach equilibrium again. Our results seem to confirm model predictions that N-rich grasslands respond quickly with an increase in net primary- and plant biomass production, while N-poor systems may undergo a prolonged transition period, during which there is little response (Cannell and Thornley, 1998). In the long run and with the ongoing increase in pCO_2, it is predicted that N-poor ecosystems may respond even more strongly to elevated pCO_2 that N-rich ecosystems. This may be

due to slow N accumulation, resulting from a decrease in leaching, decreased gaseous losses and increased N_2 fixation. A key question is: How rapidly do the rates of processes governing N cycling in the ecosystem respond relative to the rate of the actual pCO_2 increase? In the present experiment, the step increase in the atmospheric pCO_2 resulted in a large time lag between C enrichment and N acquisition, which leads to a negative yield response of pure grass swards and an increase in N_2 fixation by the legumes. Given the slow increase in the global atmospheric pCO_2, it is suggested that N cycling in grassland ecosystems can potentially adapt to the increasing atmospheric pCO_2 without dramatic changes in plant growth, as was observed during the first three years of the Swiss FACE experiment.

5.3 Recommendations for future research

This research aimed at contributing to our understanding of the underlying processes of C and N cycling in the ecosystem under the actual global increase in the atmospheric CO_2 concentration. Based on these results, the investigation of the below-mentioned issues would add substantially to the current knowledge of ecosystem functioning under elevated pCO_2.

A key questions that has yet to be answered is: Does the increase in atmospheric pCO_2 induce a substantial, long-lasting net sequestration of C (and N) into the soil? Thus, the extent to which managed grasslands may contribute to the 'missing' C-sink in the global carbon cycle, remains unclear. The relatively small potential increases in the N and C in the large pool of SOM are hardly detectible; future investigations should thus focus on processes determining the potential net sequestration of C and N. An important factor determining the size of the mineral N pool in the soil and, thus, the availability of N for plant growth is the loss of nitrate through leaching. There are hardly any reliable data on nitrate leaching in grassland soils with regard to elevated pCO_2 in particular. This is due the fact that there is a lack of experimental methodology for the determination of leaching in heterogeneous soils. The improvement of ^{15}N techniques may prove a promising approach in this respect.

As predicted by the use of models as well as by the results of these studies, N-poor ecosystems may respond very slowly to elevated pCO_2. Further long-term investigations should therefore focus on the pCO_2 response in nutrient-limited systems.

Processes in the rhizosphere were assumed to play a major role in the pCO_2 response of soil biota and, thus, in nutrient cycling in the ecosystem in general. However, it is not understood how the composition and function of the microbial communities can change in response to altered substrate availability under elevated pCO_2. Therefore, investigations of the input of organic substrates (from plant production as a result of changes in rhizodeposition and root exudation) and their influence on the microbial community in the soil and subsequent changes in the cycling and economy of nutrients would be particulary interesting. Molecular techniques for studying the composition of microbial community and the use of stable isotopes provide a means for determining how changes in root exudation, rhizodeposition and root turnover influence the function and composition of the microbial community.

Simulation models are an important tool for predicting the ecosystem response to increasing pCO_2. Improving these models based on the experimental data reported here and in related reports and the integration of all the variables affecting the N cycle in the soil would help to improve our understanding of the ecosystem response, especially of nutrient-poor systems, which have been shown to respond very slowly to elevated pCO_2.

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A Appendix

A.1 Denitrification in grass swards is increased under elevated atmospheric CO_2

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E.M. Baggs*, M. Richter†, G. Cadisch* and U.A. Hartwig†

Abstract Emissions of N_2O and N_2 were measured from *Lolium perenne* L. swards under ambient (36 Pa) and elevated (60 Pa) atmospheric CO_2 at the Swiss FACE experiment following application of 11.2 g N m^{-2} as $^{15}NH_4^{15}NO_3$ or $^{14}NH_4^{15}NO_3$ (1 atom %-excess ^{15}N). Total denitrification ($N_2O + N_2$) was increased under elevated pCO_2 with emissions of 6.2 and 19.5 mg ^{15}N m^{-2} measured over 22 d from ambient and elevated pCO_2 swards, respectively, supporting the hypothesis that increased belowground C allocation under elevated pCO_2 provides the energy for denitrification. Nitrification was the predominant N_2O producing process under ambient pCO_2 whereas denitrification was predominant under elevated pCO_2. The N_2-to-N_2O ratio was often higher under elevated pCO_2 suggesting that previous estimates of gaseous N losses based only on N_2O emissions have greatly underestimated the loss of N by denitrification.

There is uncertainty about the effect of increasing atmospheric concentrations of CO_2 on the processes of denitrification and nitrification that result in emissions of N_2O and N_2 from soils. Baggs *et al.* (2003) measured increased annual emissions of N_2O from high N fertilised *Lolium perenne* L. swards under elevated pCO_2 and Ineson *et al.* (1998) measured extremely high short-term N_2O emissions under elevated pCO_2. However, the effects of elevated pCO_2 on total denitrification still remain to be verified by measurement of emissions of N_2 as well as N_2O.

The effects of elevated pCO_2 on total denitrification and the respective contributions of nitrification and denitrification to N_2O emissions from *Lolium perenne* swards were determined over a 3 week sward re-growth period following application of ^{15}N-labelled fertiliser at the Swiss Free Air Carbon dioxide Enrichment (FACE) experiment. The hypothesis was that increased below ground allocation of C under elevated pCO_2 would increase total loss

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of N by denitrification and result in a greater contribution of denitrification to N_2O production.

The FACE experiment at Eschikon (47°27′N and 8°41′E; 550 m asl), Switzerland, consists of three fumigated (60 Pa) and three ambient (36 Pa) CO_2 experimental rings, each with a diameter of 18 m, established in an open field situation in 1993 (Hebeisen *et al.*, 1997). The soil is a clay loam (sand 36 %, silt 33 %, clay 28 %, organic matter 2.9–5.1 %, pH 6.5–7.6) classified as a Eutric Cambisol (FAO classification). The experiment was fertilised with NH_4NO_3 in solution ($1\,l\,m^{-2}$) at a rate of 11.2 g N m^{-2}, applied to *Lolium perenne* cv Bastion swards on 21 June 2001, two days after cutting of the sward to 5 cm height. The fertiliser was applied as (a) $^{14}NH_4^{15}NO_3$ or (b) $^{15}NH_4^{15}NO_3$ (1 atom %-excess ^{15}N) to different treatment replicates within each ring.

Gas samples were taken from closed flux chambers (3 replicates per plot) using gas-tight syringes and stored in 12 ml evacuated gas vials, according to Baggs *et al.* (2003). Samples were analysed for N_2O and CO_2 in an Agilent 6890 gas chromatograph fitted with an electron capture detector, flame ionisation detector and methaniser (column and detector temperatures 40 and 250°C, respectively). Samples for ^{15}N-N_2O and ^{15}N-N_2 determination were stored in 125 ml gas-tight glass bottles (Supelco, UK) and their isotopic enrichment determined on a Europa 20/20 isotope ratio mass spectrometer following condensing/cryofocusing of the sample in an ANCA TGII (PDZ/Europa, Crewe, UK) gas module. ^{15}N-enriched gas fluxes were calculated from the atom %-excess of samples, taking account of the atom % excess of the fertiliser applied. ^{15}N-N_2O and ^{15}N-N_2 fluxes measured from (a) treatment replicates were attributed to denitrification, and ^{15}N-N_2O fluxes from (b) treatment replicates minus ^{15}N-N_2O from (a) treatment replicates were attributed to nitrification, as tests had shown that there was no dissimilatory NO_3^- reduction or immobilisation and subsequent re-mineralisation of $^{15}NO_3^-$. To minimise any effects of diurnal variation in emissions, as far as possible, samples were taken at the same time of day (10:00–12:00 GMT) on each occasion. Linearity of gas diffusion into the headspace over this closure period had previously been determined, so that each flux could be calculated from a single determination at the end of closure. Five soil auger samples (0–0.25 m) from each plot were bulked, extracted with 1 M KCl and NH_4^+-N and NO_3^--N in the extract were determined colorimetrically. The isotopic enrichment of the NH_4^+ and NO_3^- were determined by diffusion methodology (Brooks *et al.*, 1989).

Table A.1: Total ^{15}N-N_2O emissions from nitrification and denitrification (mg ^{15}N-N_2O-N m^{-2}), CO_2 emissions (mg CO_2-C m^{-2}) and gross nitrification (mg N kg dry $soil^{-1}d^{-1}$) over the first week after application of 11.2 g N m^{-2} (1 atom %-excess ^{15}N) to *Lolium perenne* swards under ambient (36 Pa) and elevated (60 Pa) atmospheric CO_2. Values in parentheses represent ± 1 standard error of the mean.

	36 Pa	60 Pa
	mg ^{15}N-N_2O-N m^{-2}	
Total ^{15}N-N_2O from nitrification	6.3 (± 1.3)	0.9 (± 0.4)
Total ^{15}N-N_2O from denitrification	3.0 (± 0.9)	3.2 (± 0.8)
	mg CO_2-C m^{-2}	
Total CO_2 emission	633.3 (± 91.6)	857.0 (± 115.1)
	mg N kg dry $soil^{-1}$ d^{-1}	
Gross nitrification	9.2 (± 2.8)	5.4 (± 0.9)

A.1.1 Total denitrification

Total denitrification (^{15}N-N_2O + ^{15}N-N_2) emissions of 6.2 and 19.5 mg ^{15}N m^{-2} ($p < 0.05$) were measured over 22 d from ambient and elevated pCO_2 *Lolium perenne* swards, respectively, and accounted for 5.5 and 17.4 % of fertiliser ^{15}N applied (Fig. A.1). Emissions of CO_2 were also higher ($p < 0.05$; Table A.1) under elevated pCO_2. This supports the hypothesis that increased below ground C allocation associated with increased root biomass, root turnover and root exudation in elevated pCO_2 *Lolium* swards (Hartwig *et al.*, 2000; van Ginkel *et al.*, 2000) provided the energy for denitrification in the presence of high available N, or that there was increased oxygen consumption under elevated pCO_2.

Nitrous oxide accounted for 99 % of the denitrification product from both ambient and elevated pCO_2 swards on the first day after fertiliser application. However, the denitrified ^{15}N-N_2O flux was short-lived with 56 % of the total denitrified ^{15}N-N_2O emission (3.2 mg ^{15}N-N_2O-N m^{-2} over 7 d, Table A.1) being lost on d 1. Fluxes of ^{15}N-N_2 were low from both ambient and elevated pCO_2 swards over the first week following fertiliser application, but significantly increased after rainfall on d 8 and 9. Fluxes under elevated pCO_2 were strongly positively correlated with both available NO_3^- and rainfall (r = 0.73 and 0.62, respectively; $p < 0.05$) over this 9 d

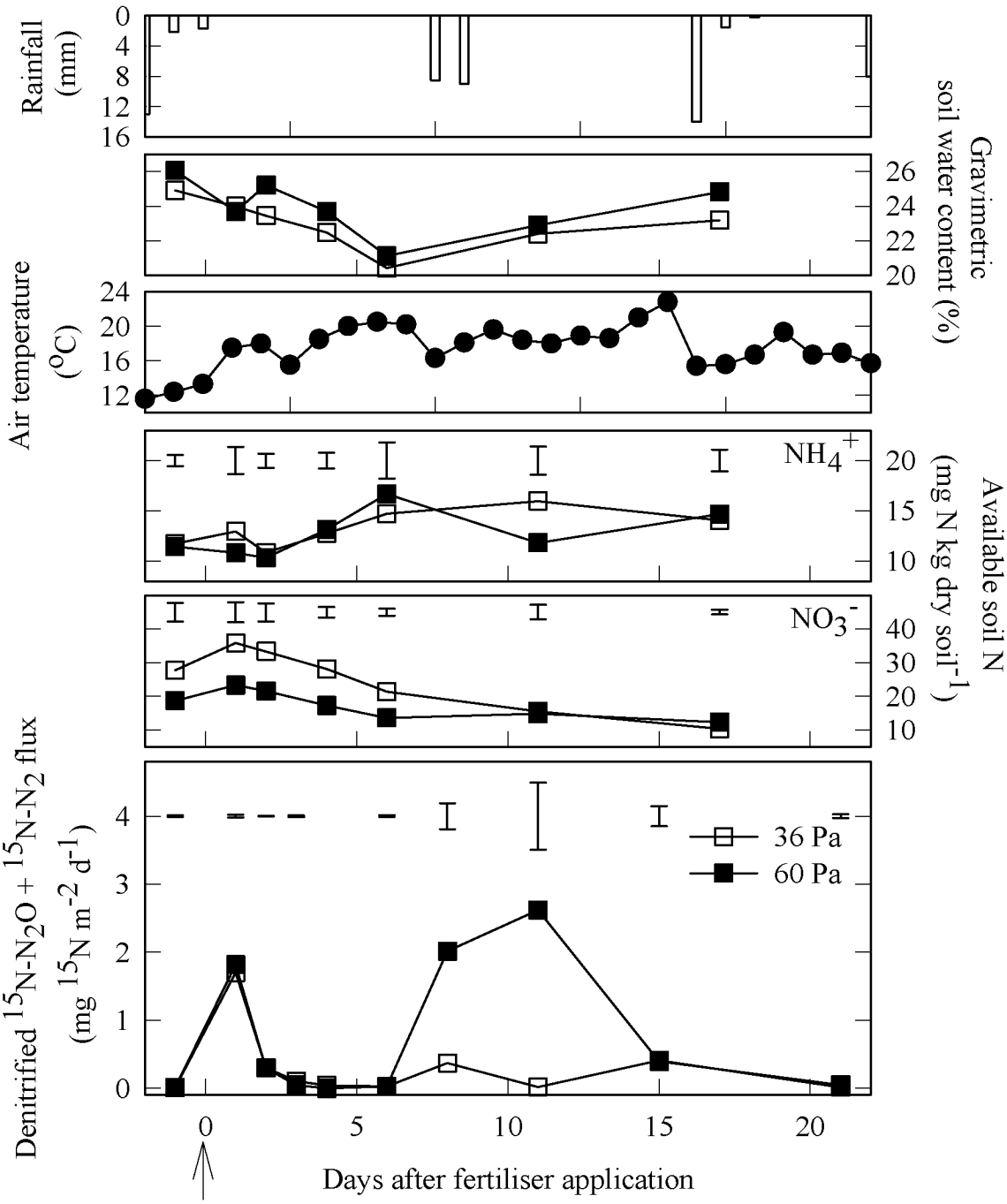

Figure A.1: Total denitrification (^{15}N-N_2O + ^{15}N-N_2; mg ^{15}N $m^{-2}d^{-1}$), available soil NH_4^+ and NO_3^- (mg N kg dry $soil^{-1}$), rainfall (mm), gravimetric soil water content (%) and air temperature (°C) following application of 11.2 g N m^{-2} (1 atom %-excess ^{15}N) to *Lolium perenne* swards under ambient (36 Pa; empty symbols) and elevated (60 Pa; solid symbols) atmospheric CO_2. Arrow indicates time of fertiliser application. Error bars represent ± one standard error of the difference.

period. A maximum ^{15}N-N_2 flux of 2.6 mg ^{15}N-N_2-N $m^{-2}d^{-1}$ under elevated pCO_2 was measured on d 11. Despite further rainfall, denitrified fluxes of N had decreased to 'background' levels at the end of the experiment when available NO_3^- was low and plant N demand would have been high (Ourry *et al.*, 1990).

$^{15}NH_4^+$ was not detected in the single labelled nitrate treatments indicating that dissimilatory reduction of NO_3^- to NH_4^+ or immobilisation of $^{15}NO_3^-$ and subsequent re-mineralisation of this ^{15}N were negligible. Thus ^{15}N-N_2O fluxes from $^{15}NH_4^{15}NO_3$ treatments minus ^{15}N-N_2O fluxes from $^{14}NH_4^{15}NO_3$ treatments could be attributed to nitrification. Nitrification was the predominant N_2O producing process under ambient pCO_2 during the first week whereas denitrification was predominant under elevated pCO_2 (Table A.1). In accordance with this, gross nitrification, calculated from the single labelled treatments (Davidson *et al.*, 1991), was higher ($p < 0.05$) in ambient swards (Table A.1). Thus during particular periods the effect of elevated pCO_2 on total N_2O emissions can be masked by a significant contribution from nitrification, e. g. in the case of lower plant N demand and lower soil water content. Although elevated pCO_2 increased total denitrification emissions later in the experiment it had no significant effect on denitrification during the first week after fertiliser application. It is likely that C became available from root turnover or recent increased root exudates later in the re-growth, but the often reported flush of soluble C after sward cutting may have occurred before fertiliser application.

A.1.2 N_2–to-N_2O ratio

The ratio of ^{15}N-N_2-to-^{15}N-N_2O during denitrification was higher under elevated pCO_2 than under ambient pCO_2 on d 8 and 11, with ratios of 345 and 410 under elevated pCO_2 on these days, respectively (Fig. A.2). The ratios under elevated pCO_2 were higher than ranges typically reported of 0.1 to 40 (e. g. Rolston *et al.*, 1976) but lower than the ratio of 549 measured by Weier *et al.* (1993) at a time of high C availability and high soil water content. Ineson *et al.* (1998) found that denitrification at this site was dominated by N_2O losses with no reduction of N_2O to N_2 on d 6 after fertiliser application. This confirms our results within the first week, but we found that N_2 emissions, and the N_2-to-N_2O ratio, were later increased, re-enforcing the need for longer-term measurements.

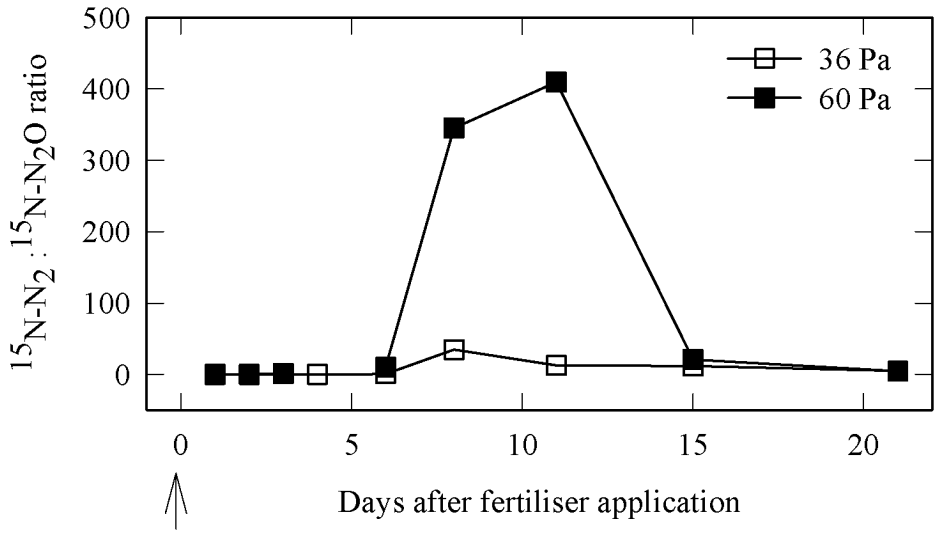

Figure A.2: The ratio of ^{15}N-N_2-to-^{15}N-N_2O denitrification products following application of 11.2 g N m^{-2} (1 atom %-excess ^{15}N) to *Lolium perenne* swards under ambient (36 Pa; empty symbols) and elevated (60 Pa; solid symbols) atmospheric CO_2. Arrow indicates time of fertiliser application.

Prior to d 8, ^{15}N-N_2-to-^{15}N-N_2O ratios were low reflecting a lag in N_2 production behind that of N_2O. Application of fertiliser NO_3^- to the *Lolium* swards may have temporarily inhibited the conversion of N_2O to N_2 because NO_3^- is preferred over N_2O as an electron acceptor at concentrations of $>10\,\mu g\,g^{-1}$ (Blackmer and Bremner, 1978). Thus it is possible that initial concentrations of up to $45\,\mu g\,g^{-1}$ $^{14+15}NO_3^-$ may have been inhibitive for reduction of N_2O to N_2 in the first few days following fertiliser application. Increased C availability under elevated pCO_2 and rainfall on d 8 and 9 would have subsequently increased reduction of N_2O to N_2 (Firestone and Davidson, 1989). The higher N_2-to-N_2O ratio under elevated pCO_2 means that previous estimates of N losses based only on measurements of N_2O emissions (e. g. Baggs *et al.*, 2003b; Ineson *et al.*, 1998) may have greatly underestimated the loss of N by denitrification.

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A.2 Nitrous oxide emissions from grass swards during the eighth year of elevated atmospheric pCO_2

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Abstract Emissions of N_2O were measured during the growth season over a year from grass swards under ambient ($360\,\mu l\,l^{-1}$) and elevated ($600\,\mu l\,l^{-1}$) CO_2 partial pressures at the Free Air Carbon dioxide Enrichment (FACE) experiment, Eschikon, Switzerland. Measurements were made following high ($56\,g\ N\ m^{-2}yr^{-1}$) and low ($14\,g\ N\ m^{-2}yr^{-1}$) rates of fertiliser application, split over 5 re-growth periods, to *Lolium perenne*, *Trifolium repens* and mixed *Lolium/Trifolium* swards. Elevated pCO_2 increased annual emissions of N_2O from the high fertilised *Lolium* and mixed *Lolium/Trifolium* swards resulting in increases in GWP (N_2O emissions) of 179 and 111 g CO_2 equivalents m^{-2}, respectively, compared with the GWP of ambient pCO_2 swards, but had no significant effect on annual emissions from *Trifolium* monoculture swards. The greater emissions from the high fertilised elevated pCO_2 *Lolium* swards were attributed to greater belowground C allocation under elevated pCO_2 providing the energy for denitrification in the presence of excess mineral N. An annual emission of 959 mg N_2O-N $m^{-2}yr^{-1}$ (1.7 % of fertiliser N applied) was measured from the high fertilised *Lolium* sward under elevated pCO_2. The magnitude of emissions varied throughout the year with 84 % of the total emission from the elevated pCO_2 *Lolium* swards measured during the first two re-growths (April–June 2001). This was associated with higher rainfall and soil water contents at this time of year. Trends in emissions varied between the first two re-growths (April–June 2001) and the third, fourth and fifth re-growths (late June–October 2000), with available soil NO_3^- and rainfall explaining 70 %, and soil water content explaining 72 % of the variability in N_2O in these periods, respectively. Caution is therefore required when extrapolating from short-term measurements to predict long-term responses to global climate change. Our findings are of global significance as increases in atmospheric concentrations of CO_2 may, depending on sward composition and fertiliser management, increase greenhouse gas emissions of N_2O, thereby exacerbating the forcing effect of elevated CO_2 on global climate. Our results suggest that when applying high rates of N fertiliser to grassland systems, *Trifolium repens* swards, or a greater component of *Trifolium* in mixed swards, may minimise the negative effect of continued increasing atmospheric CO_2 concentrations on global warming.

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A.2.1 Introduction

During the past few decades atmospheric concentrations of CO_2 and N_2O have been increasing at annual rates of 0.5 and 0.3 %, respectively (Mosier *et al.*, 1998). This is of concern due to the high global warming potentials of these gases, and N_2O is of additional concern due to its involvement in the destruction of stratospheric ozone. The effects of increasing atmospheric concentrations of CO_2 on soil N cycling through alterations in the quantity and quality of belowground plant biomass are still uncertain. Whilst much is known about aboveground plant responses to elevated atmospheric CO_2, comparatively little information is available on belowground processes. There is uncertainty about the effect of elevated CO_2 on the processes of nitrification, denitrification and resulting emissions of N_2O from soils. Validation data of N_2O emissions under elevated CO_2 are required to assist in the prediction of emissions under continued global warming. Estimates of emissions from fertilised grasslands under increasing atmospheric concentrations of CO_2 are of particular importance as managed grassland accounts for 20–30 % of land cover in Europe, and therefore will have a significant effect on global warming.

Emissions of N_2O have previously been shown to increase after application of inorganic fertiliser (e. g. Mosier, 1994; Clayton *et al.*, 1997). The magnitude of emissions varies depending on type and timing of inorganic fertiliser application, soil temperature, moisture content, aeration, soil type and cultivation. Increasing concentrations of atmospheric CO_2 are likely to effect the production of N_2O following fertiliser application. Greater aboveground biomass production under elevated pCO_2 (Stitt and Krapp, 1999) may lead to increased N uptake, reducing the potential for production of N_2O during nitrification and/or denitrification. However, the effects of elevated CO_2 on N availability are uncertain, with suggestions of increased availability (Zak *et al.*, 1993), reduced availability of N due to a higher rate of microbial immobilisation of soil N (Díaz *et al.*, 1993; Hartwig *et al.*, 1996), or no significant effect (Gloser *et al.*, 2000). Alternatively, the increased belowground C allocation under elevated CO_2 in *Lolium perenne* swards (van Kessel *et al.*, 2000) may increase the potential for denitrification by providing energy for this process. Thus it is likely that the effect of elevated CO_2 on N_2O emissions will differ between different sward types.

Ineson *et al.* (1998) measured extremely high short-term N_2O emissions from *Lolium perenne* L. swards (averaging 82 mg N_2O-N $m^{-2}d^{-1}$) after ap-

plication of 14 g N m^{-2} NH_4NO_3 fertiliser under elevated pCO_2. These emissions were on average 14 mg N_2O-N $m^{-2}d^{-1}$ higher than those measured under ambient pCO_2. These short-term emissions were some of the highest to be reported in the literature and suggest that current predictions may underestimate future N_2O emissions from soils under continued increasing atmospheric concentrations of CO_2. Longer-term measurements of N_2O are now required to verify the effects of increasing atmospheric concentrations of CO_2 on the atmospheric loading of this greenhouse gas under continued global warming.

A one year investigation was made of the effect of elevated pCO_2 on N_2O emissions following fertiliser application to *Lolium perenne*, *Trifolium repens* and mixed *Lolium/Trifolium* swards at the Swiss Free Air Carbon dioxide Enrichment (FACE) experiment. The hypothesis was that increased belowground allocation of C under elevated pCO_2 would result in greater emissions of N_2O through increased denitrification. It was expected that application of fertiliser N in the presence of this additional belowground C would drive denitrification thereby resulting in higher N_2O emissions than under ambient pCO_2. Comparisons were made between monoculture *Trifolium repens*, monoculture *Lolium perenne* and mixed *Lolium/Trifolium* swards. The extent of the response to elevated atmospheric CO_2 was expected to vary with different sward composition due to additional N inputs through biological N_2 fixation in legume-based (i. e. *Trifolium* and mixed *Lolium/Trifolium*) swards.

A.2.2 Materials and Methods

The experiment was undertaken from June 2000–June 2001 in the Free Air Carbon dioxide Enrichment (FACE) experiment at Eschikon (47°27′N and 8°41′E; 550 m above sea level), Switzerland. The FACE technology is described in Hebeisen *et al.* (1997) and Zanetti *et al.* (1996) and consists of six experimental rings, each with a diameter of 18 m, established in an open field situation in 1993. Three of the rings are fumigated with CO_2 enriched air (60 Pa; 1.7 times todays atmospheric concentration) and three are at ambient CO_2 (36 Pa). The fumigated rings are enriched during the day from March–November each year. Fumigation is stopped in the autumn when temperature drops below 5°C and is restarted in the spring when the temperature rises above 5°C.

The experiment was fertilised with NH_4NO_3 in solution ($1\,l\,m^{-2}$) at two N application rates, 14 and 56 g N $m^{-2}yr^{-1}$, applied to *Lolium perenne* cv Bastion monoculture, *Trifolium repens* cv Milkanova monoculture and mixed *Trifolium* and *Lolium* swards in plots measuring 2.8×1.9 m within each experimental ring. The fertiliser rates represent low and high applications within the range recommended for forage pasture systems in Switzerland. All plots were watered with $2\,l\,m^{-2}$ of water following the N application. Swards were harvested four times throughout each year prior to fertiliser application, giving a total of 5 re-growth periods in each year, in accordance with common practice in intensive managed pastures. At each harvest swards were cut to approximately 5 cm above ground level. The fertiliser application was split 30, 20, 20, 15 and 15 % between each re-growth in accordance with expected plant demand. Applications were made on the second day after harvesting of each re-growth, and on 2 April 2001 for the first re-growth period of 2001.

Measurements of N_2O emissions, available soil N and gravimetric soil water content were made prior to and periodically following harvest and fertilisation of each re-growth from June–October 2000, and April–June 2001. Measurements of CO_2 emissions were made in April–June 2001. In both years gas flux measurements were made from high and low fertilised *Lolium perenne* cv Bastion monoculture, *Trifolium repens* cv Milkanova monoculture and mixed *Lolium* and *Trifolium* swards. Rainfall, air and soil temperature data were obtained from a meteorological station 200 m from the experimental site.

Gas fluxes

Gas samples for N_2O and CO_2 analysis were taken from closed flux chambers (0.2 m height by 0.1 m diameter), using gas-tight syringes, as described by Smith *et al.* (1995) and stored in evacuated gas vials. Chambers were inserted to a soil depth of 50 mm one day prior to fertilisation and remained *in situ* following harvest and fertilisation of each re-growth. Four chambers were placed in each plot and samples were bulked prior to analysis. Care was taken to minimise disruption to the soil, particularly to that inside of the chamber, during insertion. Chambers were closed one hour before sampling. To minimise any effects of diurnal variation in emissions, as far as possible, samples were taken at the same time of day (10:00–12:00 GMT) on

each occasion. Linearity of gas diffusion into the headspace over this closure period had previously been determined, so that each flux could be calculated from a single determination at the end of closure. In 2000 samples were analysed for N_2O in a Pye Unicam gas chromatograph fitted with an electron capture detector (column and detector temperatures were 50 and 250°C, respectively). In 2001 samples were analysed for N_2O and CO_2 in an Agilent 6890 gas chromatograph fitted with an electron capture detector, flame ionisation detector and methaniser (column and detector temperatures were 40 and 250°C, respectively). Total emissions over specified periods of time were calculated by linear interpolation between daily fluxes.

Soil mineral N

Soil was sampled twice per re-growth period. Five auger samples (0–0.25 m) from each treatment in each ring were bulked. Subsamples (40 g) of the fresh soil were extracted with 1 M KCl in a 1:5 ratio of soil to extractant, and filtered through Whatman No. 1 filter paper. The concentrations of NH_4^+-N and NO_3^--N in the extract were determined colorimetrically by continuous flow analysis on a Burkard SFA2 autoanalyser.

Statistical analysis

All data were analysed using the MINITAB statistical package. Prior to analysis of variance all N_2O and CO_2 data were tested for normality and were log-transformed (Parkin and Robinson, 1993).

A.2.3 Results

Annual N_2O emissions

Total annual N_2O emissions (June–October 2000; April–June 2001) from the *Lolium* and mixed *Lolium/Trifolium* swards were significantly increased ($p < 0.05$) under elevated pCO_2 following application of 56 g N $m^{-2}yr^{-1}$ (Table A.2). Elevated pCO_2 had no significant effect on emissions following application of 14 g N $m^{-2}yr^{-1}$, although the trend was towards lower emissions under elevated pCO_2 following this application. Elevated pCO_2 had no significant effect on total annual emissions from the *Trifolium* monoculture swards following both high and low fertiliser applications. The greatest

Table A.2: Total annual emissions (June–October 2000; April–June 2001) of N_2O (mg N_2O-N $m^{-2}yr^{-1}$) following low (14 g N $m^{-2}yr^{-1}$) and high (56 g N $m^{-2}yr^{-1}$) fertiliser application to ambient (36 Pa) and elevated (60 Pa) pCO_2 swards. Values in parentheses represent ± one standard error of the mean.

	Total emission (mg N_2O-N $m^{-2}\,yr^{-1}$)	
Treatment	36 Pa	60 Pa
14 g N $m^{-2}\,yr^{-1}$		
Lolium	446 (± 159)	330 (± 124)
Lolium/Trifolium	436 (± 204)	343 (± 218)
Trifolium	531 (± 155)	501 (± 137)
56 g N m^{-2} yr^{-1}		
Lolium	591 (± 262)	959 (± 258)
Lolium/Trifolium	576 (± 159)	805 (± 234)
Trifolium	908 (± 224)	921 (± 373)

annual emission of 959 mg N_2O-N $m^{-2}yr^{-1}$ was measured from the high fertilised elevated pCO_2 *Lolium* sward and was significantly higher ($p < 0.05$) than that measured from the high fertilised ambient pCO_2 *Lolium* sward and the high fertilised elevated and ambient pCO_2 mixed *Lolium/Trifolium* swards.

Total annual N_2O emissions from all swards following application of 56 g N $m^{-2}yr^{-1}$ were significantly higher than following application of 14 g N $m^{-2}\,yr^{-1}$. This was most apparent in the *Lolium* swards where the emission of 959 mg N_2O-N $m^{-2}yr^{-1}$ measured from the high fertilised elevated pCO_2 sward was almost 3 times greater than that measured from the low fertilised elevated pCO_2 *Lolium* sward. Fertiliser application rate had less effect on the ambient pCO_2 *Lolium* and mixed *Lolium/Trifolium* swards with emissions following the high application being only 1.3 times greater than following the low fertiliser application. There was no interaction in the effect of fertiliser rate on N_2O emissions between elevated and ambient pCO_2 *Trifolium* swards.

Total N_2O emitted within different re-growth periods

The magnitude of N_2O emissions varied between re-growth periods with the greatest emissions measured in the first and second annual re-growth periods (April–June 2001) and the lowest emissions measured in the third

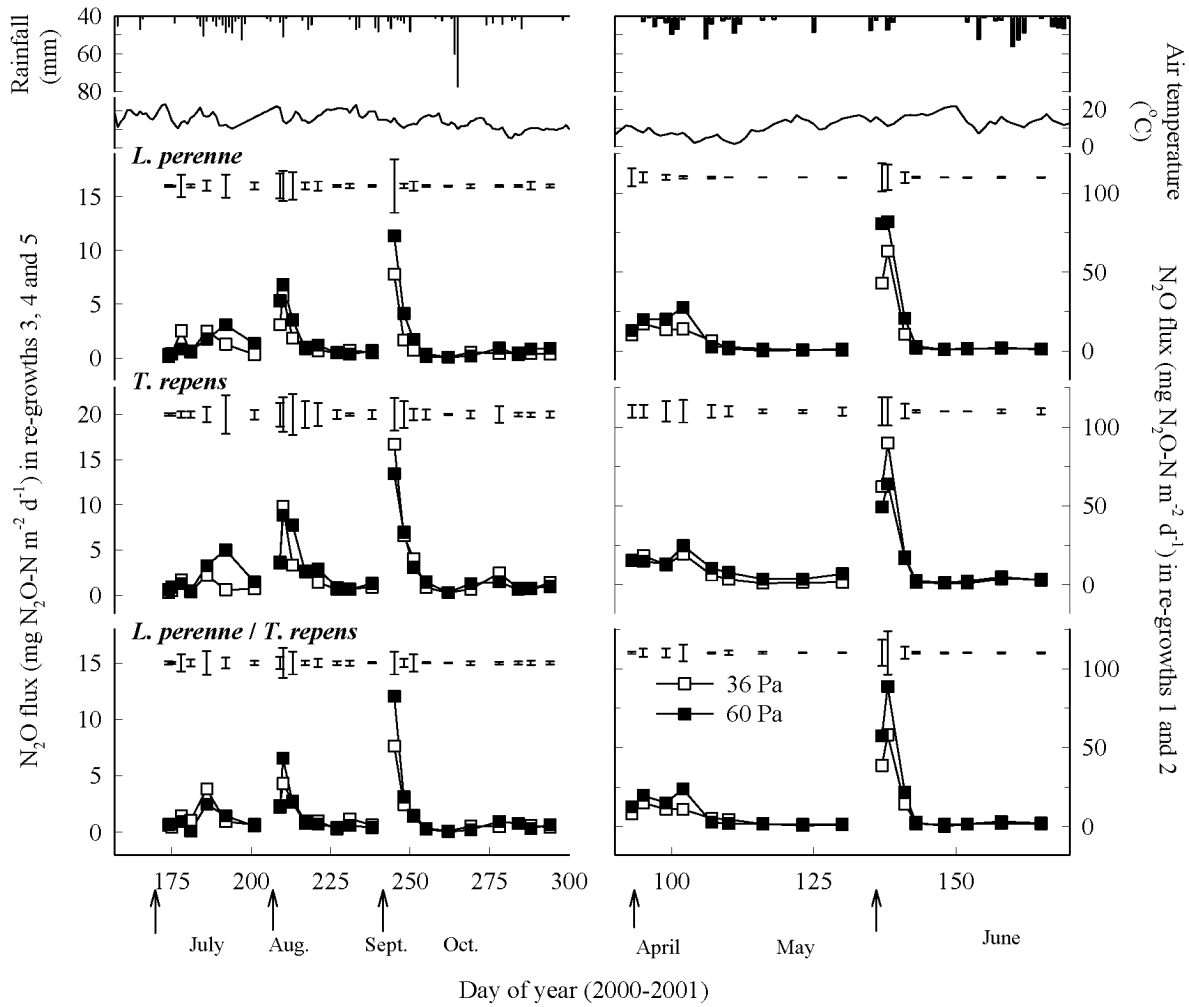

Figure A.3: Rainfall (mm), air temperature (°C) and daily fluxes of N_2O (mg N_2O-N $m^{-2}d^{-1}$) following application of 56 g N $m^{-2}yr^{-1}$ to *Lolium perenne*, *Trifolium repens* and mixed *Lolium/Trifolium* swards in 2000/2001. Arrows indicates times of fertiliser application to each re-growth. Error bars represent ± one standard error of the deviation.

re-growth (June–July 2000) (Fig. A.3). In the second re-growth 82–90 % of N_2O was lost in the first 7 days after the high fertilisation. An average total emission per re-growth of 405 mg N_2O-N m^{-2} was emitted from the high fertilised elevated pCO_2 *Lolium* sward over 65 days in the first and second re-growths, but an average of only 50 mg N_2O-N m^{-2} was emitted from this treatment over 106 days in the third, fourth and fifth re-growth periods (Table A.3).

Emissions from *Lolium* swards averaged over the first and second re-growth periods showed the same trend as annual emissions with significantly higher ($p < 0.05$) emissions under elevated pCO_2 following high fertiliser ap-

Table A.3: Total emissions of N_2O averaged over re-growths 1 and 2 (April–June 2001) and re-growths 3, 4 and 5 (late June–October 2000) and CO_2 emissions averaged over re-growths 1 and 2 following application of 14 and 56 g N $m^{-2}yr^{-1}$ to ambient (36 Pa) and elevated (60 Pa) pCO_2 swards. Values in parentheses represent ± one standard error of the mean.

		Low N ($14\,g\,N\,m^{-2}\,yr^{-1}$)		High N ($56\,g\,N\,m^{-2}\,yr^{-1}$)		
Re-growths	Sward	36 Pa	60 Pa	36 Pa	60 Pa	Days
1 and 2	*Lolium perenne*	156 (±57)	107 (±40)	237 (±116)	405 (±102)	65
	Lolium/Trifolium	140 (±79)	105 (±80)	222 (±70)	340 (±133)	65
	Trifolium repens	184 (±57)	156 (±37)	350 (±92)	334 (±133)	65
	s.e.d.	38	33	55	71	
3, 4 and 5	*Lolium perenne*	45 (±15)	39 (±12)	39 (±10)	50 (±11)	106
	Lolium/Trifolium	52 (±16)	45 (±19)	44 (±6)	42 (±10)	106
	Trifolium repens	54 (±14)	63 (±21)	70 (±13)	84 (±36)	106
	s.e.d.	9	10	6	13	
		Total CO_2 emission ($g\,CO_2\text{-}C\,m^{-2}$ re-$growth^{-1}$)				
1 and 2	*Lolium perenne*	143 (±9)	165 (±19)	136 (±10)	131 (±8)	65
	Lolium/Trifolium	154 (±9)	192 (±34)	127 (±16)	143 (±12)	65
	Trifolium repens	127 (±9)	106 (±10)	122 (±9)	195 (±75)	65
	s.e.d.	7	13	9	68	

plication, but no significant effect of elevated pCO_2 following low fertiliser application. The average concentrations of available NH_4^+ over re-growths 1 and 2 were significantly higher ($p < 0.05$) in elevated than in ambient pCO_2 *Lolium* swards following both high and low fertiliser applications (Table A.4). In these two re-growth periods elevated pCO_2 had no significant effect on N_2O emissions from *Trifolium* or mixed *Lolium/Trifolium* swards. Elevated pCO_2 also had no significant effect on CO_2 emissions during these two re-growth periods although the trend was towards increased emissions in the high fertilised *Lolium/Trifolium* and *Trifolium* monoculture swards under elevated pCO_2 (Table A.3). N_2O ($\log_e$) emissions were positively correlated with CO_2 ($\log_e$) emissions. This relationship was stronger under elevated (r = 0.5-0.9; $p < 0.05$) than under ambient pCO_2 (r = 0.5-0.9; $p < 0.05$).

Elevated pCO_2 had no significant effect on N_2O emissions averaged over the third, fourth and fifth re-growth periods (Table A.3). Available NO_3^- was significantly lower ($p < 0.05$) in low fertilised elevated than in ambient pCO_2 *Lolium* swards in these re-growth periods (Table A.4).

Daily N_2O fluxes

Daily fluxes of N_2O increased following fertilisation and declined throughout each re-growth period (Fig. A.3). The highest fluxes were measured after high fertilisation of swards in the second re-growth (May 2001). A flux of 90 mg N_2O-N $m^{-2}d^{-1}$ was measured from the high fertilised ambient pCO_2 *Trifolium* sward two days after fertiliser application in this re-growth. Fluxes of 82 and 89 mg N_2O-N $m^{-2}d^{-1}$ were measured from high fertilised elevated pCO_2 *Lolium* and mixed *Lolium/ Trifolium* swards on this day.

Concentrations of mineral N at the end of each re-growth period were significantly lower ($p < 0.05$) than at the start of each re-growth after fertiliser application (data not shown). Daily N_2O ($\log_e$) fluxes were positively correlated with available soil N (r = 0.2-0.7, available NH_4^+; r = 0.1-0.7, available NO_3^-; $p < 0.05$) throughout the year. Correlation between N_2O ($\log_e$) and available soil N was stronger under ambient pCO_2 (r = 0.5-0.7; $p < 0.05$) than under elevated pCO_2 (r = 0.3-0.6; $p < 0.05$) following the high fertiliser application. This was best exemplified in the high fertilised *Lolium* swards (Fig. A.4a and b).

Table A.4: Soil mineral N (mg N kg dry soil^{-1}) averaged over re-growths 1 and 2 (April–June 2001) and re-growths 3, 4 and 5 (late June–October 2000) following application of 14 and 56 g N m^{-2}yr^{-1} to ambient (36 Pa) and elevated (60 Pa) pCO_2 swards. Values in parentheses represent ± one s.e.m.

		Low N (14 g N m^{-2} yr^{-1})		High N (56 g N m^{-2} yr^{-1})	
Re-growths	Sward	36 Pa	60 Pa	36 Pa	60 Pa
		Available NH_4^+ (mg NH_4^+–N kg dry soil^{-1} re-growth^{-1})			
1 and 2	*Lolium perenne*	7 (±1)	12 (±1)	11 (±1)	17 (±4)
	Lolium/Trifolium	7 (±2)	9 (±2)	14 (±3)	16 (±4)
	Trifolium repens	7 (±2)	9 (±2)	14 (±4)	15 (±3)
3, 4 and 5	*Lolium perenne*	6 (±1)	7 (±1)	11 (±3)	12 (±3)
	Lolium/Trifolium	7 (±2)	6 (±2)	16 (±4)	15 (±5)
	Trifolium repens	4 (±1)	6 (±1)	12 (±2)	12 (±4)
		Available NO_3^- (mg NO_3^-–N kg dry soil^{-1} re-growth^{-1})			
1 and 2	*Lolium perenne*	14 (±5)	11 (±5)	43 (±12)	38 (±12)
	Lolium/Trifolium	11 (±3)	12 (±5)	35 (±10)	34 (±13)
	Trifolium repens	18 (±5)	22 (±7)	44 (±6)	43 (±7)
3, 4 and 5	*Lolium perenne*	12 (±3)	8 (±3)	34 (±6)	41 (±13)
	Lolium/Trifolium	16 (±8)	18 (±8)	37 (±7)	35 (±12)
	Trifolium repens	23 (±6)	20 (±5)	39 (±6)	42 (±12)

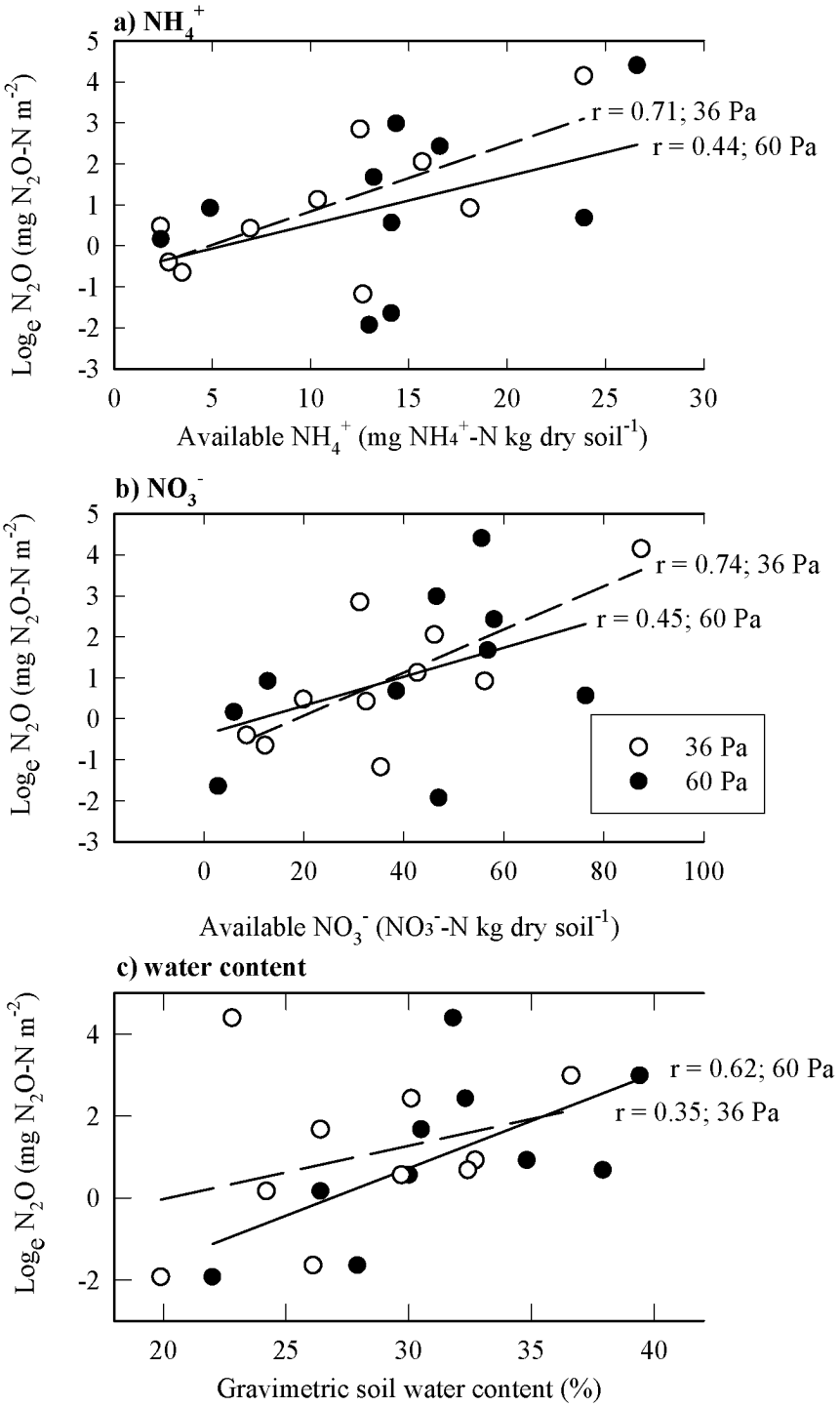

Figure A.4: Relationship between N_2O ($\log_e$) emissions from high fertilised (56 g N $m^{-2}yr^{-1}$) ambient (36 Pa; empty symbols, dashed line) and elevated (60 Pa; filled symbols, solid line) pCO_2 *Lolium* swards and a) available soil NH_4^+ b) available soil NO_3^- and c) gravimetric soil water content over the whole growth season (June–October 2000; April–June 2001).

Emissions over the first 7 days after fertiliser application were negatively correlated with air temperature in all re-growths (r = −0.2 to −0.9; $p < 0.05$) except in the fifth re-growth (r = 0.7-0.9; $p < 0.05$), but positively correlated with rainfall in all re-growths (r = 0.3-0.9; $p < 0.05$). Elevated pCO_2 only appeared to affect these correlations in the third re-growth where stronger relationships were found between N_2O ($\log_e$) emissions and rainfall under elevated pCO_2 *Trifolium* and *Lolium* swards (r = 0.9; $p < 0.05$) than under ambient pCO_2 swards (r = 0.6-0.7; $p < 0.05$) following high N application. Emissions were positively correlated with soil water content and more strongly under elevated than under ambient pCO_2 (r = 0.4-0.7, ambient pCO_2; r = 0.6-0.9, elevated pCO_2; $p < 0.05$; Fig. A.4c).

Stepwise multiple regression indicated that 38 % of the variation in daily N_2O ($\log_e$) fluxes from high fertilised Lolium swards under elevated pCO_2 was explained by soil water content and 44 % ($p < 0.05$) explained by soil water content and rainfall. In the first two re-growth periods available soil NO_3^- explained 62 % of the variability in N_2O emission and 70 % ($p < 0.05$) was explained by available soil NO_3^- and rainfall. However, in the third, fourth and fifth re-growths 72 % ($p < 0.001$) of variability was explained by soil water content.

A.2.4 Discussion

Effect of sward type and elevated pCO_2 on annual N_2O emissions

Total annual N_2O emissions from high fertilised *Lolium* and mixed *Lolium*/ *Trifolium* swards were increased under elevated pCO_2 compared with emissions measured from ambient pCO_2 swards. The high N application (56 g N $m^{-2}yr^{-1}$) was in excess of the *Lolium* requirement for N of 29 g N $m^{-2}yr^{-1}$ (Richter, pers. comm.) resulting in high availability of mineral N for nitrification and denitrification. Annual emissions from elevated pCO_2 *Lolium* swards were three times greater following high fertiliser application (959 mg N_2O-N m^{-2}) than following low fertiliser application (330 mg N_2O-N m^{-2}). Such a response to fertiliser application is well documented as N is applied in a form readily available for nitrification and denitrification (e. g. Mosier, 1994; Clayton *et al.*, 1997), as corroborated by our mineral N data.

We originally hypothesised that belowground C allocation would be increased under elevated pCO_2, and that application of fertiliser N in the

presence of this belowground C would drive denitrification resulting in increased N_2O emissions. The effect of elevated pCO_2 in increasing belowground C allocation associated with increased root biomass, root turnover and root exudation is well documented (Rogers *et al.*, 1994; Fitter *et al.*, 1997; Hartwig *et al.*, 2000; van Ginkel *et al.*, 2000; van Kessel *et al.*, 2000). In this Swiss FACE experiment van Kessel *et al.* (2000) measured greater *Lolium* root biomass under elevated ($7.6\,g\,kg\,soil^{-1}$) than under ambient pCO_2 ($3.7\,g\,kg\,soil^{-1}$) four years following fumigation and application of $56\,g\ N\ m^{-2}yr^{-1}$. Similarly, following application of ^{14}C to *Lolium perenne* in a controlled environment experiment, van Ginkel *et al.* (2000) reported a 41 % greater root biomass under elevated than under ambient pCO_2 and an increase in ^{14}C-labelled C in the soil solution, microbial biomass and soil residues under elevated pCO_2. Belowground C was not directly measured in our study but we found positive correlations between N_2O and soil CO_2 emissions under elevated pCO_2 that were indicative of increased C cycling (van Kessel *et al.*, 2000) and suggest that C availability was providing the energy for denitrification in this system. The stronger correlation between N_2O and CO_2 under elevated pCO_2 suggested that C availability was greater under elevated than under ambient pCO_2 resulting in the higher N_2O emissions measured from the high fertilised *Lolium* and mixed *Lolium/Trifolium* swards under elevated pCO_2.

Despite the expected increase in belowground C, elevated pCO_2 had no significant effect on N immobilisation during this study, but gross N mineralisation was increased by 30 % in the *Lolium perenne* swards (Richter *et al.*, 2003). In agreement with Gloser *et al.* (2000) and Zanetti *et al.* (1997), Richter (pers. comm.) found no significant increase in N uptake from the soil by *Lolium* under elevated pCO_2 (25 and $29\,g\ N\ m^{-2}yr^{-1}$ under ambient and elevated pCO_2, respectively) during the period of this study, and Schortemeyer *et al.* (1996) found no evidence of significantly increased microbial biomass under elevated pCO_2 *Lolium* and *Trifolium* swards in this experiment. Thus N applied would have been available for nitrification and denitrification with the typically reported increased C availability under elevated pCO_2 driving denitrification. Higher soil water resulting from lowered water use of plants under elevated pCO_2 (Morison, 1985; Baggs *et al.*, 2003), along with increased O_2 consumption during microbial activity and by the root biomass (Jongen *et al.*, 1995) may lower the O_2 partial pressure in soil, also favouring denitrification.

Elevated pCO_2 had no significant effect on annual N_2O emissions from

Trifolium monoculture swards, or on mineral N concentrations in these swards. It is possible that any effect of elevated pCO_2 on belowground C allocation was less in *Trifolium* swards than in *Lolium* swards as the C:N ratio of plant litter is thought to only increase in legume-free grass monocultures under elevated pCO_2 (Hartwig *et al.*, 2000).

Short- versus longer-term responses to elevated pCO_2

The magnitude of emissions varied throughout the year with the greatest emissions measured in the first and second re-growth periods (April–June 2001) and the lowest emissions measured in the third re-growth period (late June–July 2000). The high emissions in April–June were most likely due to the high soil water content and rainfall of 59 mm over 65 days, the relatively high soil temperature (10–20°C), the beginning of decomposition of newly grown roots and the fact that 30 and 20 % of the annual fertiliser application was applied in April and May, respectively. Available soil NO_3^- accounted for 62 % of the variability in N_2O emissions during these first two re-growths, and both available soil NO_3^- and rainfall accounted for 70 % of the variability. Increasing air temperatures in May would have resulted in a flush of microbial activity following fertiliser application to the second re-growth, and the consequent high fluxes of N_2O measured over the first week after fertilisation. Low emissions in the third re-growth most likely reflected the low rainfall at the start of this period and following fertiliser application resulting in gravimetric soil water contents of less than 20 %. Hence in the third, fourth and fifth re-growths soil water content explained 72 % of the variability in emissions. The strong relationship between N_2O emissions and soil water content and rainfall suggests that denitrification, rather than nitrification, may have been the predominant process contributing to N_2O emissions from these swards. In accordance with this Baggs *et al.* (2003), using stable isotope techniques in a short-term study at this site in June/July 2001, showed that denitrification was the predominant process contributing to $^{15}N\text{-}N_2O$ and $^{15}N\text{-}N_2$ emissions from high fertilised (56 g N $m^{-2}yr^{-1}$) elevated pCO_2 *Lolium* swards.

Although over the whole year atmospheric pCO_2 had no significant effect on N_2O emissions from *Trifolium* swards different trends were seen in the short-term within particular re-growth periods. Such differences in trend from individual re-growth totals to annual estimates means that caution is required when extrapolating short-term measurements, such as made by

Ineson *et al.* (1998) from *Lolium* swards, to predict long-term atmospheric loading. In the third, fourth and fifth re-growths (June–October 2000) emissions from the *Trifolium* swards were higher than from the *Lolium* and mixed *Lolium/Trifolium* swards. *Trifolium* has a greater ability to fix atmospheric N_2 in June to September (re-growths 3–5) (Zanetti *et al.*, 1996; Zanetti and Hartwig, 1997) and this was reflected in significantly higher available NO_3^- and higher total dry matter yield (298 and 239 g dry matter m^{-2}, respectively; Richter, pers. comm.) in low fertilised *Trifolium* than in low fertilised *Lolium* swards during these re-growths.

In all re-growths, except the third, response to fertiliser application in elevated pCO_2 swards was more rapid than in ambient pCO_2 swards, with emissions from elevated pCO_2 treatments tending to be higher than from ambient swards over the first two days after fertiliser application. Available soil N decreased throughout each re-growth period with the increase in plant N uptake reducing the potential over time for nitrification and denitrification, but elevated pCO_2 had in most cases no significant effect on N availability in any of the re-growth periods. This rapid response to fertiliser application under elevated pCO_2 corroborates the extremely high short-term emissions of up to 168 mg $m^{-2}d^{-1}$ measured from the high fertilised *Lolium* swards at this site in July 1996 by Ineson *et al.* (1998), although our emissions measured in July 2000 were not as high as these. These extremely high emissions were most likely a result of fertiliser being applied after addition of simulated rainfall to swards at a time of unusually high temperature.

A.2.5 Conclusions

These results indicate that increases in atmospheric concentrations of CO_2 may, depending on fertiliser management, increase greenhouse gas emissions of N_2O from *Lolium* swards and mixed *Lolium/Trifolium* swards, potentially exacerbating the forcing effect of elevated CO_2 on global climate. Over the whole growing season (June–October 2000; April–June 2001) elevated pCO_2 resulted in increases in Global Warming Potential (GWP) from N_2O emissions of 179, 111 and 7 g CO_2-equivalents m^{-2} from high fertilised *Lolium*, *Lolium/Trifolium* and *Trifolium* swards, respectively, compared with the GWP of ambient pCO_2 swards. However, when taking account of both CO_2 and N_2O emissions over the first two re-growths, the increases in GWP under elevated pCO_2were 78, 74 and 65 g CO_2-equivalents m^{-2} from high fertilised *Lolium*, *Lolium/Trifolium* and *Trifolium* swards, respectively. Un-

der ambient pCO_2 and following low rates of fertiliser application there is a tendency for *Trifolium* swards to emit more N_2O than *Lolium* or mixed *Lolium/Trifolium* swards. However, following high rates of fertiliser application *Trifolium* monoculture swards, or a greater component of *Trifolium* in mixed swards, appear to result in the least increase in GWP under continued increasing atmospheric CO_2 concentration and may help to maintain N_2O emissions at todays rates. This has important implications for management of grassland systems in order to mitigate greenhouse gas emissions under continued global warming and is helped by the apparent competitive advantage of *Trifolium* in mixed swards under elevated CO_2.

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