Input of Nitrogen from N_2 Fixation to Northern Grasslands

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Abstract

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Forage legumes form N_2-fixing symbioses with rhizobia and may thus make substantial contributions to the N pool in grasslands. However, to optimize their use as sources of N, it is important to elucidate the effects of management factors that influence their N_2 fixation rates, and to develop convenient methods for measuring N_2 fixation quickly and reliably.

An analysis of published data on N_2 fixation in the field showed that lucerne (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), and white clover (*T. repens* L.) grown in mixtures with grasses derived most of their N from N_2 fixation, irrespective of geographic location and management practices – and despite large inter-annual variations in legume dry matter yield (kg ha^{-1} $year^{-1}$). Consequently, there were strong correlations between legume dry matter yield and amounts of N_2 fixed (kg N ha^{-1} $year^{-1}$), which can be used very simply to obtain estimates of N_2 fixation in these legumes.

In experimental grassland plots where the species richness of neighbouring vegetation was varied, alsike clover (*T. hybridum* L.), red clover, and white clover consistently derived at least half of their N from N_2 fixation, measured by the ^{15}N natural abundance (NA) method using three different reference plants. This method is sensitive to the degree of discrimination against ^{15}N in the N_2-fixing plant (B value) and the choice of reference plant. B values were therefore established for each of the three clover species in symbioses with different Scandinavian *Rhizobium leguminosarum* bv. *trifolii* genotypes.

In red clover, reductions following cutting in the activity of the N_2-fixing enzyme, nitrogenase, and the rate of shoot regrowth were dependent on the cutting height. The recovery in nitrogenase activity after cutting followed the rate of leaf area increment, which confirms the correlation between N_2 fixation and growth found in field experiments.

The results of the work underlying this thesis show that perennial forage legumes growing in grasslands are highly dependent on N_2 fixation. Awareness of this should facilitate the development of resource-efficient management regimes for northern grasslands.

Key words: Acetylene reduction activity, clover, cutting height, $\delta^{15}N$, forage, legume, methods, N_2 fixation, perennial, *Rhizobium*, species richness.

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Units and abbreviations

SI units have been used throughout the text, with the following exceptions: temperature is given in degrees Celsius (°C), and data expressing herbage yield, amounts of N_2 fixed, and N fertilizer applications, are given in kg ha^{-1}.

For conversion:

1 ha = 10,000 m^2
1 kg ha^{-1} = 0.1 g m^{-2}

ARA, Acetylene reduction activity
B, The $\delta^{15}N$ of a plant that has derived all its N from atmospheric N_2 fixation
C, Carbon
$\delta^{15}N$, Parts per thousand deviation from the $^{15}N/^{14}N$ ratio of atmospheric N_2
DM, Dry matter yield as kg ha^{-1} $year^{-1}$
ID, ^{15}N Isotope dilution
N, Nitrogen
NA, ^{15}N Natural abundance
ND, Nitrogen difference
Ndfa, N_2 fixation as the proportion of N derived from atmospheric N_2 fixation
Nfix, N_2 fixation as kg N ha^{-1} $year^{-1}$.
Rlt, *Rhizobium leguminosarum* bv. *Trifolii*

Cover photograph: my dad's cows in Oviken, Jämtland, northern Sweden; the first subject for my interest in sustainable food production.

Contents

Appendix

Papers I-IV

This thesis is based on the following papers, which are referred to by the corresponding Roman numerals:

I. Carlsson, G. & Huss-Danell, K. 2003. Nitrogen fixation in perennial forage legumes in the field. *Plant and Soil 253*, 353-372.

II. Carlsson, G., Palmborg, C. & Huss-Danell, K. 2005. Discrimination against ^{15}N in three N_2-fixing *Trifolium* species as influenced by *Rhizobium* strain and plant age. *Acta Agriculturae Scandinavica Section B. Soil and Plant Science* (in press).

III. Carlsson, G., Palmborg, C., Jumpponen, A., Högberg, P. & Huss-Danell, K. N_2 fixation in three perennial clover species in communities of varied plant species diversity. *Submitted*.

IV. Carlsson, G. & Huss-Danell, K. Dynamics in nitrogenase activity and growth after cutting red clover (*Trifolium pratense*) at different heights. *Submitted*.

Introduction

Plants are the primary food producers on earth. Plant products are consumed either directly, *e.g.* as grains and vegetables, or indirectly, *e.g.* as bread, milk, meat, and eggs. In addition, plant production is used to meet large proportions of human requirements for fibre, fuel and other materials (*e.g*. cotton, wool, biofuels, pulp, paper and timber). The world's demand for plant products is rapidly increasing, due both to population growth and increasing prosperity (leading, for instance, to greater incorporation of meat in human diets). In the mid-1990s, a third of global grain production was fed to livestock, and it takes about seven units of grain to produce a single unit of meat (Sinclair & Gardner, 1998). Thus, increasing the proportion of fodder from grasslands in ruminant diets would make milk- and meat-production systems less reliant on grains and vegetables, more of which could then be used in human diets, increasing the overall efficiency of resource use in food production.

In northern areas, cultivation of cereals for direct human consumption is constrained by the short growing seasons. Consequently, northern agriculture is largely based on ruminant animal production, *i.e.* milk and meat. Plants grown at high latitudes, where days are long and temperatures low during the growing season, attain relatively high concentrations of simple carbohydrates, resulting in high feeding values (van Soest, Mertens & Deinum, 1978; Deinum *et al.*, 1981). It should therefore be possible to produce milk and meat in northern areas efficiently by supporting the livestock almost entirely with locally grown forage crops. For such production to be economically sustainable, with minimal needs for imported fodder, grasslands must produce high and predictable forage yields. One of the most crucial limitations to plant production in temperate and northern areas is the amount of plant-available nitrogen (N).

Forage legumes are valuable in agriculture from more than one perspective. Apart from providing very important inputs of N to grasslands via N_2 fixation in symbioses with rhizobia, forage legumes have been shown to have a positive influence on soil structure, and to have a high feeding value in ruminant diets. Compared to grasses, legumes generally have lower contents of structural fibre, higher protein contents and greater digestibility, resulting in higher nutrient intake rates and animal production when they are used as fodder (Frame, Charlton & Laidlaw, 1998). Furthermore, forage legumes are widespread, and have the potential to give high yields over a range of climatic conditions; the four major forage legumes lucerne (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), subterranean clover (*T. subterraneum* L.), and white clover (*T. repens* L.) together cover grasslands from hot and dry regions of Australia and New Zealand to the arctic regions of northern Scandinavia.

The studies underlying this thesis comprised a literature review in which field measurements of N_2 fixation in the three perennial forage legumes lucerne, red clover, and white clover were summarized and analyzed (I), methodological considerations regarding the ^{15}N natural abundance method (II; III), a field study of the effects of the species richness of neighbouring vegetation on N_2 fixation in

the perennials alsike clover (*T. hybridum* L.), red clover, and white clover (III), and an investigation of the effect of cutting height on N_2 fixation and regrowth in greenhouse- and field-grown red clover plants (IV).

Plants and nitrogen

Nitrogen, being a constituent of proteins, nucleic acids, and chlorophyll, is essential for plant growth and functions. After photosynthesis, N acquisition is considered the second most important process for plant growth and development (Vance, 1997), and plant production in temperate ecosystems is often limited by the amount of plant-available N (Whitehead, 1995).

Nitrogen as a limiting resource for plant growth

It may seem paradoxical that N availability can limit plant growth, since N is highly abundant in the atmosphere. The atmosphere contains about 78% N_2, corresponding to 2300 kg N ha^{-1} in typical soils, assuming that 25% of the soil pore space is air-filled (Myrold, 1998). However, N_2 cannot be used directly by organisms, but has to be combined with hydrogen before it can be incorporated into amino acids and further into other essential organic compounds. The two N atoms in the N_2 molecule are held together by a very stable triple bond. Thus, the reactions in which this triple bond is broken and N is combined with hydrogen or oxygen require substantial inputs of energy, and only occur under highly specific conditions, mainly in the industrial manufacture of N fertilizers, during thunderstorms, in combustion engines, and in biological systems where the bacterial enzyme nitrogenase is expressed (Sprent & Sprent, 1990).

Plant N requirements

Plant tissues typically contain about 10 – 50 g N kg dry $matter^{-1}$ (1 – 5% N). Thus, in a field producing a yield of 10,000 kg plant dry matter ha^{-1} $year^{-1}$ (DM), several hundred kg N ha^{-1} $year^{-1}$ will be removed in harvested plant parts. Plants take up and assimilate N only in the forms of nitrate (NO_3^-), ammonium (NH_4^+) and, to some extent, simple organic N-containing molecules, *e.g.* amino acids (Näsholm, Huss-Danell & Högberg, 2000, 2001). While the N in NH_4^+ and amino acids is readily incorporated into a plant's organic components, NO_3^- must first be reduced to NH_4^+, which is an energy-demanding process.

Even though agricultural soils may contain several thousand kg N ha^{-1}, at any given time only a small fraction of it is present in forms that plants can take up. Inorganic N (NO_3^- and NH_4^+) typically constitutes less than 5% of total soil N, the remainder being present in complex mixtures of many different N-containing organic compounds (Whitehead, 1995; Tate, 2000). While some of these compounds are available for plant uptake, or readily accessible for soil organisms to degrade into smaller molecules and inorganic N, other fractions of the soil organic N pool are more stable, being resistant to varying degrees to degradation (Tate, 2000). Since the rate of degradation of organic N is not usually high enough to sustain high crop yields, intensive crop production systems rely on inputs of N, via N fertilization or biological N_2 fixation (Whitehead, 1995).

N and crop yields

In agricultural ecosystems, the availability of N is tightly coupled to plant productivity. The tremendous increases in cereal yields that occurred in the 1950's and 1960's, during the "green revolution", were achieved by the concurrent development of high-yielding crop varieties and their cultivation with high applications of N fertilizers (Bohlool *et al.*, 1992; Vance, 1997). In many grassland systems without legumes, plant DM increases linearly with the amount of N fertilizer applied, up to about 300 kg N ha^{-1} $year^{-1}$ (Whitehead, 1995).

Biological N_2 fixation

Biological N_2 fixation refers to the bacterial conversion of atmospheric N_2 to ammonia (NH_3), catalyzed by the enzyme nitrogenase. The nitrogenase reaction is supplied with energy in the form of ATP and reducing power from electron (e^-) carriers, usually ferredoxin (Marschner, 1995):

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP \quad \text{(equation 1)}$$

Fixed NH_3 is rapidly transformed into plant-available NH_4^+ at neutral and acidic pH. As shown in equation 1, a quarter of the electrons involved in the reaction are used to reduce H^+ to H_2. H_2 production is an inherent part of the nitrogenase-catalysed reaction mechanism, and under suboptimal conditions it may consume considerably more than the minimum 25% of the energy and electrons allocated to nitrogenase. However, some N_2-fixing organisms express an uptake hydrogenase, catalyzing the oxidation of H_2 to H_2O coupled to ATP production. Thus, uptake hydrogenase activity can recycle some or most of the energy 'lost' in H_2 production (Simpson, 1987; Sprent & Sprent, 1990; Marschner, 1995). Nitrogenase is very sensitive to O_2, so biological N_2 fixation generally occurs under anaerobic or microaerobic conditions (Sprent & Sprent, 1990).

Free-living N_2-fixing bacteria

Biological N_2 fixation is a strictly prokaryotic process, since the ability to express nitrogenase and fix N_2 is found only in certain bacteria. Nevertheless, free-living N_2-fixing bacteria occupy a wide range of habitats (*e.g.* soil, seawater, freshwater and animal guts) and are highly metabolically diverse, including heterotrophic, autotrophic, aerobic, microaerobic and anaerobic organisms (Zuberer, 1998). Heterotrophic N_2-fixing bacteria such as *Azotobacter*, *Azospirillum*, *Bacillus*, *Clostridium,* and *Pseudomonas* depend indirectly on energy derived from photosynthesis. Thus, they derive their Carbon (C) sources in competition with other heterotrophic organisms, and have a competitive advantage in any habitat that is rich in organic C but low in combined N (Sprent & Sprent, 1990). Autotrophic bacteria generally fix N_2 under strictly anaerobic conditions, and do not evolve O_2 while they are photosynthesising. Examples of bacteria that exploit this strategy include the genera *Rhodospirillum* and *Chromatium*. Another autotrophic N_2-fixing bacterium, *Arthrobacter fluorescens*, does not rely on

photosynthesis, but can use hydrogen gas as an energy source for fixing CO_2 and N_2 (Sprent & Sprent, 1990).

Cyanobacteria

As in higher plants, photosynthesis in cyanobacteria results in the formation of O_2. The ability to fix N_2 is found in several cyanobacterial genera, and N_2 fixation often occurs in special cells, heterocysts, which do not photosynthesize and have modified cell walls that restrict O_2 diffusion. N_2 fixation by cyanobacteria (both free-living and in associations with fungi and plants) occurs in a wide range of habitats. Of particular agricultural importance is the association between cyanobacteria and water ferns in the genus *Azolla*, which can supply valuable inputs of N to rice fields (Sprent & Sprent, 1990; Zuberer, 1998).

Root nodule symbioses

Two groups of N_2-fixing microorganisms, the phylogenetically diverse group of bacteria referred to as rhizobia and the actinomycete genus *Frankia*, have the ability to induce a highly specialized structure in plants: root nodules (Fig. 1). The formation of root nodules leads to a symbiotic relationship between the plant (host) and the N_2-fixing organism (symbiont), in which the plant derives NH_4^+ and in return supplies the symbiont with energy. Rhizobia nodulate plants belonging to the legume family *Fabaceae*, and plants in the non-legume genus *Parasponia* (family *Ulmaceae*) (Sprent & Sprent, 1990). *Frankia* strains reportedly form symbioses with plants belonging to 25 genera in eight different plant families, commonly referred to as actinorhizal plants (Huss-Danell, 1997). According to a phylogenetic analysis of chloroplast gene sequence data, legumes and actinorhizal plants occur in a single clade, suggesting that all plants forming root nodule symbioses have a common ancestor (Soltis *et al.*, 1995).

The root nodule offers a very favourable environment for the symbiont, including release from competition with other soil microorganisms for reduced C and nutrients, allowing it to reach high population densities and express nitrogenase. Supported with carbohydrates derived from the plants' photosynthesis, symbiotic microorganisms inside root nodules may fix N_2 at much higher rates (expressed as kg N ha^{-1} $year^{-1}$) than free-living heterotrophic N_2-fixing organisms (Marschner, 1995). Unlike most rhizobia, *Frankia* genotypes are also known to fix N_2 when free-living. Like cyanobacteria, *Frankia* can localize nitrogenase in specialized cells, so-called *Frankia* vesicles, where it is protected from O_2 (Huss-Danell, 1997). Rhizobia on the other hand, depend on O_2 exclusion mechanisms provided by the host plant in order to express nitrogenase (Fig. 1).

Legumes-rhizobia

Fabaceae, the family of legumes, is the third largest family of flowering plants, including about 650 genera and 18,000 species (Sprent, 1995). Leguminous trees and shrubs are highly abundant in the tropics and subtropics, but the family also contains a large number of annual and perennial herbaceous plant species that are distributed in both tropical and temperate regions. Investigations of nodulation

have found that far from all of the taxa examined (covering about 57% of the genera and 20% of the species in the family) are nodulated. *Fabaceae* can be divided into three sub-families. In the sub-family *Caesalpinioidae*, which comprises about 1900 mainly woody and tropical species, 23% of the examined species have nodules. A higher proportion, about 90% of examined species, is nodulated in the sub-family *Mimosoidae*, comprising about 2700 mainly woody species in the tropical, sub-tropical, and temperate regions. The third, and largest, sub-family, *Papilionoidae*, comprises about 13,000 woody and herbaceous species, including the forage legumes investigated in studies I-IV (Table 1). In *Papilionoidae*, about 97% of the examined species are nodulated (de Faria *et al.*, 1989; Sprent, 1995).

A wide range of legumes are cultivated around the world, either for their protein-rich seeds (grain legumes) or for entire shoots (forage legumes). Grain legumes contribute 25-35% of the global human protein intake (Vance, 1997; Graham & Vance, 2003). The most common legumes included in human diets are bean (*Phaseolus vulgaris* L.), broad bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), cowpea (*Vigna unguiculata* L.), lentil (*Lens esculenta* L.), pea (*Pisum sativum* L.), and pigeon pea (*Cajanus cajan* L.), while peanut (*Arachis hypogea* L.) and soybean (*Glycine max* L.) are commonly used sources of vegetable oil, and of proteins for the chicken and pork industries (Graham & Vance, 2003).

Table 1. *Common names, Latin binomials and nodulating organisms of forage legumes investigated in the studies underlying this thesis, and some of the other forage legumes used in temperate and northern grasslands acording to Frame, Charlton & Laidlaw (1998). a, annual; p, perennial;* Rlt, Rhizobium leguminosarum *bv.* trifolii.

Common name	Scientific name	Nodulated by
Species investigated in studies I-IV:		
Alsike clover (p)	*Trifolium hybridum* L.	*Rlt*
Lucerne (p)	*Medicago sativa* L.	*Sinorhizobium meliloti*
Red clover (p)	*T. pratense* L.	*Rlt*
White clover (p)	*T. repens* L.	*Rlt*
Others:		
Arrowleaf clover (a)	*T. vesiculosum* Savi.	*Rlt*
Birdsfoot trefoil (p)	*Lotus corniculatus* L.	*Mesorhizobium loti*
Caucasian/kura clover (p)	*T. ambiguum* M. Bieb.	*Rlt*
Crimson clover (a)	*T. incarnatum* L.	*Rlt*
Greater lotus (p)	*L. pedunculatus* Cav.	*M. lotii*
Sainfoin (p)	*Onobrychis viciifolia* Scop.	e.g. *S. meliloti*
Strawberry clover (p)	*T. fragiferum* L.	*Rlt*
Subterranean clover (a)	*T. subterraneum* L.	*Rlt*
Sulla (p)	*Hedysarium coronarium* L.	*Rhizobium hedisari*
Tagasaste (p)	*Chamaecytisus palmensis* Christ.	e.g. *M. loti*
Zigzag clover (p)	*T. medium* L.	*Rlt*

The most widely used forage legumes in temperate and northern areas are the perennials birdsfoot trefoil, lucerne (syn. alfalfa), red clover, white clover, and, less commonly, alsike clover. In areas that have a pronounced dry period, *e.g.* Australia and drier areas of New Zealand, the winter annual subterranean clover is very commonly used (Frame, Charlton & Laidlaw, 1998). In addition to these species, other forage legumes that are important in certain areas, are used in niche situations, or have interesting potential, are also listed in Table 1. N_2 fixation in legume symbioses has been estimated to amount to approximately 70 – 90 Tg N $year^{-1}$ globally (Brockwell, Bottomley & Thies, 1995; Vance, 1997), with cultivated crops contributing about 40 Tg $year^{-1}$ (Danso, 1995).

N_2-fixing legume root nodules (Fig. 1) are the products of intricate processes at the legume-rhizobial interface. Among the many interrelated steps are recognition of molecules on the rhizobial cell wall by the host legume, activation of specific nodulation genes in both the plant and the rhizobia, and morphological changes in the legume root as rhizobia invade root cells. As a consequence of these intimate interactions, there is often a high degree of legume-rhizobial genotype specificity (Vance, 1996; Graham, 1998) and large variations in nodulation and N_2 fixation efficiency among different combinations of legumes and rhizobia (Marschner, 1995).

Investigations of host specificity have played an important role in the classification of rhizobia (Table 1). In recent years new methods, especially DNA sequence analyses, have been used to explore the taxonomy of rhizobia further and partly revised the classification based on host specificity (Young & Haukka, 1996; Young *et al.*, 2001). According to the current taxonomy, the group of bacteria classified as rhizobia belong to the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium*, and *Sinorhizobium* in the α-subclass of proteobacteria, and the genera *Burkholderia* and *Ralstonia* in the ß-subclass of proteobacteria (Sy *et al.*, 2001; Young *et al.*, 2001; Chen *et al.*, 2003). Characterization of bacteria isolated from nodules of as yet unexplored legumes may result in the classification of further bacterial species as rhizobia (Moulin *et al.*, 2001).

Methods to measure N_2 fixation

Reliable measurements of N_2 fixation are essential for any attempt to elucidate why activities vary in different conditions. The most direct method for measuring N_2 fixation in plants nodulated by N_2-fixing bacteria (for simplicity: N_2-fixing plants) is to incubate plants in N_2 gas enriched with the heavier stable N isotope, ^{15}N, and subsequently analyse the $^{15}N/^{14}N$ ratio in their tissues by mass-spectrometry. However, since plants can only be incubated in sealed containers for short durations and since this approach is not convenient in field situations, $^{15}N_2$ incubation has very limited use in the field (Danso, 1995). Cultivating N_2-fixing plants with N_2 in air as the only N-source is also a direct method, since the plant N content will correspond to the amount of N_2 fixed. However, grassland soils that are totally free of plant-available N are very rare or non-existent (Whitehead, 1995), so the method has little relevance for forage legumes in the field.

For these reasons, several alternative methods have been developed and used in field measurements of N_2 fixation, including the N difference (ND), ^{15}N natural abundance (NA), and ^{15}N isotope dilution (ID) methods. The NA and ID methods are suitable for integrated estimations of N_2 fixation in the field over relatively long time periods, *e.g.* seasons or years. While ID measures N_2 fixation over a defined time period, from the application of ^{15}N to harvest, NA measures N_2 fixation over a longer time period, up to a plants' entire lifetime (Huss-Danell & Chaia, 2005). NA and ID are also considered to be more reliable and precise than ND measurements (Danso, 1995). Acetylene reduction activity (ARA, see below) measurements give point-in-time estimates of N_2 fixation but are not easily applied in the field (Danso, 1995). A thorough description of these four methods is given in Paper I.

The acetylene reduction activity (ARA) method

The ARA method relies on the fact that nitrogenase, among for several alternative substrates, also has a high affinity for acetylene, C_2H_2. Thus, ARA is an indirect method, it does not measure the actual N_2 fixation process but the activity of the N_2-fixing enzyme. When nitrogenase is exposed to an atmosphere containing 10% C_2H_2, by enclosing the nodulated roots in a gas-tight vessel, the entire electron flow through the enzyme will be directed to the reduction of C_2H_2 to etylene, C_2H_4. Nitrogenase activity can then be assayed by measuring the production of C_2H_4 over time, using gas chromatography (Ledgard & Steele, 1992; Vessey, 1994; IV). ARA is relatively cheap and gives rapid results of analyses, as compared to isotope-based methods. It is non-destructive, which makes it very suitable for following changes in nitrogenase activity in plant individuals (Warembourg, Lafont & Fernandez, 1997; IV). The method has however several shortcomings. The acetylene reduction rate needs to be converted to N_2 fixation (the C_2H_2/N_2 ratio) through calibration with a direct method (*e.g.* $^{15}N_2$ incorporation), which is expensive and laborious. ARA can only measure N_2 fixation over short time periods, and is therefore not suitable for whole-season estimations. Furthermore, the change in N metabolism following incubation in acetylene can cause a decline in nitrogenase activity, leading to underestimation of actual N_2 fixation (Minchin *et al.*, 1983). The method is therefore only recommended for comparative studies, *e.g.* for following relative changes in nitrogenase activity over time, and when the acetylene-induced decline is the same for all treatments (Minchin *et al.*, 1983).

The NA method

The heavier stable N isotope, ^{15}N, is present in nature in small amounts; atmospheric N_2 contains only 0.3663% ^{15}N. The relative abundance of ^{14}N and ^{15}N in the biosphere varies as a result of discrimination against the heavier isotope during biological, chemical, and physical processes. Since such natural variations are generally very small, ^{15}N natural abundance is commonly expressed as $\delta^{15}N$, defined as the parts per thousand deviation from the $^{15}N/^{14}N$ ratio (R) of atmospheric N_2 (Hauck, 1973):

$$\delta^{15}N = ((R_{sample} - R_{atm}) / R_{atm}) * 1000 \qquad \text{(equation 2).}$$

In situations where the $\delta^{15}N$ of plant-available soil N is different from 0 ($\delta^{15}N$ of atmospheric $N_2 = 0$), the $\delta^{15}N$ in an N_2-fixing plant ($\delta^{15}N_{fix}$) provides a measure to calculate the proportion of N derived from N_2 fixation (Ndfa, Fig. 2) in the N_2-fixing plant as follows (Amarger *et al.*, 1979; Shearer & Kohl, 1986):

$$Ndfa = (\delta^{15}N_{ref} - \delta^{15}N_{fix}) / (\delta^{15}N_{ref} - B) \qquad \text{(equation 3)},$$

where $\delta^{15}N_{ref}$ is the $\delta^{15}N$ of the non-N_2-fixing reference plant and B is the $\delta^{15}N$ of the N_2-fixing plant when relying on atmospheric N_2 as the sole N source (Fig. 2). B is included in the caclulation to account for ^{15}N discrimination in the N_2-fixing plant (Yoneyama *et al.*, 1986; Högberg, 1997; Evans, 2001).

The method (used in study III) is convenient since there is no requirement to add ^{15}N-enriched fertilizer to the soil, thus minimizing disturbance to the plant-soil system. It is also a reliable and precise method, provided that the difference in $\delta^{15}N$ between the soil and atmosphere is sufficiently large ($\geq$ 5 parts per thousand), that care is taken to account for ^{15}N discrimination in the N_2-fixing plants (B in equation 3; II), and that appropriate reference plants are used.

Both the NA and ID methods are based on the assumption that the N_2-fixing plant and the non-N_2-fixing reference plant take up soil N with an identical $^{15}N/^{14}N$ ratio. However, the $^{15}N/^{14}N$ ratio of soil N can vary between N pools (*e.g.* organic *vs.* inorganic soil N, NH_4^+ *vs*. NO_3^-), soil depths, and over time (Högberg, 1997), and adding ^{15}N-enriched fertilizer may lead to uneven distributions of ^{15}N in the soil (Witty, 1983; Danso, Hardarson & Zapata, 1993). It is therefore important for the reference plant and the N_2 fixing plant to have similar N uptake dynamics and for them to acquire N from the same soil depth.

Grassland N dynamics

Productive grasslands are often sustained with N from industrial fertilizers, but efficient legume-rhizobia symbioses may provide sufficient N to partly or entirely replace the need for N fertilization. In addition, the pool of plant-available soil N may be refilled via atmospheric N deposition, degradation of soil organic N, circulation of N via grazing animals, and application of organic and green manure (Fig. 3).

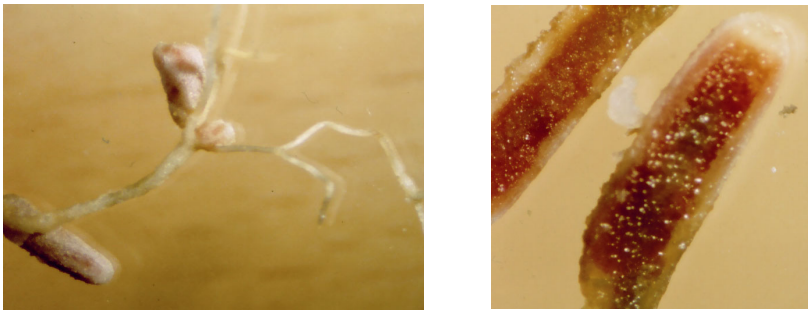

Fig. 1. Nodules on a red clover root (left) and sections of red clover nodules cut longitudinally (right). The expression of leghaemoglobin, which is a part of the O_2 exclusion mechanism, gives rise to the pink colour of the nodule surface (left) and the red colour of the internal tissues (right), and indicates that there is N_2 fixation activity in the nodules. Photographs taken under a stereomicroscope by Ann-Sofi Hahlin.

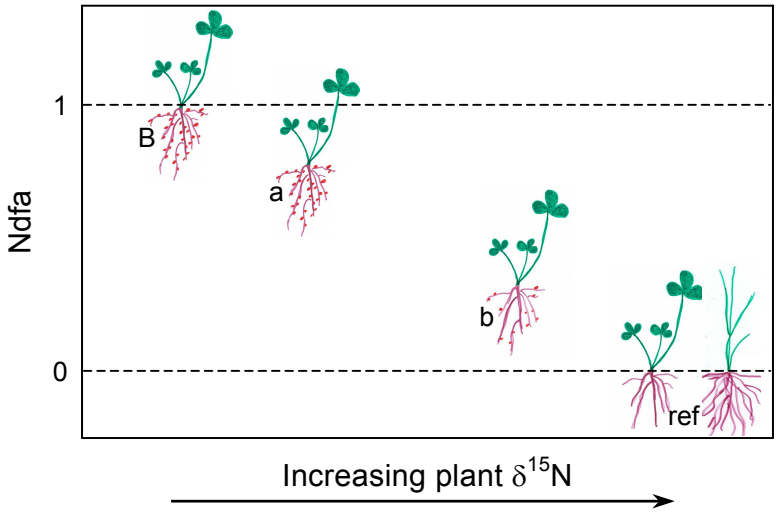

Fig. 2. Illustration of the relationship between plant $\delta^{15}N$ and the proportion of plant N derived from N_2 fixation, Ndfa, in a situation where the plant-available soil N has a higher $\delta^{15}N$ than atmospheric N_2. The B value is the $\delta^{15}N$ of a plant that derives all its N from N_2 fixation (B). Plant (a) derives most of its N from N_2 fixation and has a low $\delta^{15}N$. Plant (b) derives most of its N from the soil and has a higher $\delta^{15}N$, more similar to the reference plant (ref). The reference plant, i.e. the non-nodulated legume or a non-legume, derives all its N from the soil.

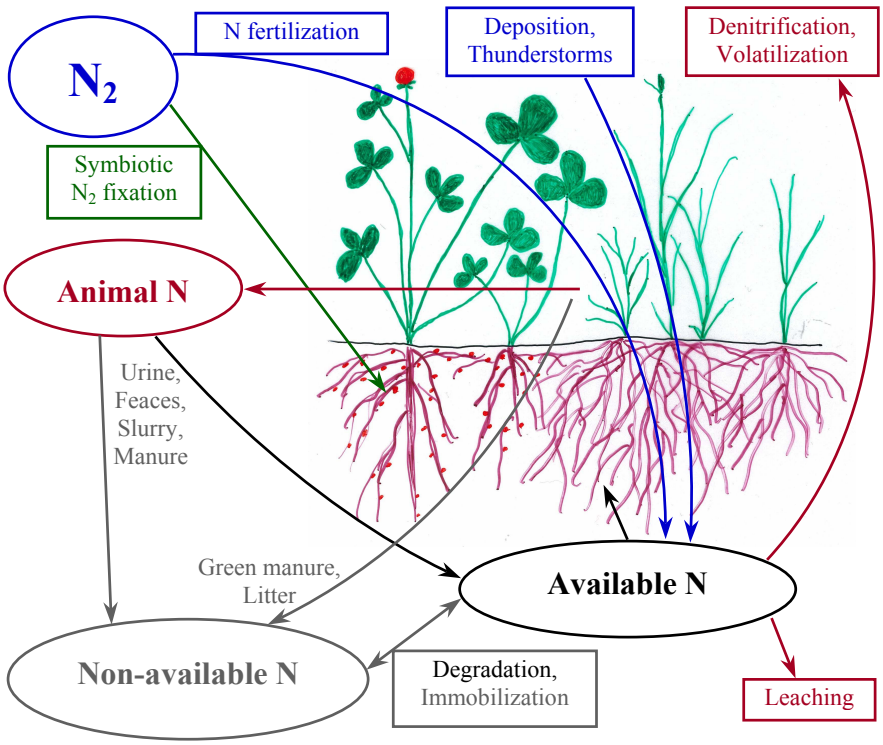

Fig. 3. Inputs and outputs of N to and from the pool of plant-available soil N. Boxes symbolize processes, and circles symbolize pools of N. Green and blue arrows show inputs, black and grey arrows show transformations, and red arrows show losses.

Inputs of N

Industrial N fertilizers

The Haber-Bosch process, where N_2 reacts with H_2 to form NH_3 (Fig. 3), requires high pressure (10-30 MPa) and temperature (up to 1200 °C) and consumes large amounts of energy, mainly derived from natural gas. In 1988 to 1989, global fertilizer N consumption amounted to 78 Tg $year^{-1}$, at an energy cost of 6.6 EJ. Most of the energy (83%) was used in production, and the rest for packaging, transport, and application of the fertilizers (Jensen & Hauggaard-Nielsen, 2003). The energy consumed in the production and application of N fertilizer has been estimated to be equivalent to about 70 and 33% of the total energy inputs in conventional production of grass/clover silage and barley, respectively (Jensen & Hauggaard-Nielsen, 2003). Increasing public concerns about the use of fossil fuels has led to doubts regarding the heavy use of N fertilizers (Peoples, Herridge & Ladha, 1995). Biological N_2 fixation in forage legumes could help address these concerns as a resource-efficient alternative, or complement, to the use of industrial N fertilizers, thereby reducing the consumption of fossil fuels involved in grassland plant production.

N_2 fixation in forage legumes

The N_2 fixation efficiency of legume-rhizobia symbioses is affected by three sets of variables: the plant genotype, the rhizobial genotype and the environment (Whitehead, 1995; Unkovich & Pate, 2000). The plant genotype can be manipulated by selecting plant species or varieties with high N_2 fixation capacity. Given the commonly observed variation in N_2 fixation efficiency among rhizobial genotypes nodulating the same host species, the introduction of effective strains is another frequently-proposed way to increase N_2 fixation (Russel & Jones, 1975; Jones & Hardarson, 1979; Taylor & Quesenberry, 1996). However, this has proved difficult in practice, since many grassland soils host large and diverse populations of rhizobia with which introduced strains have to compete for nodulation of the host legume (Brockwell, Bottomley & Thies, 1995; Hagen & Hamrick, 1996; Barran & Bromfield, 1997).

The third important set of variables influencing N_2 fixation in grassland legumes (the environment) includes a range of abiotic and biotic factors such as temperature, rainfall, drainage, soil pH, soil nutrient status (including N), competition between plants, pests, diseases, and the cutting/grazing regime. These factors are here divided into two sub-classes; purely environmental, referring to factors beyond cultivation control (*e.g.* weather and geographic location), and management, referring to factors that may be manipulated in practical grassland production. For instance, nutrient status and soil properties can be influenced by fertilization and cultivation methods, competition from neighbouring plants can be manipulated by the choice of seed mixtures and weed control, and defoliation severity can be controlled by adjusting the grazing intensity or cutting regimes.

Effects of N fertilization

It has been established that nodulation and N_2 fixation activity in both legumes and actinorhizal plants are strongly inhibited by high levels of plant-available N (Streeter, 1988; Huss-Danell, 1997). In experiments in controlled environments, N has been shown to reduce both nodule biomass plant^{-1} and specific N_2 fixation rates (N_2 fixation in relation to plant or root biomass) (see, for instance, Svenning & Macduff, 1996; Hellsten & Huss-Danell, 2000). In field experiments with legume/grass mixtures, inorganic N fertilization with up to 160 kg N ha^{-1} year^{-1} caused large reductions in N_2 fixation expressed as kg N ha^{-1} year^{-1} (Nfix) and in legume proportion of DM, while the effects on N_2 fixation expressed as Ndfa were small and inconsistent: Ndfa ranged from 0.80 to 0.98 without N fertilization and from 0.68 to 0.93 with N fertilization (Boller & Nösberger, 1987, 1994; Nesheim & Øyen, 1994). In contrast, with higher rates of inorganic N fertilization, also Ndfa was markedly reduced: Ndfa ranged from 0.73 to 0.96 at 20 kg fertilizer N ha^{-1} year^{-1}, and from 0.50 to 0.64 at 400 kg fertilizer N ha^{-1} year^{-1} (Høgh-Jensen & Schjørring, 1994). Even stronger reductions in Ndfa were observed after applications of cow urine corresponding to about 450 to 750 kg N ha^{-1} year^{-1}: Ndfa was in the range 0.8 to 0.9 in untreated plots, and in the range 0.2 to 0.4 in urine-treated plots (Vinther, 1998; Menneer *et al.*, 2003).

Species composition

Many grasses have been found to be strong competitors for soil N, causing legumes to rely more on N_2 fixation as an N source when grown in mixtures with grasses, than when grown alone (Zanetti *et al.*, 1996; Loiseau *et al.*, 2001). Moreover, plant diversity has been shown to increase the soil N use efficiency of the plant community (Hooper & Vitousek, 1998; Zak *et al.*, 2003; Spehn *et al.*, 2005). Thus, neighbouring plants could be expected to oblige the legumes to rely on N_2 fixation to varying degrees depending on the abundance of available N and the competitive ability of the plants involved.

Cutting/grazing regime

A commonly observed response to defoliation, in forage legumes and grasses, is to release stored C and N compounds from roots and other storage tissues, which support the production of new stems and leaves (Gordon *et al.*, 1986; Kim *et al.*, 1991; Ta, MacDowall & Faris, 1990; Volenec, Ourry & Joern, 1996; Louahlia *et al.*, 1999; Morvan-Bertrand *et al.*, 1999). The remaining leaf area is also important for supporting regrowth in cut plants (Cralle & Heichel, 1981; Kim *et al.*, 1991, 1993; Menneer *et al.*, 2004). Thus, removal of all or most of the leaf area by cutting (or grazing) very close to the ground makes regrowth completely dependent on reserves stored below ground. Cutting experimental fields containing lucerne, red clover, timothy, meadow fescue (*Festuca pratensis* L.) and legume/grass mixtures at 12 cm resulted in lower quantities but higher quality (higher leaf/stem ratios) of harvested herbage, and higher DM production in red clover and red clover/grass mixtures during the next growing season, compared to cutting at 4 cm in a study by Fagerberg (1979).

Greenhouse experiments have shown that defoliation of legumes causes a marked decline in the activity of the N_2-fixing enzyme, nitrogenase, followed by a recovery as the shoot regrows (Moustafa, Ball & Field, 1969; Vance *et al.*, 1979; Cralle & Heichel, 1981; Ryle, Powell & Gordon, 1985; Kim *et al.*, 1993). Defoliation intensity has been shown to influence the severity of the decline in nitrogenase activity in white clover (Hartwig *et al.*, 1994), but the possible effects of cutting at different heights, or different frequencies, on nitrogenase activity in red clover are not known. In field experiments, on the other hand, neither cutting frequency (three or six cuts during the growing season, red and white clover) nor cutting height (4 or 10 cm, white clover) has been found to have a significant effect on Ndfa or Nfix in red and white clover grown in mixtures with grasses (Farnham & George, 1994; Høgh-Jensen & Schjørring, 1994; Seresinhe *et al.*, 1994; Høgh-Jensen & Kristensen, 1995).

The response to cutting and grazing of white clover growing in the field together with grass depends on several factors. Due to the production of stolons, which keep their growing points very close to the ground (Fig. 4c), white clover is tolerant to cutting and grazing, but is also subject to shading by taller plants. Frequent cutting or grazing of white clover/grass mixtures may therefore improve white clover performance due to reduced shading by the grass (Whitehead, 1995; Frame, Charlton & Laidlaw, 1998; Menneer *et al.*, 2004). Furthermore, repeated cutting of a white clover/grass field at 3.5 cm stubble height increased both Ndfa

and Nfix compared to cutting at 8.5 cm in a study by Menneer *et al.* (2003), and this was suggested to be caused by more active growth of weeds decreasing the level of plant-available soil N. However, intensive continuous grazing, especially in winter and early spring, may have severe negative effects on white clover growth and N_2 fixation (Frame, Charlton & Laidlaw, 1998; Menneer *et al.*, 2004).

Inter-annual variations

In addition to the variables listed above, all of which influence the legume-rhizobia symbiosis during the growing season, frost hardiness and plant survival rates during winters are very important for N_2 fixation in perennial legumes in temperate and northern areas (Svenning, Rosnes & Junttila, 1997; Frankow-Lindberg, 1999). In a study of overwintering in white clover/perennial ryegrass (*Lolium perenne* L.) mixtures performed at 12 sites across Europe, temperature was found to have a direct effect on the content of white clover in the mixtures and its leaf area index. In addition, white clover performance in spring was positively influenced by its leaf area in the previous autumn (Wachendorf *et al.*, 2001). In an Australian study, legume growth and N_2 fixation were found to be less variable between years in perennial pastures including lucerne than in annual pastures including subterranean clover (Peoples *et al.*, 1998). Developing management practices and legume varieties that minimize the negative effects of such year-to-year variations must be considered a challenge of great importance for researchers and plant breeders.

Pests and diseases

In general, forage legumes are attractive not only to livestock but also to undesirable organisms, i.e. pests. Forage legumes are also susceptible to infestation by diverse pathogens, and a range of slugs, insects, mites, nematodes, fungi (especially *Fusarium* and *Sclerotinia*), bacteria, and viruses may have severe negative effects on production and N_2 fixation in lucerne and perennial clovers (Frame, Charlton & Laidlaw, 1998).

Other N sources: atmospheric deposition, thunderstorms

Plant available N can be carried between locations in the atmosphere, mainly as NH_3 volatilized from areas of intensive livestock production and (to lesser degrees) NO and NO_2, collectively referred to as NO_x, formed by combustion processes in vehicle engines and power plants. In the atmosphere, NH_3 and NO_x may react with water to form NH_4^+, HNO_2, and HNO_3, which may then be deposited with rainfall (Fig. 3; Whitehead, 1995). The magnitude of N deposition varies geographically and temporally, depending on agricultural and industrial activities in the surrounding area. In north-west Sweden, about 1 – 2 kg N ha^{-1} $year^{-1}$ comes from atmospheric deposition, while more than 20 kg N ha^{-1} $year^{-1}$ may be deposited in the south-eastern parts of the country (Lövblad *et al.*, 1992).

Thunderstorms release large amounts of energy, and may cause N and O to combine, resulting in the formation of plant-available NO_3^- (Fig. 3). The amounts of N_2 'fixed' during thunderstorms are probably highly variable, but have been

proposed to be of the order of 30 kg N ha^{-1} in some parts of the world (Sprent & Sprent, 1990).

Turnover of soil N

The term N mineralization refers to the conversion of organically bound N to inorganic N (NH_4^+ and, via nitrification, NO_3^-). Since plants also take up amino acids, the wider term degradation is used here to describe the release of plant-available N from soil organic N (Fig. 3). Soil organic N is contained in plant, animal, and microbial biomass, litter, and humus. Consequently, all nitrogenous compounds found inside living cells are also present in the soil organic N fraction (Tate, 2000). A wide range of heterotrophic soil microorganisms express the enzymes (proteases, amidases and deaminases) needed to hydrolyse the most common soil organic N compounds (amino acids, amino sugars, and polymers of these compounds) to simple organic compounds and NH_4^+. Microorganisms degrading organic N may use the C in the hydrolysed organic molecules as an energy source and assimilate much of the released N, so only the N that is surplus to their requirements will be available for plant uptake (Whitehead, 1995).

N assimilation by soil microorganisms, often termed immobilization, reduces the amount of plant-available soil N (Fig. 3). As a general principle, net immobilization occurs when N, rather than C, is limiting for microbial growth, otherwise net release of plant-available N occurs (Myrold, 1998). N degradation and immobilization has been suggested to be in equilibrium when the C/N ratio in the decomposing organic material is around 20 (Myrold, 1998), implying that decomposition of material with a C/N ratio < 20 generally results in net N degradation.

Nitrification

Nitrification, the process in which NH_4^+ is converted to NO_3^-, proceeds in two steps: oxidation of NH_3 to NO_2^- followed by oxidation of NO_2^- to NO_3^- (Myrold, 1998). The two processes are carried out by specific autotrophic soil bacteria that derive energy for growth from the oxidation of NH_3 or NO_2^-, respectively. In addition, a variety of bacteria and fungi are “heterotrophic nitrifiers”, i.e. they can oxidise NH_3, but cannot utilize the released energy for growth. Nitrification is an aerobic process, and occurs at highest rates at neutral pH (Whitehead, 1995; Myrold, 1998).

Cycling of N in grassland systems

Although the immediate effect of N uptake by plants is to reduce the pool of plant-available soil N, there are several ways in which plant-bound N can be returned to the soil. About 75-95% of the N consumed passes through grazing cattle and sheep (depending on the physiological state of the animals) and becomes available for plant uptake via urine and faeces (Fig. 3; Whitehead, 1995). Furthermore, much of the N removed in harvested plant tissues can be returned to the soil via applications of urine, manure, slurry, and green manure (Fig. 3).

N in urine and faeces

Most of the N in urine from cattle and sheep is present as urea and other water-soluble organic molecules, which can be converted within days to plant-available forms of N (Whitehead, 1995; Vinther, 1998; Menneer *et al.*, 2003). Compared to urine, a higher proportion of the N in faeces is bound in insoluble organic compounds, and is more slowly converted to plant-available N (Menneer *et al.*, 2004). While dietary N concentration has little effect on faecal N excretion, urinary N excretion increases with increasing N concentration in the diet, and varies from about 45 to 80% of total excreted N (Ledgard & Steele, 1992; Whitehead, 1995).

Grazing by large mammalian herbivores such as cattle and sheep results in plant-bound N being released and concentrated in patches of urine and faeces. The concentration of plant-available N in such patches can be very high: the amounts of NH_4^+ in soil a few days after applications of urine corresponded to 350 to 600 kg N ha^{-1} in studies by Vinther (1998) and Menneer *et al.* (2003). In contrast to N excreted during grazing, urine and faeces from livestock kept in buildings or feedlots can be collected and distributed more evenly on a field. The proportion of plant-available N in manure from housed animals is strongly influenced by the handling and storage of the manure. If urine and faeces are separated and the solid fraction is mixed with a bedding material (straw or sawdust), most of the N will be present in organic forms. On the other hand, mixtures of urine and faeces (slurry) contain high proportions of plant-available N, mainly in the form of NH_4^+ originating from the hydrolysis of urea (Whitehead, 1995). During storage of slurry and solid manure, organic N decomposes, causing increases in the proportion of plant-available N. However, NH_4^+ in urine, slurry, and solid manure is subject to substantial losses via NH_3 volatilization both during storage and at the time of application.

Plant physiological responses to defoliation

Besides clearly removing above-ground plant tissue, grazing and cutting also induce physiological responses in plants, such as increasing root exudation of carbohydrates and nutrients (short-term) and reducing root biomass (long-term). Carbon exuded from roots serves as an energy source for heterotrophic soil microorganisms, and defoliation has been shown to increase both the biomass and activity of the soil microbial community (Bardgett, Wardle & Yeates, 1998). The soil microbial activity is also likely to be influenced by changes in soil temperature and humidity following reductions in the vegetation cover. Like above-ground herbivory, below-ground herbivory (by root-feeding nematodes, for instance) can lead to increased root C exudation and stimulation of microbial activity (Bardgett *et al.*, 1999). In turn, soil bacteria and fungi are consumed by animals such as nematodes and springtails (Collembola), leading to the release of nutrients previously immobilized by soil microorganisms (Bardgett & Chan, 1999; Bardgett *et al.*, 1999). In this context, Bardgett, Wardle & Yeates (1998) suggested that, as a response to defoliation, trophic interactions between plants, microorganisms, and soil animals may increase soil N availability and plant N uptake.

Decomposition of plant residues, green manure

N is continuously returned to the soil via various types of plant litter, *e.g.* shed leaves, dead plant parts, damaged roots, and harvest spillage (Fig. 3). The amounts of N contributed by plant litter are generally small, but may become substantial at times of widespread plant death (*e.g.* severe winter damage or pathogen outbreaks). Application of harvested plant tissues directly to a field, green manure, is commonly used in the cultivation of vegetables and crop production systems that do not involve animals, and can provide substantial amounts of plant-available N (Wivstad, 1999). In addition, large amounts of plant litter are incorporated into the soil when grasslands are ploughed, leading to considerable releases of plant-available N (Whitehead, 1995; Vinther & Jensen, 2000).

Nitrogen in plant tissues is mainly bound in proteins, and returns to the pool of plant-available soil N during the microbial degradation of plant residues incorporated into the soil. The rate of N degradation depends to a high degree on the C/N ratio and N concentration of the plant residues (Quemada & Cabrera, 1995; Breland, 1996; Kuo, Sainju & Jellum, 1997). In general, legumes have lower C/N ratios (usually < 25) than grasses (usually > 25) (Whitehead, 1995; Gil & Fick, 2001), but the C/N ratio also varies among plant parts and developmental stages (Quemada & Cabrera, 1995; Wivstad, 1999). A negative relationship between plant C/N ratio and net N mineralization rate has been found in grasslands where lucerne, red clover, and eastern gamagrass (*Tripsacum dactyloides* L.) were grown alone and in mixtures (Gil & Fick, 2001). The critical C/N ratio of 20 (see '*Turnover of soil N*' above) should be considered as a general guideline for the net degradation threshold, but the critical C/N ratio may vary depending on the time-scale (Breland, 1996) and composition (leaf/stem ratio) of the litter (Bloemhof & Berendse, 1995). In a comparison of the degradation of residues from four different legume species, Frankenberger Jr & Abdelmagid (1985) found the critical C/N ratio to be in the range 15 to 33, while Marstorp & Kirchmann (1991) found a critical C/N ratio of about 15 in a similar comparison of six different legumes.

Although the incorporation of plant material with a high C/N ratio (*e.g.* grass herbage) may result in net N immobilization in the short term, the resulting increase in soil organic N may lead to valuable releases of plant-available N in the long term (Breland, 1996; Kuo, Sainju & Jellum, 1997). In addition, short-term N immobilization may be desirable in un-vegetated fields in order to reduce the risk of N losses (Breland, 1996).

The C/N ratio is not the only factor that affects the decomposition of plant residues (Wivstad *et al.*, 2003). Plants that have high digestibility when consumed by animals are also readily decomposed by soil microorganisms, while high contents of complex structural carbohydrates (cell walls) and secondary defence compounds (*e.g.* phenolics) decrease the decomposition rate (Bardgett, Wardle & Yeates, 1998). Thus, legumes are very valuable as green manure crops, due both to their symbiotic N_2 fixation, which adds N to the system, and their relatively low cell wall contents and C/N ratios, resulting in the rapid release of N from legume litter.

Losses of N from grasslands

As well as the 5 – 25% of consumed N that is assimilated by livestock, large losses of N often occur during the recycling of excreted N. Hydrolysis of urea releases NH_4^+, which is rapidly converted to NH_3 due to the basic pH in urine and slurry. Volatilization of this NH_3 (Fig. 3) may lead to the loss of as much as 50% of the NH_4^+ during the storage and application of animal-excreted N (Whitehead, 1995). If the soil conditions favour nitrification (if the soil is well aerated and its pH is neutral for instance) application of organic N at high rates will lead to high concentrations of soil NO_3^-. As NO_3^- is more mobile in the soil than NH_4^+, NO_3^- is subject to losses via movements of soil water, *i.e.* leaching (Fig. 3; Myrold, 1998). In addition, if the levels of O_2 are low, *e.g.* in very wet soils, NO_3^- can be converted to N_2O and N_2, a process termed denitrification that is carried out by certain heterotrophic soil bacteria (Fig. 3; Whitehead, 1995; Myrold, 1998). Loss of inorganic N via complete denitrification, releasing N_2, is the only process that returns N_2 to the atmosphere, closing the cycle that starts by biological or industrial N_2 fixation. The other main product of denitrification, N_2O, has a greenhouse effect that is 180 times stronger than that of CO_2 and also contributes to the destruction of ozone in the stratosphere. The ratio between N_2O and N_2 released during denitrification tends to decrease with increasing proportions of water-filled pore space in the soil, increasing availability of organic C, and decreasing soil NO_3^- concentrations (Whitehead, 1995).

Plant diversity and grassland N dynamics

The BIODEPTH project

In 1995-1996, experimental grassland communities with varying degrees of plant diversity were established at eight sites along north-south and east-west transects across Europe, comprising the BIODEPTH (BIODiversity and Ecological Processes in Terrestrial Herbaceous ecosystems) project. The sites were located in Germany, Greece, Ireland, Portugal, Sweden, Switzerland (one site in each), and the United Kingdom (two sites). Plant diversity gradients were obtained by manipulating species richness and functional group (grasses, legumes, non-legume herbs) richness based on locally, naturally occurring plant species. A major aim of the BIODEPTH project was to investigate whether diversity effects on ecosystem functions were consistent over space and time.

The results have shown positive overall relationships between species richness and above-ground plant DM, and between functional group richness (at a given level of species richness) and above-ground plant DM (Hector *et al.*, 1999). Spehn *et al.* (2005) found that diverse communities exploited more resources than species-poor communities by intercepting more light and taking up more N, concluding that these effects were mainly due to complementarity, *i.e.* niche differentiation in resource use. These findings are consistent with results from several other plant diversity experiments (Tilman, Wedin & Knops, 1996; Tilman *et al.*, 1997, 2001; Hooper & Vitousek, 1998; Zak *et al.*, 2003).

At the BIODEPTH sites in Germany and Sweden, higher soil NO_3^- concentrations were found under pure legume communities than under non-legume and mixed communities (Scherer-Lorenzen *et al.*, 2003; Palmborg *et al.*, 2005), and complementarity was suggested to lead to increases in inorganic N uptake in species-rich communities lacking legumes (Palmborg *et al.*, 2005). In addition, legumes had positive effects on total plant community DM and N yield. Furthermore, ^{15}N analyses of plant tissues showed that the legumes fixed N_2 and indicated that fixed N was transferred from legumes to non-legumes (Mulder *et al.*, 2002; Spehn *et al.*, 2002). Although these studies have highlighted the importance of N_2-fixing legumes in unfertilized grasslands, and the positive relationship between plant species richness and plant N uptake efficiency has been well established, little is known about specific effects of plant species richness on legume N_2 fixation.

Aims and hypotheses

The overall aim of the work underlying this thesis was to increase our understanding of factors that affect N_2 fixation in perennial forage legumes, and thus help identify management practices that maximize the utilization of this resource in northern grasslands. An additional aim was to improve knowledge about N_2 fixation in alsike and red clover. These two species are important in northern grasslands, but have been less intensively studied than white clover.

The aims of study I were (i) to provide an updated overview of published data on N_2 fixation in temperate and northern grasslands, (ii) to compare estimates obtained with different methods, and (iii) to identify factors responsible for variations in the N_2 fixation rates in field situations, *e.g.* legume DM, plant genotype, environmental variables (such as geographic location and inter-annual variations in growth conditions), and management practices (such as N fertilization, the cutting/grazing regime and measures to control species composition). In study II, B values were established for alsike, red, and white clover in symbioses with different Scandinavian *Rhizobium leguminosarum* bv. *trifolii* (*Rlt*) genotypes with the aim to increase the precision of the NA method when applied to northern grasslands. The main aim of study III was to investigate whether N_2 fixation in alsike, red, and white clover is influenced by the species richness and composition of neighbouring vegetation, by applying the NA method to the Swedish BIODEPTH plots. In addition, the effect of using three different reference species with the NA method was also tested in study III. In study IV, red clover was grown in the greenhouse and field and cut at different heights to investigate changes in nitrogenase activity and regrowth following cutting at different heights.

The main hypotheses tested were the following:

1) There would be a positive relationship between cutting height and regrowth rate, and a negative relationship between cutting height and loss of nitrogenase activity in red clover.

2) Increasing species richness would lead to increased Ndfa in alsike, red, and white clover.

3) The overview of data on N_2 fixation measured in experiments with a wide range of N fertilization levels was expected to show that N fertilization has a negative effect on N_2 fixation (both Ndfa and Nfix).

4) Analysis of field experiments performed in a range of locations, from New Zealand to northern Scandinavia, was expected to reveal that Nfix decreases as latitude increases, due to factors such as the lower temperatures and shorter growing seasons at higher latitudes.

5) Negative correlations were expected between N_2 fixation efficiency and discrimination against ^{15}N during N_2 fixation among *Rlt* genotypes isolated from alsike, red, and white clover when inoculated in the different hosts.

Materials and methods

Studied legumes

Frame, Charlton & Laidlaw (1998) state that lucerne is the highest-yielding temperate forage legume. The plant has a large tap root that can reach water deep in the soil profile, allowing the plant to grow in relatively dry soils. It is widely used in Argentina, Canada, China, Italy, the former Soviet Union, and the USA. In southern Scandinavia, lucerne is successfully cultivated in dry soils. Although the cultivation of lucerne is limited in northern areas, it is highly important in grasslands producing silage and hay in warmer temperate areas, and its growth and N_2 fixation have been extensively studied. Due to its wide use in temperate grasslands and the abundance of published field estimates of N_2 fixation in lucerne under various management regimes, the species was included in the analysis of literature data on N_2 fixation (I).

Alsike clover (Fig. 4a) is relatively little used, compared to red and white clover. Nevertheless, it is a legume of considerable potential interest in northern grasslands where it may complement red clover. Alsike clover grows well in cool temperate conditions, it is well adapted to sites that are too wet, acid, or infertile for red clover, and it has a similar feeding value to that of red clover (Frame, Charlton & Laidlaw, 1998). Limited information is available about N_2 fixation in alsike clover.

Fig 4. Alsike clover, *Trifolium hybridum* L., (a), red clover, *T. pratense* L., (b), and white clover, *T. repens* L., (c). The figures are taken from *Temperate Forage Legumes* by J. Frame, J.F.L. Charlton and A.S. Laidlaw (1998), and reproduced with kind permission of CAB International.

Red clover (Fig. 4b) is the dominant forage legume in Scandinavia, and is also widely used in silage- and hay-producing grasslands in other parts of the world (Frame, Charlton & Laidlaw, 1998). Red clover is commonly recognized as having a high feeding value for dairy cows (see, for instance, Frame, Charlton & Laidlaw, 1998; Broderick, Walgenbach & Maignan, 2001). Although red clover has been studied more than alsike clover, there have been few reports of N_2 fixation in red clover under various management regimes.

Globally, white clover (Fig. 4c) is the most widespread clover species used in agriculture (Frame, Charlton & Laidlaw, 1998). It is of particular value in pasture management, due to its high tolerance to frequent defoliation. Although northern grasslands are used mainly for the production of silage and hay, rather than grazing, white clover is also of considerable interest in northern areas. It is adapted to a wide range of climates, including the arctic climate in northern Scandinavia (Svenning *et al.*, 2001), and its ability to spread vegetatively makes it attractive as a constituent in species mixtures for perennial grasslands since it can fill vegetation gaps caused by the death of plants such as red clover. Growth, adaptation to cold climates, and N_2 fixation in white clover have been extensively studied in a wide range of climatic and management situations

The clover varieties (alsike clover cv. Stena, red clover cv. Betty, white clover cv. Undrom) used in studies II – IV are all relatively new, Swedish varieties that have been developed to suit the climate in northern Scandinavia.

Field site and greenhouse plant cultivation

The Swedish BIODEPTH site (III) and the site where field-grown red clover plants were cut at different heights (IV) were located in close proximity to each other in Umeå (63°49'N, 20°17'E), northern Sweden, on an estate belonging to the Swedish University of Agricultural Sciences. The field site has been established on agricultural land and is situated in the boreal zone, where the winters are long and cold, and the topsoil is frozen in the winter. The soil is a fine silty sand with low clay contents (about 4% clay, 58% silt, 38% fine sand) and a pH (H_2O) around 6.0. The fields have been included in a crop rotation including barley, potato, and clover-grass leys, and have been regularly fertilized with N, P, K, solid manure, urine, and slurry.

The Umeå BIODEPTH plots

In early June 1996, 72 plots, each measuring 2.2 by 5 m, were established in Umeå as part of the BIODEPTH project (Mulder *et al.*, 2002). Six plots were not sown with any species: two of these plots were non-vegetated controls, and four were left for invasion by naturally occurring plant species. The remaining 66 plots were sown with 1, 2, 4, 8, or 12 species in 28 unique species mixtures (Table 2). The 12 plant species were selected from perennial species that naturally occur in the area, and were chosen to represent three functional groups: legumes, grasses, and non-leguminous herbs. Each seeded plot received 2000 seeds, amongst which each of the species in the plot was represented in equal numbers. The species composition of the plots was maintained by hand weeding and resowing when necessary. Fertilizer was last added in 1995, and the plots received no fertilizer from 1996 to 2001.

Every year in mid-August, plant biomass higher than 5 cm above ground was sampled from an area of 20 x 50 cm in the centre of each plot. After sampling, the remaining area of the plots was cut at 5 cm height and clippings were removed. DM and N concentrations in the sampled plant material, sorted to species, were determined in 1996, 1998, 2000, and 2002. In plant samples from 1996, 1998, and 2000, ^{15}N abundance was also analyzed and used to calculate Ndfa and Nfix in the clovers by the NA method (III).

Table 2. *Experimental design of the 72 plots at the Swedish BIODEPTH site. Species were: TP,* Trifolium pratense *L. cv. Betty; TH,* Trifolium hybridum *L. cv. Stena; TR,* Trifolium repens *L. cv. Undrom; LC,* Lotus corniculatus *L.; DG,* Dactylis glomerata *L.; PA,* Phalaris arundinacea *L.; PP,* Phleum pratense *L. cv. Jonatan; FO,* Festuca ovina *L.; LV,* Leucanthemum vulgare *Lam.; Ra,* Ranunculus acris *L.; Ru,* Rumex acetosa *L.; AM,* Achillea millefolium *L.*

Legumes				Grasses				Herbs				No. of sown species	No. of replicates
TP	TH	TR	LC	DG	PA	PP	FO	LV	Ra	Ru	AM		
												0[a]	6
x												1	2
	x											1	2
		x										1	2
			x									1	2
				x								1	2
					x							1	2
						x						1	2
							x					1	2
								x				1	2
									x			1	2
										x		1	2
											x	1	2
x						x						2	3
	x	x										2	2
			x					x				2	2
				x	x							2	2
							x				x	2	2
									x	x		2	3
x			x		x	x						4	3
x						x			x	x		4	3
	x	x					x				x	4	2
	x	x						x	x			4	2
			x	x	x			x				4	2
				x			x			x	x	4	2
x	x	x	x	x			x			x	x	8	2
x			x		x	x		x	x	x	x	8	3
	x	x		x	x	x	x	x	x			8	3
x	x	x	x	x	x	x	x	x	x	x	x	12	6

[a] 2 weeded plots, kept without vegetation; 4 unweeded plots, left for weed invasion

Rhizobium leguminosarum bv. *trifolii* (*Rlt*) genotypes

In June 1999, *Rlt* genotypes were isolated according to standard methods (Somasegaran & Hoben, 1994) from alsike, red, and white clover nodules sampled from a clover-dominated plot sown with eight plant species in the BIODEPTH experiment. Thirty *Rlt* isolates were characterized with a genome fingerprinting method (deBruijn, 1992; Carlsson, Wiklund & Huss-Danell, 2005). Three of these isolates were used in the investigation of ^{15}N discrimination in the three clovers (II) and a mixture of six of these isolates was used in the study of nitrogenase activity in red clover cut at different heights (IV).

Greenhouse plant cultivation

The greenhouse used in all the experiments was located in Umeå. The plants were grown under diurnal cycles with 17 h of supplemental light from Philips HPI/T 400 W lamps at approx. 25º C and 7 h without supplemental light at approx. 15º C. Perlite was chosen as growth substrate for all of the greenhouse experiments, since it is an inert substrate with good water-holding capacity, and has been found to be suitable for the studied clover species. Although it provides a controlled environment, the temperature, light, and humidity in a greenhouse are influenced by the outdoor conditions. However, variations in the greenhouse environment were not considered to constrain the reliability of the experiments. Rather, the variations were used to test the hypotheses under different growth conditions.

Results and discussion

High dependence on N_2 fixation in perennial forage legumes

The studies reported in Paper I showed that lucerne, red clover, and white clover all gained a large proportion of their N from N_2 fixation (high Ndfa). When these species were grown in mixtures with grasses Ndfa was even higher; on average around 0.8. This finding was confirmed by the results from a field study performed in grasslands of different ages (the first, second, and third years after establishment): Ndfa in red clover growing in mixtures with grass, sampled in various parts of the growing season, was consistently high, usually ≥ 0.8 (Huss-Danell & Chaia, 2005). Ndfa values found in alsike, red, and white clover in study III were on average around 0.7, irrespective of the level of plant species richness. Data presented in Paper I were assembled from a large number of studies, representing a wide range of localities with differing climatic conditions, soil properties, management regimes etc. In contrast, the management of the experimental field plots in study III was different from that of typical forage-producing grasslands; the plots were harvested only once, late in the season, and comprised species that are not typical forage plants. A conclusion from studies I and III is that management practices do not affect the consistently high Ndfa values in these three perennial forage legumes.

Coupling N_2 fixation and legume growth

In study I, N_2 fixation on an areal basis ranged from just over zero to 350 kg N ha^{-1} $year^{-1}$ in lucerne, up to around 370 kg N ha^{-1} $year^{-1}$ in red clover, and up to 545 kg N ha^{-1} $year^{-1}$ in white clover. The range was smaller in study III; from just over zero up to about 100 kg N ha^{-1} $year^{-1}$ in all three clover species. These figures are based on aboveground plant parts above stubble. The large variations in Nfix were due to large variations in legume DM: since Ndfa was consistently high, there were strong positive correlations between Nfix and DM (I; III).

Estimating N_2 fixation in field situations

For lucerne, alsike clover, red clover, and white clover, formulas were developed for predicting N_2 fixation based on legume DM (Table 3). No variable other than plant DM needs to be measured to use these formulas, which can be applied in practical situations to obtain rough estimates of the amount of N_2 fixed *e.g.* in a farmer's field. This may be of great value for establishing on-farm N budgets or N fertilization plans. Moreover, for establishing N budgets at field or farm levels, and to estimate needs for extra N fertilization, ways of obtaining quick estimates of N_2 fixation might be more valuable than less convenient methods that give the highest possible precision.

Compared to data in the review (I), N_2 fixation rates expressed as kg N kg DM^{-1} were significantly lower in red and white clover growing in the BIODEPTH plots (III; Table 3, two-sample t-test, $P < 0.005$). In study III, the N concentrations in alsike and red clover were significantly lower in plots with more than 1000 kg clover DM ha^{-1} $year^{-1}$, compared to plots with less than 1000 kg clover DM (two-sample t-test, $P < 0.0025$). In plots with < 1000 kg clover DM the N concentration was on average 2.9% both in alsike (n = 14) and red (n = 19) clover, while in plots with > 1000 kg clover DM the N concentration was on average 3.6% in alsike clover (n = 19) and 3.4% in red clover (n = 47). Consequently, if the regression analysis between DM and Nfix was based on plots with < 1000 kg clover DM, N_2 fixation expressed as kg N kg DM^{-1} was higher than when the entire data-set was used, and in red clover it was similar to the value obtained in study I (Table 3). In study III, the white clover N concentration was not lower in plots with > 1000 kg white clover DM, but the overall N concentration (range 1.9 to 5.4%, mean 3.6%) was lower than in the papers cited in Paper I (range 3.0 to 6.0%, mean 4.4%).

Table 3. *Amounts of N_2 fixed (kg N kg DM^{-1}) derived from linear regression analyses between legume DM (kg ha^{-1} $year^{-1}$) and Nfix (kg N ha^{-1} $year^{-1}$).*

Species	Paper I	Paper III	Paper III, < 1000 kg DM
Alsike clover		0.019	0.024
Red clover	0.026	0.018	0.025
White clover	0.031	0.026	0.027
Lucerne	0.021		

Thus, the lower rates of N_2 fixation expressed as kg N kg DM^{-1} found in study III could have been due to N concentrations being lower in the harvested clover biomass, as a consequence of the single harvest late in the season of the

BIODEPTH plots. Leaf/shoot ratios and protein concentrations are known to decrease over time in forage legumes as they mature, flower and set seed (Fagerberg, 1979, 1988; Wivstad, 1997), and this effect may have been more pronounced in alsike and red clover in plots with high clover DM.

In practice, when estimating Nfix in a farmers field, the formulas presented in Paper I should be used rather than the formulas based on study III, since the literature data (I) were derived mainly from productive grasslands with management regimes similar to those applied to cultivated fields. In Paper I, these formulas were obtained by including an intercept, in order to get the best fit to the data. However, such an intercept implies that N_2 fixation occurs even in the absence of legume biomass, which has no relevance. The formulas should therefore be used without the intercept, as presented here in Table 3. Since the formulas have been obtained from data representing a wide range of environmental conditions and management regimes, the formulas can be used to obtain estimates of N_2 fixation in many different situations.

The formulas in Table 3 are based on harvested DM, and thus only provide estimates of the amount of fixed N allocated to plant parts above cutting height. Few studies have measured total Nfix, including stubble and roots, and reports of the proportion of fixed N allocated to plant parts above cutting height range from about 35 to 90% (I). The root/shoot ratio in red clover in red clover/grass leys of differing ages at differing times of the growing season also varies widely, from about 0.3 to 3 (Huss-Danell & Chaia, 2005).

Nitrogenase activity and regrowth of shoots after cutting

N_2 fixation rates, measured in terms of acetylene reduction activity (ARA), were also tightly coupled to growth during regrowth in cut red clover plants (IV). Both the magnitude of the decline in ARA and the rate of leaf area regrowth were influenced by cutting height, in accordance with the fiirst hypothesis listed in '*Aims and hypotheses*' above. From about eight days after cutting, ARA per unit leaf area was fairly constant over time and across cutting treatments. The relatively long period of decline in ARA following cutting indicated that the plants' energy reserves could support nitrogenase activity for some time, or that a strong N sink other than leaves maintained the plants' demand for fixed N. When ARA started to recover, it followed the growth rate of leaf area, illustrating the link between legume growth and N_2 fixation. In spite of the the dramatic loss of notrigenase activity following cutting shown in study IV, Ndfa is known to be high in red clover over the entire growing season (Huss-Danell & Chaia, 2005).

The ND, NA, and ID methods all involve use of legume DM to calculate Nfix. Thus, in situations where legume N concentration and Ndfa are relatively constant (as in the legumes studied here), Nfix estimated by these methods will be inevitably correlated to legume DM. ARA on the other hand, is a yield-independent method, since it measures the actual activity of the N_2-fixing enzyme. The coupling between ARA and leaf area (IV) therefore strengthens the findings that Nfix and legume growth are strongly correlated (I; III).

Influence of neighbouring species, management, and environment

When lucerne, red clover, and white clover were grown in mixtures with grasses, Ndfa was higher than in legume monocultures (I). On the other hand, there was no consistent effect of plant species richness on Ndfa in alsike, red, and white clover (III). Thus, the hypothesis that the positive correlation between plant N uptake and species richness (as noted *e.g.* by Hooper & Vitousek, 1998; Spehn *et al.*, 2005) would lead to a positive relationship between species richness and Ndfa was rejected (the second hypothesis listed in '*Aims and hypotheses*'). In the same experiment (III), no significant effects of species richness on soil NO_3^- and NH_4^+ pools in mixed legume/non-legume communities were found, and the higher DM in species-rich communities was suggested to be supported by leguminous N_2 fixation (Palmborg *et al.*, 2005). It is also possible that increased N degradation at high levels of species richness (Zak *et al.*, 2003) supports the increases in DM and N yield observed with increasing species richness (Mulder *et al.*, 2002; Spehn *et al.*, 2005), and that the amount of soil inorganic N remains more or less unaffected by species richness.

In red and white clover grown in mixtures with grasses, N fertilization up to about 200 kg N ha^{-1} $year^{-1}$ had no effect on Ndfa. Nor was there any apparent relationship between Nfix and N fertilization in these species (I). It was concluded that forage grass species are generally highly efficient in taking up N from the soil, depleting plant-available soil N below levels where suppression of N_2 fixation occurs. The effect of N fertilization up to 200 kg N ha^{-1} $year^{-1}$ was to increase grass DM and consequently reduce the legume proportion of DM in the legume/grass mixtures, rather than to decrease Ndfa in the legumes. On the other hand, higher rates of N fertilization (400 kg N ha^{-1} $year^{-1}$) did decrease Ndfa and Nfix in white clover grown in mixture with grasses (I).

There was no correlation between latitude and Nfix (I), and it was concluded that lucerne, red clover, and white clover were well adapted to the cold climate at high latitudes (up to around 60 ºN), aided by successful plant breeding. Cutting or grazing regimes did not influence Nfix. However, Ndfa in white clover was higher in grazing than in cutting experiments (I), mainly because grazing experiments did not include any white clover monocultures. These findings partly conflict with the third and fourth hypotheses stated in this thesis: Ndfa and Nfix in legume/grass mixtures were negatively affected by N fertilization only at fertilizer applications higher than 200 kg N ha^{-1} $year^{-1}$, and Nfix was not negatively correlated to latitude.

Importance of legume over-wintering

The most important factor influencing Nfix in the literature data was the inter-annual variation in legume DM (I). Legume DM was also important in the species richness experiment, where winter damage had strong effects on legume survival, and consequently on legume DM and N_2 fixation (III). Visual inspection of the BIODEPTH plots in the spring following years with severe winter damage indicated that alsike clover had higher survival rates than red clover. On the other

hand, mildew infestation of alsike clover and drought stress in white clover potentially reduced growth and N_2 fixation in these species in dry years (Palmborg, Carlsson & Huss-Danell, 2004). However, in conditions with sufficient water supply, white clover has the ability to fill damaged patches by vegetative growth. Thus, the studied clovers seem to respond differently to environmental variables that affect clover performance during winters as well as during the growing seasons, and could be expected to complement each other when grown together. In addition, in study III, the presence of grass stubble in the field may have reduced winter damage in the studied clover species. Growing several clovers together, in mixture with grass, in productive grasslands should, therefore, provide a way of increasing the yield stability of perennial clovers, and thus increase the contribution of N_2 fixation in northern grasslands.

Precision of the NA method

Discrimination against ^{15}N in N_2-fixing plants

When using the NA method, B is included to take into account isotopic discrimination during N_2 fixation, N translocation and N transformation within the plant (equation 3), and the results obtained with the NA method are sensitive to the B value used (Høgh-Jensen & Schjørring, 1994; Carranca, de Varennes & Rolston, 1999). In several legumes, B is known to be influenced by the rhizobial genotype (Steele *et al.*, 1983; Bergersen *et al.*, 1986; Ledgard, 1989). The results presented in Paper II showed that B in alsike, red, and white clover were influenced by the *Rlt* genotype, and confirmed that they had a strong influence on Ndfa estimates obtained by the NA method. On the other hand, there was no consistent correlation between B and N_2 fixation efficiency among the different clover/*Rlt* symbioses (II), and the fifth hypothesis was not confirmed. In Paper II it was suggested that the mean B value obtained with the different *Rlt* genotypes should be a representative value of ^{15}N discrimination in the field. Furthermore, there was very little variation in B with plant age or before and after over-wintering, so it was proposed that the same B value could be used when calculating N_2 fixation rates in successive growing seasons with the NA method (II).

In study III, the lowest values of $\delta^{15}N$ in clover shoots sampled in 1996 and 1998 were similar to the B values presented in Paper II. In 2000, however, several clover samples had lower $\delta^{15}N$ than the measured B values. The lowest detected $\delta^{15}N$ in each clover species was therefore used as the respective B value for the whole experiment, as suggested in Paper II and by Hansen & Vinther (2001). B in white clover has been shown to be influenced by nutrition and water conditions during plant cultivation (Ledgard, 1989). Thus, as a consequence of different plant growth conditions, the B values may differ between field-grown and greenhouse-grown plants. Since agricultural soils always contain plant-available soil N, it is impossible to determine the 'true' B value in the field. The difficulties involved in determining an appropriate B value impose some uncertainty on Ndfa values obtained with the NA method. However, these uncertainties should be minimized by using B values established with local *Rlt* strains (as in Paper II) and plants

cultivated in an environment resembling, in terms of light, moisture, nutrition and temperature regimes, that of the field.

Reference species

In Paper III, the use of *Phleum pratense* as a reference species in the calculations of Ndfa in alsike, red, and white clover with the NA method gave significantly higher results than the use of *Leucanthemum vulgare* or *Ranunculus acris* (paired t-test, Fig. 5). However, although the differences were significant, the estimates obtained with the different reference plants were highly correlated and very similar (Fig. 5). There were no significant differences in the estimates of Ndfa obtained using *L. vulgare* and *R. acris* as reference plants (paired t-test; Fig. 5).

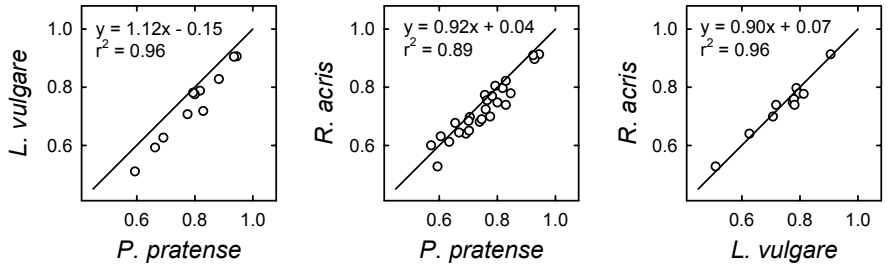

Fig. 5. Proportion of N derived from N_2 fixation, Ndfa, calculated with three different reference species according to the NA method, including data from alsike, red, and white clover. The lines show the 1:1 ratio.

The different results obtained with the different reference plants in study III are in accordance with results of uptake experiments in the field, in which uptake rates of NO_3^-, NH_4^+, and glycine in alsike clover and red clover were found to differ from those in *P. pratense* and *R. acris* (Näsholm, Huss-Danell & Högberg, 2000). Furthermore, uptake of ^{15}N injected as NH_4^+ to different soil depths in the field differed between red clover and *P. pratense*: the latter used ^{15}N in deeper horizons to a greater extent (Jumpponen *et al.*, 2002). These findings show that the assumption that the N_2-fixing plant and the reference plant take up forms of soil N with identical ^{15}N abundance profiles is not strictly valid, and may introduce potential errors in results obtained with the NA method. This problem may be overcome, or at least decreased, by using several different reference plants simultaneously. Using the mean $\delta^{15}N$ of several reference plants reduces the risk of comparing the N_2-fixing plant with a plant that has very different soil N uptake characteristics. However, grasslands may not always contain a large number of non-legume species that can be selected as reference crops. In particular, grasslands managed for silage or hay production usually only comprise one or two grass species in mixtures with one legume. In such cases, weeds, if present, could be sampled and used as additional reference plants.

Use of the recommended B values established in Paper III, and several reference plants, when N_2 fixation in alsike, red, and white clover is measured with the NA method will thus improve the precision and reliability of this method. However, the method is recommended only in situations where the difference between B and the $\delta^{15}N$ in reference plants is ≥ 5 parts per thousand.

Conclusions

Due to their high dependence on N_2 fixation, perennial forage legumes have the potential to provide large inputs of N (> 100 kg N ha^{-1} $year^{-1}$) to northern grasslands. In addition, N fixed by legumes becomes available to neighbouring and succeeding plants via animal excreta and legume litter (Fig. 3). Thus, leguminous N_2 fixation is a resource-efficient alternative, or complement, to the application of N fertilizers in northern grasslands.

The work presented in this thesis has shown that the studied legumes derive at least half, and often more than three quarters, of their N from N_2 fixation when grown in mixtures with grasses or herbs. Fertilizer N applications up to 200 kg N ha^{-1} $year^{-1}$, geographic location, and species richness of neighbouring vegetation all had only marginal effects on Ndfa (I, III). Consequently, Nfix was positively correlated to legume growth, both on an areal basis (kg N ha^{-1} $year^{-1}$; I, III) and on a plant basis (nitrogenase activity during regrowth in cut red clover plants; IV). Rough but useful estimates of Nfix can be obtained with a simple calculation based on legume DM (Table 3). More precise estimates of Ndfa and Nfix can be obtained with the NA method by using appropriate B values (II) and several reference plant species, provided that the difference between B and $\delta^{15}N$ in the reference plants is large enough (≥ 5 parts per thousand).

Alsike clover, red clover, and white clover responded differently to abiotic and biotic factors, and should therefore be expected to complement each other when they are grown together. Thus, including more than one clover species in the seed mixtures could be a way to achieve higher and more stable yields of perennial clovers, and therby increasing the reliance on N_2 fixation in northern grasslands. This interesting possibility stresses the need for further research about the sustainability of northern grasslands.

Future research

Diversity of clover-nodulating rhizobia in northern grasslands

Rhizobial populations in perennial clover nodules are known to be diverse (Leung *et al.*, 1994), and perennial clovers are likely to utilize a broad spectrum of *Rlt* genotypes. It has been shown that certain strains in mixtures of *Rlt* strains can out-compete others for nodulation of the same host, or be preferentially selected by the host (Robinson, 1969; Masterson & Sherwood, 1974; Russel & Jones, 1975; Jones & Hardarson, 1979; Leung *et al.*, 1994; Zeze, Mutch & Young, 2001). Clover-nodulating rhizobia have also been found to exhibit considerable phenotypic diversity, *i.e.* variation in N_2 fixation efficiency. For instance, in a soil population of rhizobia nodulating subterranean clover, an abundant *Rlt* genotype was found to fix N_2 less efficiently than less abundant genotypes (Leung, Wanjage & Bottomley, 1994).

Wide genotypic diversity was found among *Rlt* genotypes isolated from nodules of alsike, red, and white clover growing in the same plot within the Swedish BIODEPTH experiment (Carlsson, Wiklund & Huss-Danell, 2005). In addition, large variations were found in N_2 fixation efficiency between different clover x *Rlt* symbioses, estimated by cultivating the clovers in the greenhouse with no N in the nutrient solution. However, the importance of host selection by perennial clovers in field situations is largely unknown, and there is a need for studies of the distribution and N_2-fixing potential of *Rlt* genotypes in northern grassland soils.

In common experiments performed in Umeå and Tromsø, northern Norway, *Rlt* genotypes have been sampled using red clover trap plants in different parts of the growing season. A total of 429 *Rlt* isolates have been characterized with several DNA fingerprinting methods. These studies have found large genotypic diversity, higher frequencies of certain genotypes, and some evidence that different genotypes may be active in different parts of the season. Selected genotypes are currently being phenotypically characterized: the N_2 fixation efficiency will be measured in greenhouse-grown red clover plants that have been inoculated with common and rare *Rlt* genotypes and supplied with ^{15}N in the nutrient solution (S. Duodu, G. Carlsson, K. Huss-Danell & M.M. Svenning; in thesis by Duodu, 2005).

A new method to study rhizobial diversity

Comparisons of the diversity of rhizobia in nodules with the diversity of rhizobia in soil should increase our understanding of intra- and inter-population competition in field situations. However, making such comparisons has not previously been possible, since acquiring data on the diversity of rhizobia in soil has been difficult, partly because they are difficult to distinguish from many other soil bacteria (Zeze, Mutch & Young, 2001). However, the problem of separating rhizobia from other soil bacteria can now be solved by specifically amplifying sequences that occur only in rhizobia from total DNA extracts from soil samples using the polymerase chain reaction (PCR) and primers with specificity for the

respective sequences. Rhizobial Nod genes are required for host infection and nodule formation, and they offer suitable target sequences since they exist only in rhizobia. Furthermore, studies have shown considerable variation between rhizobial genotypes in the nucleotide sequences of Nod genes (Laguerre *et al.*, 2001; Zeze, Mutch & Young, 2001).

A promising approach for examining diversity within a sample of PCR-amplified fragments is Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis (Clement *et al.*, 1998). In T-RFLP analyses, one of the primers in the PCR reaction is fluorescently labelled. The PCR products are then digested with restriction enzymes and the length heterogeneity of terminal fragments is examined. The number of distinguished terminal fragments is used as a measure of the diversity in the sample. Data outputs from T-RFLP analyses are well suited for multivariate data analyses, e.g. principle component analysis (PCA), for quick and reliable comparisons of samples. So far, T-RFLP has not been applied to rhizobial markers. Analyses based on this method would allow comparisons of rhizobial diversity in nodules and soil, and the investigation of possible relationships between rhizobial diversity and N_2 fixation efficiency.

Other legumes: birdsfoot trefoil, *Lotus corniculatus*

Birdsfoot trefoil is an interesting plant that has a different rhizobial symbiont and differs in nodule anatomy (determinate nodules) from clovers (indeterminate nodules). *Lotus* species also contain condensed tannins, which potentially inhibit animal gut parasites. In addition, condensed tannins may protect herbage proteins from degradation in the rumen, thereby improving the amino acid supply in the ruminant small intestine (Frame, Charlton & Laidlaw, 1998). Birdsfoot trefoil is currently only marginally used in Scandinavian grasslands. When contemplating the possibility of cultivating a legume for the first time, an issue that needs to be considered is whether or not there is a need to inoculate it with a suitable symbiont. Birdsfoot trefoil was included in the BIODEPTH experiment at the Swedish site, and seeds were inoculated with *Mesorhizobium loti*. In the summer of 2003, seven years after establishment, *M. loti* was present in very high population densities in plots containing birdsfoot trefoil, but not in soils where the host plant was not growing (G. Carlsson, J. Lysholm, C. Palmborg & K. Huss-Danell, unpublished).

Birdsfoot trefoil established slowly in the BIODEPTH plots in Umeå; during the first three years it was almost absent in species mixtures. However, from the fifth year onwards it has established well in all of the plots where it was sown, and samples are currently being analyzed for ^{15}N in order to calculate Ndfa with the NA method.

Plant diversity in productive grasslands

The BIODEPTH experiment has established a correlation between plant productivity and species richness in unfertilized grassland communities that are harvested once per growing season and include 'non-forage' species. Further issues to consider are whether or not the effect is also present in productive grasslands harvested twice or three times per season, and managed to provide high-quality forage. These issues could be addressed in field experiments in which several different species/varieties of grasses, legumes, and non-legume herbs with potentially high feeding values are grown across a diversity gradient. The experimental plots could be managed as productive grasslands, with applications of different levels of N fertilization, slurry, and/or manure in order to achieve the desired legume proportions in harvested DM, and with several harvests to optimise the quality of the harvested fodder. To adopt a whole-system approach, measured response variables could include Ndfa, DM production, over-wintering, feeding value, N losses, and the occurrence of pests and diseases.

Substantial evidence indicating that fixed N is transferred from legumes to neighbouring species is provided by many of the studies cited in Paper I (Boller & Nösberger, 1987; Heichel & Henjum, 1991; Farnham & George, 1993, 1994; Walley *et al.*, 1996; Elgersma & Hassink, 1997; Elgersma, Nassiri & Schlepers, 1998), and the species richness experiment described in Paper III (Mulder *et al.*, 2002). Data on plant DM and pools of soil inorganic N in the species richness experiment (III) indicate that N acquisition is optimised in productive grasslands by N_2 fixation in legumes and efficient N uptake by non-legumes (Palmborg *et al.*, 2005). All these findings highlight not only the important benefits of legumes to neighbouring plants, but also the positive effect of non-legumes in reducing levels of plant-available N, thereby obliging the legumes to acquire most of their N from N fixation. Nevertheless, measured rates of N transfer from legumes to grass are highly variable, and the factors that most strongly affect N transfer have not yet been elucidated. N transfer could potentially be influenced by management practices, *e.g.* the cutting regime and choice of seed mixtures. Investigations of the effects of such factors could also be included in the experiment outlined above.

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Sammanfattning

Kväve (N) är det näringsämne som behövs i störst mängd och som ofta begränsar växtproduktionen i tempererade och nordliga områden. Luften består till ca 80 % av kvävgas, kväve som inte kan nyttjas direkt av växter utan måste omvandlas till en kväveform som växter kan ta upp. Kvävgas används både i den industriella framställningen av kvävegödsel, med hög energiåtgång, och i biologisk kvävefixering som utförs av vissa bakterier. Symbiotisk kvävefixering sker i rotknölar hos växter som har infekterats av kvävefixerande markbakterier. Växten gynnas då av att den får tillgång till en kvävekälla, ammonium, som hela tiden fylls på. Även biologisk kvävefixering kräver stora mängder energi, och bakterier som lever i symbios med växter får energi i form av kolhydrater från växtens fotosyntes.

Växter odlade i nordliga områden, med ljusa svala somrar, innehåller relativt höga halter av enkla kolhydrater, vilket ger dem goda foderegenskaper. Foderbaljväxter, t.ex. klöver och lusern, har särskilt höga fodervärden p.g.a. höga proteinhalter och låga halter av komplexa kolhydrater. Foderbaljväxter kan odlas i ett stort geografiskt område, från varma och torra delar av Australien och Nya Zeeland till arktiska delar av norra Skandinavien. Dessutom kan de bilda kvävefixerande symbioser med marklevande rhizobium-bakterier. Denna avhandling beskriver studier som har syftat till att öka kunskapen om faktorer som påverkar foderbaljväxters kvävefixering, med fokus på perenna foderbaljväxter i nordliga områden, för att maximera utnyttjandet av denna resurs i odling av betes- och slåttervallar.

En genomgång av publicerade studier av kvävefixering i perenna foderbaljväxter i fält, i huvudsak i norra Europa och Nordamerika, visade att lusern, rödklöver, och vitklöver erhöll huvudparten, minst hälften och ofta mer än tre fjärdedelar, av sitt kväve från kvävefixering, särskilt då de odlades i blandningar med gräs. Den stabilt höga andelen kväve från kvävefixering hos dessa tre baljväxter påverkades inte av omvärldsfaktorer såsom väder- och odlingsförhållanden. Detta innebär att det fanns ett starkt positivt samband mellan kvävefixering (kg N per ha och år) och skördad baljväxtbiomassa (ton torrvikt per ha och år). Enligt sambanden fixerade lusern 21 kg N, rödklöver 26 kg N, och vitklöver 31 kg N per ton torrvikt. Dessa samband kan användas till grova men enkla och snabba uppskattningar av kvävefixeringens storlek i respektive baljväxt, som kan vara av stort värde för att kunna uppskatta vallars gödslingsbehov samt för att kunna göra kvävebalansberäkningar på fält- och gårdsnivå. Eftersom de data som har använts för att ta fram sambanden kommer från många studier med skilda väder- och odlingsförhållanden kan sambanden användas för uppskattning av kvävefixering i många olika situationer.

Även i ett fältförsök där antalet samodlade växtarter varierades erhöll alsikeklöver, rödklöver och vitklöver åtminstone hälften av sitt kväve från kvävefixering, oavsett artantal. I detta försök beräknades kvävefixeringen med hjälp av en metod som baseras på naturliga skillnader i förekomsten av den stabila kväveisotopen ^{15}N, den så kallade naturlig abundans-metoden (NA-metoden). I

jordbruksjord är förekomsten av ^{15}N ofta något högre än i atmosfärens kvävgas, varför växter som fått allt sitt kväve från jorden har högre ^{15}N-förekomst än kvävefixerande växter. Metoden är relativt enkel, men kräver tillgång till masspektrometeranalys av ^{15}N. Eftersom de naturliga skillnaderna i ^{15}N-förekomst är väldigt små är metoden känslig för graden av diskriminering mot ^{15}N i den kvävefixerande växten (det så kallade B-värdet), samt för valet av icke-kvävefixerande referensart. I en växthus-baserad studie bestämdes B-värden för alsike-, röd- och vitklöver i symbios med olika skandinaviska rhizobium-stammar, i syfte att öka precisionen när NA-metoden tillämpas i skandinaviska vallar. I fältstudien användes tre olika referensarter för att öka metodens pålitlighet.

I en fjärde studie följdes kvävefixeringsaktivitetens dynamik efter skörd hos rödklöverplantor som skördades med olika stubbhöjd. Resultaten visade ett negativt samband mellan stubbhöjd och förlust av kvävefixeringsaktivitet, samt att kvävefixeringsaktivitetens återhämtning följde bladens återväxt. Vidare påvisades ett positivt samband mellan stubbhöjd och bladåterväxtens hastighet, både hos plantor odlade i växthus och i fält.

Studierna som denna avhandling baseras på har visat att de perenna foderbaljväxterna alsikeklöver, lusern, rödklöver och vitklöver, tack vare den stabilt höga andelen kväve från kvävefixering, potentiellt kan bidra med ansenliga mängder kväve (> 100 kg N per ha och år) till vallar i nordliga områden. Kväve som fixerats av baljväxterna kommer dessutom samodlade och efterföljande grödor till godo, t.ex. via nedbrytning av döda växtdelar, via boskapens avföring och urin, och via gröngödsling. Sammanfattningsvis konstateras att foderbaljväxters kvävefixering utgör ett alternativ eller komplement till industriellt framställd kvävegödsel, och kan därför bidra till ökad resurseffektivitet i jordbruket. För att baljväxters kvävefixering helt ska kunna ersätta industriellt framställd kvävegödsel krävs dock att man kan uppnå höga, stabila, och förutsägbara skördar av de odlade baljväxterna, vilket förutsätter fortsatt forskning och utveckling inom nordlig växtodling.

A personal outlook

The earth's human population is growing, and the amount of available agricultural land is decreasing. This imposes high demands on agriculture around the world to increase its resource use efficiency. Increasing the contribution of biological N_2 fixation in agriculture, with concomitant replacement of fossil fuels by photosynthesis-derived ATP, could play a major role in such improvements. I hope that this thesis will provide some guidance on how to measure the N supply from N_2 fixation in agriculture, and how to increase the use of this resource.

In a wider sense, the great challenge for plant and animal production systems around the world is to develop ways to remain economically and ecologically sustainable while meeting constantly increasing demands for agricultural products. In northern countries, however, the high demand for agricultural products may not always be apparent (instead, the industrialization of agriculture, aided by ambitious subsidies, has resulted in highly efficient farming systems with the potential to produce more food than is consumed within them). In addition, producers in northern areas may feel threatened by competing imports from areas with lower production costs, and the efforts to produce cheap products challenges both the economic and ecological sustainability of farms in industrialized parts of the world.

On the other hand, 800 million people on the earth are hungry. It is a tragic irony that farmers in one part of the world are straining to produce food at increasingly low costs in order to sell it in a well-supplied market, while farmers in other parts of the world face the frustration of not being able to feed their hungry. Before farmers in the third world can even start to think about ecological sustainability, their farms have to attain economic sustainability, *i.e.* their land must produce reliable yields that are large enough both to feed the family and to give a surplus that can be sold for profit. This situation imposes very important challenges for farmers, governments, non-governmental organizations and agricultural scientists around the world.

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Finally, in the context of writing a doctoral thesis in agricultural science, I would like to borrow these few words by "the greatest writer of our time"*:

He said "What do doctors know about farms, pray tell?"

- from the song *Motorpsycho Nightmare*,
released on the Bob Dylan album *Another side of Bob Dylan*, 1964

*Johnny Cash at San Quentin 1969, introducing a song written by Cash/Dylan.