

Nitrogen fixation by legumes in Australian dairy pasture systems: review and prospect

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Executive Summary

Quantitative measurement of N₂ fixation has rarely been conducted in Australian dairy pastures. However, from the available data it is quite clear that annual N₂ fixation rates in Australian dairy pastures are generally low, due to low pasture legume content. With typical legume contents of grazed pastures less than 30% of total pasture biomass production, annual N₂ fixation in herbage is of the order of only 50 kg ha⁻¹ or less. Other factors which are likely to be able to contribute to increased N₂ fixation input (rhizobia, mineral N management, soil acidity, soil water contents) will have little impact until such time as legume contents are increased. In contrast, for some hay systems, such as those using lucerne, N₂ fixation input is likely to be high (200–300 kg ha⁻¹ yr⁻¹).

As long as clover contents remain low there is little value in study or measurement of N₂ fixation, nor in complex modelling, as N₂ fixation will be of little quantitative importance. However, where legume contents, and thus potential N₂ fixation are increased, there is scope for investigation into potential increases in N input from this source, which is invariably linked to fertiliser application, the management of grazing and the N returns in urine and dung. These are the major influences on sward N dynamics and N₂ fixation. The inoculant rhizobia used for white clover in Australia (TA1) is likely to be suboptimal. Isolated in Tasmania in 1953 it has been shown to be inferior in N₂ fixation compared to other strains on a number of occasions.

Modelling is often used to describe the probable influence of management and/or climate on the operation of agricultural systems. Reliable modelling of N₂ fixation requires capacity to integrate the effects of grazing and pasture composition on soil mineral N dynamics, the influence of this mineral N on nodulation and on suppression of N₂ fixation, and environmental and management influences on soil rhizobial populations. Currently no models have demonstrated this capacity. At present, a suitably calibrated regression model is probably the best option for modelling N₂ fixation in dairy pastures.

Environmental benefits ensuing from increasing N₂ fixation and substituting this for fertiliser N are likely to be greater off-farm (reduced GHG emissions at site of fertiliser manufacture) than on, if fertiliser management is optimal. Nevertheless substituting fixed N for fertiliser N should have some modest environmental and feed efficiency benefits.

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1 Introduction

Nitrogen (N) in plants is the primary source for animal and milk protein production in dairy systems. Biological dinitrogen (N_2) fixation is the process whereby specialised microorganisms are able to convert N_2 from the atmosphere into ammonia (NH_3) via an enzyme called nitrogenase. This ‘fixed’ N can then be incorporated into microbial and plant protein. This is a very important process because, along with fertiliser nitrogen (industrial nitrogen fixation), it provides the main entry point for nitrogen into agricultural systems. There are four principal forms of N_2 fixation which relate to the type of N_2 -fixing bacteria and to the strength of their relationships with plants. Some bacteria fix N_2 in a *free-living* state, while others fix N_2 in association with plants. The associations with plants range from rather loose associations around plant roots (*associative*), *endophytic* N_2 -fixing bacteria residing in the vascular tissues of some grasses, and finally, highly-evolved, complex *symbioses*, involving morphological changes of both microbe and plant in specialised root structures (nodules). In legume symbioses the N_2 -fixing bacteria pass all the fixed N as NH_3 directly on to their plant hosts which incorporate it into plant protein. The N_2 -fixing symbioses with legume plants (e.g. clovers, medics, peas, beans) are the most important because they are more highly evolved and able to fix much greater amounts of N than the other associations. For example, symbiotic N_2 fixation can provide for all of the N requirements of pasture legumes, while for pasture grasses the N_2 fixing associations are unlikely to be able to provide more than 10% of grass N demand, even under optimal conditions .

Because the Australian dairy industry is primarily based on pasture resources for feed supply, the industry is concentrated in environments where there is a constant supply of water, either from rainfall or irrigation (Bethune and Armstrong 2004), thus providing for the year round feed base required. While growth of some species may be reduced by low winter temperatures there is still usually some pasture growth over the winter period, in contrast to some other regions of the world where very low winter temperatures halt clover growth (Wachendorf *et al.* 2001), there is potential for year round symbiotic N_2 fixation in Australian temperate pastures.

1.1 Scope of this review

The objective of this work is to “*Undertake and prepare a comprehensive review paper documenting the state of knowledge of N_2 fixation in dairy pastures in Australia*”

While the review is clearly directed at Australian field studies, the limited Australian research requires recourse to salient reviews or critical information from studies elsewhere, where Australian field data are not available. Unfortunately there have been very few studies measuring N_2 fixation in Australian dairy

systems, and a number have used crude estimations without measurement. There does not appear to have been a previous review specific to N₂ fixation in Australian dairy systems. While annual legumes are also important components of some dairy pastures in Australia, this review focuses primarily on perennial high rainfall, or perennial irrigated legume pastures. Thorough reviews on N₂ fixation in annual legume pastures can be found elsewhere (e.g. Peoples and Baldock 2001; Peoples *et al.* 2001; Peoples *et al.* 1998; Unkovich *et al.* 1997) and Unkovich *et al.* (1998) provide a detailed study of N dynamics in grazed annual clover pastures.

Some of the more pertinent reviews on N₂ fixation in grazed perennial pastures include Menneer *et al.* (2004), Ledgard and Steele (1992), Ledgard (2001), Jarvis *et al.* (1995) and Haynes and Williams (1993), while the reviews of Carlsson and Huss-Danell (2003) and Cuttle *et al.* (2003) are also quite useful. Eckard (1998) provides salient background to the N dynamics of dairy pastures in Australia and likely responses to N fertiliser application but does not explicitly deal with N₂ fixation.

2 Operation of the N₂ fixing legume symbiosis under field conditions

Symbiotic N₂ fixation is a complex process involving two organisms in a dynamic partnership subject to a range of environmental and management influences. While the physiological operation of the symbioses are generally understood (Neera and Geetanjali 2007; Schulze 2004), an ability to predict N₂ fixation under field conditions requires site specific knowledge of partner and symbiotic responses to relevant environmental and management parameters (Russelle 2008).

2.1 Mineral N depresses N₂ fixation

While legumes have the capacity to fix atmospheric N₂ *via* their symbioses with rhizobia, they are also able to take up soil mineral N like non-legume plants. Indeed they have a preference for use of soil mineral N such that the nodulation and N₂ fixation processes are down regulated or turned off in the presence of significant concentrations of mineral N (see Streeter 1988). The dynamic relationship between these factors is illustrated in Figure 1 for two annual pasture legumes grown under controlled (glasshouse) conditions. The figure highlights that (i) both nodulation and N₂ fixation are down regulated by available mineral N (ii) small amounts of mineral N can stimulate growth, nodulation and N₂ fixation, and (iii) there are significant differences between species in the extent of these relationships. Although it is not illustrated here, the same legume species with different rhizobia may also vary in their sensitivity to soil mineral N (Unkovich and Pate 1998).

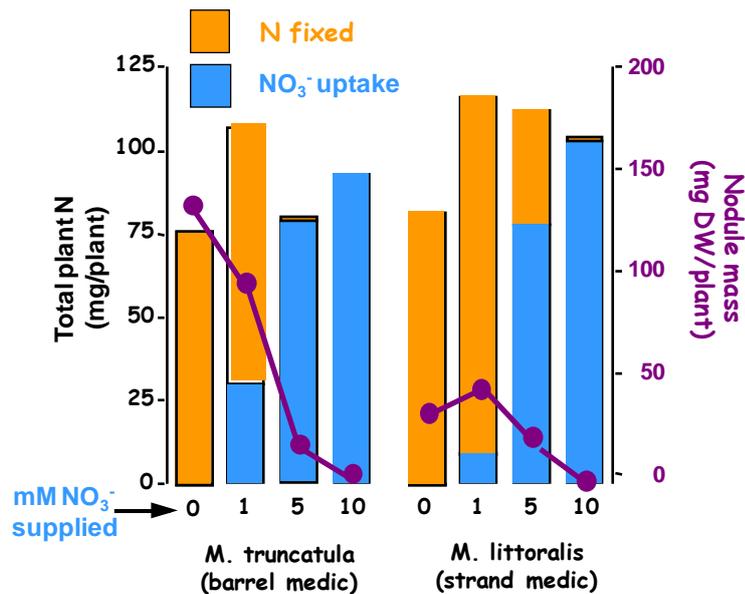


Figure 1 Relationship between mineral N (nitrate) supply and nodulation and N₂ fixation for two annual pasture legumes. From Pate and Unkovich (1999).

In the context of grazed dairy pastures, this means that returns of N in urine and dung will suppress N₂ fixation if most of the resultant mineral N is not taken up by companion grasses. Similarly, application of fertiliser N to legume pastures will suppress clover N₂ fixation (see e.g. , Ledgard *et al.* 2001; Ledgard *et al.* 1996). In the absence of fertiliser N application, this phenomenon is most likely to occur under urine patches which may result in concentrations of readily mineralisable N equivalent to $\geq 1000 \text{ kg ha}^{-1}$ (Haynes and Williams 1993), which would be expected to suppress N₂ fixation and nodulation for some months. Soil nitrate concentrations high enough to suppress nodulation and N₂ fixation may also arise in rain fed pastures at the end of summer and into autumn, particularly in pastures containing annual species.

In a study in northern Victoria Mundy (1987) used a ¹⁵N tracer to follow fertiliser uptake and N₂ fixation in an irrigated white clover/ryegrass pasture following the application of 5 or 100 kg N ha⁻¹ (Figure 2). Pasture clover content was reduced from 40% in the 5 kg N ha⁻¹ treatment to 20% with 100 kg N fertiliser applied. However, total clover soil mineral N uptake was not reduced. Fertiliser N uptake was substituted for N₂ fixation which was reduced from 74 to 45% of clover herbage N. The authors indicated that this suppression of N₂ fixation continued for at least 10 weeks. These data demonstrate the dynamic interaction between soil mineral N availability, clover and grass growth, and symbiotic N₂ fixation, and in this case, in the absence of grazing animals. Increased availability of soil mineral N reduces the competitive advantage of N₂ fixing legumes under low soil mineral N supply, switches off legume N₂ fixation and reduces pasture clover content.

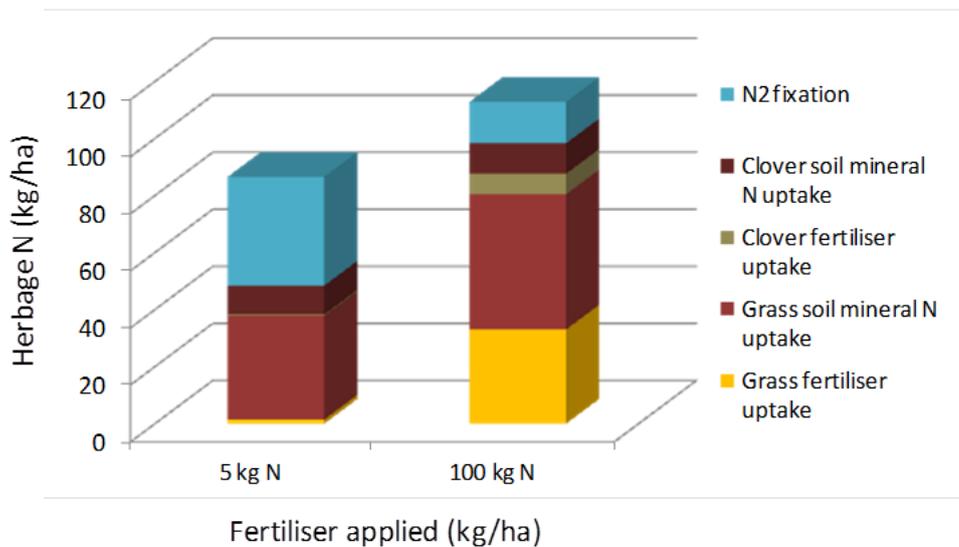


Figure 2 Sources of herbage N in an irrigated white clover/ryegrass pasture in northern Victoria 35 days after the application of 5 or 100 kg ha⁻¹ N fertiliser. (Plotted from the data of Mundy 1987).

A second example of the effect of N fertiliser application on N₂ fixation in an irrigated white clover/ryegrass dairy pasture from northern Victoria is shown in Figure 3. Following application of 100 kg N, N₂ fixation remained at about 50% of that for unfertilised pasture over the following two weeks.

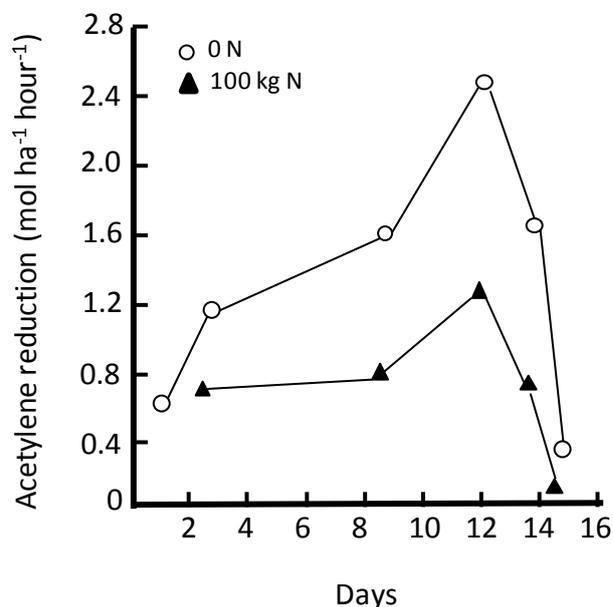


Figure 3 The sensitivity of N₂ fixation (relative nitrogenase activity) to applied nitrogen in irrigated white clover in northern Victoria. (Redrawn from Mundy *et al.* 1988).

In a study of a rain fed white clover pasture in western Victoria (McKenzie *et al.* 1998) application of 45 kg N ha⁻¹ had no measurable impact on N₂ fixation, regardless of fertiliser type (Table 1). However, in

this case, differences between treatments might not be expected since prior grazing may have provided much more mineral N than the modest fertiliser application, and this effect may last many months (Menneer *et al.* 2004). Furthermore, very low legume content (9%) and thus low N₂ fixation (2–4 kg ha⁻¹) mask potential treatment effects on N₂ fixation.

Table 1 Response of a ryegrass/white clover pasture to fertilisers measured 37 days after application. All treatments were applied at a rate of 45 kg N ha⁻¹. (From McKenzie *et al.* 1998).

Fertiliser	%Ndfa*	N fixed (kg ha ⁻¹)
none	69	3.6
Phosphorus, potassium and sulphur	60	2.5
Urea	58	1.9
Pastureboosta blend	59	2.0
Ammonium nitrate	65	3.0
Di-ammonium phosphate (DAP)	65	2.9
Ammonium sulphate	64	2.7
Ammonium nitrate and sulphur	70	4.1
Urea + PKS	69	3.5
Pastureboosta + PKS	66	3.5
Ammonium nitrate + PKS	64	2.6
DAP + PKS	64	2.7
Ammonium sulphate + PKS	61	2.6
Ammonium nitrate and sulphur + PKS	66	3.4

*% of N derived from the atmosphere

In a second experiment McKenzie *et al.* (1998) applied 0–60 kg N ha⁻¹ to the pasture and N₂ fixed ranged from 0.83–7 kg N ha⁻¹, and while they indicated a positive linear response to increasing N fertiliser, this seems an unlikely conclusion given the difficulties of measuring such small differences in N₂ fixation at the field level (Unkovich *et al.* 2008). The results of these two experiments highlight the limited value in measuring N₂ fixation in low clover content pastures.

2.2 Soil water limitations to N₂ fixation

N₂ fixation activity of legume nodules declines under high soil water content associated with flood irrigation (Mundy *et al.* 1988) or water logging, possibly as a consequence of the production of ethanol in nodules under anoxic conditions (Sprent and Gallacher 1976). Decreases in soil oxygen availability and subsequent declines in N₂ fixation may also result from pugging or increased bulk densities under grazing (Menneer *et al.* 2001). Nitrogenase activity also declines at low soil water contents, and probably more so than plant growth (Davey and Simpson 1990), although it is difficult to separate these as it is often unclear whether N₂ fixation activity reduction is due to reduced plant demand for N or a reduced supply of photosynthate to the nodules. Nodule activity declines with water stress, but can only recover if the water stress is moderate (Sprent 1971). Re commencement of N₂ fixation after more severe stress requires regrowth of existing nodules (3–4 days, Engin and Sprent 1973), but after drought initiation and growth of completely new nodules is required, which would take longer (5–10 days, Davey and Simpson 1990).

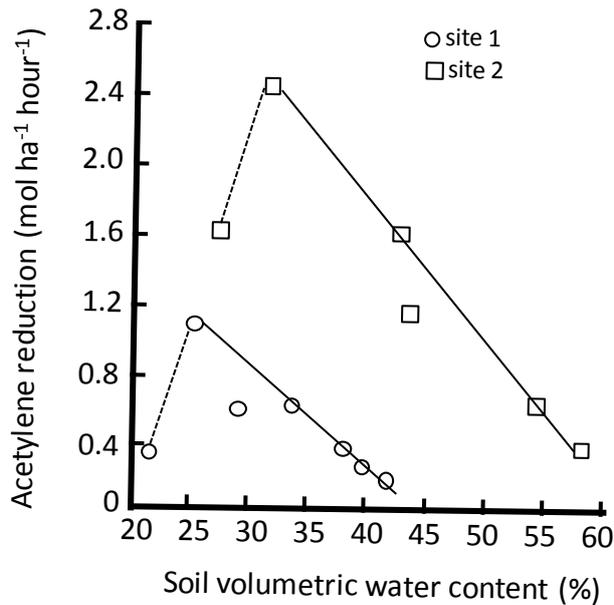


Figure 4 The sensitivity of symbiotic nitrogen fixation (relative nitrogenase activity) to soil water content. (Redrawn from Mundy *et al.* 1988).

An example of the sensitivity of the N₂ fixing nodule to soil water content is given in Figure 4 which shows nitrogenase activity for two irrigated white clover pastures in northern Victoria. The two sites had different soil bulk densities, thus different pore space, and presumably oxygen availability, but the relative effects of soil water content were maintained. For irrigated systems there is thus a challenge to maintain soil water content within the non-limiting range to maximise N₂ fixation activity.

White clover may be more susceptible to water stress than other perennial pasture legume species and lucerne more tolerant (Kelly *et al.* 1989; Neal *et al.* 2009). Ostrowski (1972) considered white clover to be more susceptible to water than heat stress. This is a probable explanation for observed increases in pasture growth in summer in high rainfall or irrigated (Kelly and O'Brien 1992) environments when clover contents are increased, and potential increases in N₂ fixation during the warmer months of the year (see Eckard 1998; Eckard 2001). The low drought tolerance of white clover may be a significant cause of its poor persistence in many systems. Even under irrigated conditions, white clover may only maintain maximal growth for 4–5 days after irrigation (Mason *et al.* 1987). Pasture clover content remains higher with more frequent irrigation (Dunbabin *et al.* 1997). Compared to other perennial legumes, lucerne may be more tolerant of water stress, producing greater biomass than five other perennial legumes when grown under deficit irrigation (Neal *et al.* 2009).

2.3 Temperature and N₂ fixation

There is considerable inconsistency in the literature relating temperature to N₂ fixation in white clover. Whitehead (1995) suggests that fixation does not occur below a soil temperature of 9°C, but other evidence indicates that it occurs over a wider range of temperatures (ca 2–40°C), and is relatively insensitive to temperature over quite a wide range (15–30 °C) (Liu *et al.* 2010). Provided that there is adequate water available, white clover can maintain a constant N₂ fixation rate over the 20–33° temperature range (Ryle *et al.* 1989), and thus the summer temperatures experienced in the Australian dairy regions should not be prohibitive to N₂ fixation. Low temperatures were suggested to affect N₂ fixation less than NO₃⁻ uptake (Hatch and Macduff 1991). While Bouchart *et al.* (1998) reported the opposite, that N₂ fixation in white clover declined more than NH₄⁺ uptake at low temperatures (6°C), they also showed that this was due to reduced clover N demand, not to a direct effect of low temperature on N₂ fixation *per se*. Temperatures as low as 7°C were not limiting to N₂ fixation in white clover (Svenning and MacDuff 1996). In the study of three white clover pastures in western Victoria (Riffkin *et al.* 1997), dependence of white clover on N₂ fixation did not decline during the winter months. Dart and Day (1971) found that most of the legume species studied (including red clover but not lucerne) continued to fix N₂ down to 2°C , and N₂ fixation in lucerne was maintained up to 37°C. Nodulation and N₂ fixation in lucerne was suggested to cease below 8°C (Bordeleau and Prévost 1994) but this is not consistent with other reports. Temperature *per se* is thus unlikely to have any significant direct influence on N₂ fixation at the field level under Australian dairy climates, although clearly it will exert indirect influence *via* effects on plant development, plant water relations, and on the mineralisation of soil N.

2.4 Rhizobia and N₂ fixation

The microsymbiont bacteria contained in commercial inoculants that partner the primary pasture legumes in Australian dairy systems are given in Table 2. Here it can be seen that while development of legume inoculants has continued for lucerne and annual *Trifolium* species, there has been no development of rhizobial inoculants for perennial *Trifolium* species since the initial release of TA1 in ca 1954.

Table 2 *Rhizobia used in Australian commercial inoculants for legumes used in dairy systems*

Inoculant group	Rhizobia	Strain	Recommended legume hosts
B	<i>Rhizobium leguminosarum</i> bv. trifolii	TA1, (since ca 1954)	Perennial <i>Trifolium</i> spp (white, red, strawberry clover)
AL	<i>Sinorhizobium meliloti</i>	RRI128 (since 2001)	Lucerne
C	<i>Rhizobium leguminosarum</i> bv. trifolii	WSM1325 (since 2005)	Annual <i>Trifolium</i> spp

2.4.1 Inoculant rhizobia for perennial *Trifolium* species

The current rhizobia (Table 2) used in the commercial inoculant for white (*Trifolium repens*), red (*Trifolium pratense*) and strawberry (*Trifolium fragiferum*) clovers in Australia was isolated in Tasmania and first tested on clovers in 1953 (Paton 1957). Initially named BA-Tas, it was renamed TA1 (Waters 1957). In combination with strain NA30 it was recommended for use as the commercial inoculant for clovers at that time (Waters 1957), primarily because it was effective on a wide range of annual and perennial *Trifolium* species (Paton 1957). Although TA1 was later shown to be poorly competitive with native rhizobia (Brockwell *et al.* 1972) on alpine soils, it appeared to fare better in agricultural soils (Dudman and Brockwell 1968). Strain NA30 was annexed from the culture (Brockwell and Gibson 1968) and TA1 remains the single strain in the Group B commercial inoculant for white clover available today (Pulsford and Bullard 1997). *Rhizobium leguminosarum* bv. trifolii strain TA1 became a benchmark organism, and studies deploying this strain of rhizobia developed into a voluminous literature internationally, but little of this relates to its field performance in N₂ fixation, particularly with the varieties of white clover grown in Australia. As far as I am able to ascertain this has in fact not been examined, although it has been shown to be less effective in N₂ fixation on clovers than a range of other field isolates on a number of occasions (see Brockwell and Gibson 1968; Riffkin *et al.* 1999a). Under laboratory conditions Gibson *et al.* (1975) found that very few field isolates could match its N₂ fixing effectiveness. Meanwhile, there is a strong tendency for self selection of suitable rhizobia in the field (Baird 1955; Brockwell *et al.* 1972), and this may be reflected in the superior performance of some field isolates in western Victoria when compared to TA1 (Riffkin *et al.* 1999b). There is little doubt that significant improvements could be made with respect to the N₂ fixation effectiveness of the microsymbiont used for white clover in Australia. However, while legume contents of dairy pastures remain low, there may be little benefit realised from such improvement.

2.4.2 Inoculant rhizobia for lucerne

Nodulation and rhizobiology of lucerne in Australia has been much better studied than that of white clover, probably because lucerne also has important roles outside of the dairy industry. These studies have generally shown lucerne to nodulate well and fix nitrogen with the range of rhizobia that persist in agricultural soils in Australia (Ballard *et al.* 2003; Bowman *et al.* 1998) and in this respect lucerne may be more gregarious than some other *Medicago* species (Ballard *et al.* 2003).

2.5 N₂ fixation, soil acidity and salinity

The average soil pH on 44 dairy farms across the country in the survey of Gourley *et al.* (2010) was 5.3 (CaCl₂), and 4.8 across 71 dairy farms in western Victoria (Riffkin *et al.* 1999a). At these low pH's rhizobia (Richardson and Simpson 1989) and nodulation (Munns 1965b) are likely to be severely

compromised. Effects may be primarily manifest through poor survival of rhizobia at low pH (Ballard *et al.* 2003; Richardson and Simpson 1989) and/or inhibition of legume nodulation by toxic aluminium (Unkovich *et al.* 1996). The white clover inoculant strain (TA1) was shown to be less persistent in acid soil than five of six other strains in a field comparison on annual clovers (Watkin *et al.* 2000), so this rhizobia may be relatively sensitive to low soil pH. Consistent with this, in the survey of Riffkin *et al.* (1999a) clover dependence on N₂ fixation was negatively correlated with rhizobial numbers on light textured soils (mean pH 4.6), but not on medium textured soils (mean pH 4.9). Similarly, the amount of N₂ fixed positively correlated with soil pH on light but not medium textured soils. Although lucerne has been shown to “select” compatible, effective rhizobia under acid soil conditions (Ballard *et al.* 2003), N₂ fixation will most likely be suboptimal under the typical soil pH of Australian dairy farms. While rhizobial partners more able to withstand acid soil conditions can be identified (Howieson *et al.* 1991), these are not a long term solution to the problem of acid soil development which requires the addition of lime to provide improved soil chemical conditions for plant growth, legume N₂ fixation (Howieson and Ballard 2004) and general soil health. Lucerne is generally considered more susceptible to problems of low soil pH than some other legume species and nodule establishment can be an issue (Munns 1965a).

Irrigation of a white clover/ryegrass pasture with saline water reduced clover growth but not grass growth (Smith *et al.* 1993), yet N₂ fixation did not appear to be impaired at the salinities encountered (5 dS m⁻¹). Similarly long-term applications of sewage sludge to soils under dairy pasture in NSW did not impair the operation of white clover symbioses or the effectiveness of the naturalised soil rhizobia (Munn *et al.* 1997).

2.6 Pests and diseases

A range of parasitic nematodes are known to infect white clover across the dairy zone, and to reduce root growth and nodulation (McLeish *et al.* 1997), with bacterial feeding nematodes being particularly important as pasture legume content increases (Yeates and Stirling 2008). Some pests feed directly on clover nodules (Gerard 2001) and in this case would severely compromise N₂ fixation capacity. Although specific, direct effects of pests and diseases on N₂ fixation have not been studied (quantified) in the field, the density of some nematode species was correlated with the amount of N₂ fixed and the dependence of white clover on N₂ fixation on light textured soils in a field survey in western Victoria (Riffkin *et al.* 1999a). This would imply that nematodes might be having a measurable (but unquantified) effect on white clover N₂ fixation in this region. If the white clover content of pastures is higher this may constitute a significant restraint on N₂ fixation potential.

3 Quantitative estimates of N₂ fixation in Australian dairy pastures

3.1 Interpreting N₂ fixation data in grazed pasture systems

Before we start examining the available quantitative data it is worth considering a framework for interpretation of symbiotic N₂ fixation field data. From a systems point of view the key elements are the interactive effects of soil mineral N, clover to grass ratio, and grazing pressure, on N₂ fixation (Figure 5).

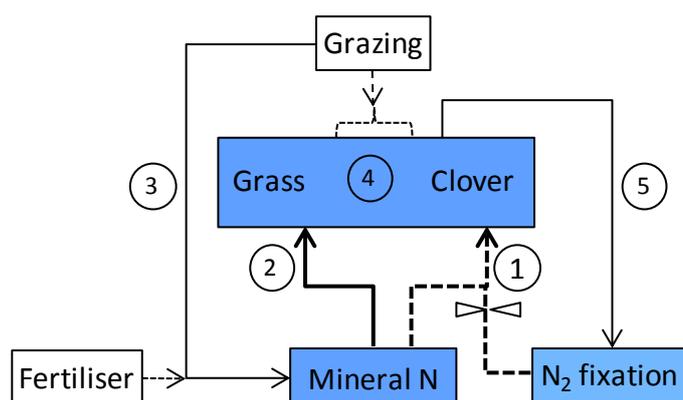


Figure 5 Key influences on N₂ fixation in a grazed clover/grass pasture

Figure 5 highlights that

- 1) N₂ fixation generally tops up clover N demand where it cannot first be satisfied by soil mineral N supply
- 2) grasses and other non-legumes are stronger competitors for mineral N than legumes and thus the mineral N demand of non-legumes tends to be met first
- 3) the N returns in urine and dung from grazing animals, and fertiliser N, result in increased mineral N in the soil which tends to favour growth of grasses over legumes and to reduce N₂ fixation directly, but contrary to this
- 4) at the lower end of the grazing spectrum, increased grazing intensity may favour the growth of clover over grass due to reduced shading of the clover
- 5) when clover content is lower it is forced to depend more on N₂ fixation for its N requirement because more of the mineral N will be taken by the larger grass component.

One must be careful when interpreting N₂ fixation data, for example a clover pasture fixing 100% of its nitrogen might be considered excellent, but if the total clover production is only say 500 kg ha⁻¹ then only the tiny amount of 12 kg N ha⁻¹ might be fixed. Conversely, if only 20 kg N were fixed this might be quite acceptable for a pasture with a clover yield of 10 t ha⁻¹, in which case %Ndfa would be low but total

clover N might be a respectable 300 kg N ha⁻¹. Unambiguous data on N₂ fixation for pastures thus includes information on clover total N or dry matter, as well as the amount of N₂ fixed, and the proportional dependence on N₂ fixation (%Ndfa).

Potential (maximum) N₂ fixation is established by legume total dry matter production (Figure 6), with the realisation of this potential primarily determined by mineral N availability, soil fertility (primarily phosphorus), and the abundance and competence of the microsymbiont rhizobia. The figure shows that a clover production of 14 t ha⁻¹ could potentially sponsor up to 700 kg of N₂ fixation annually.

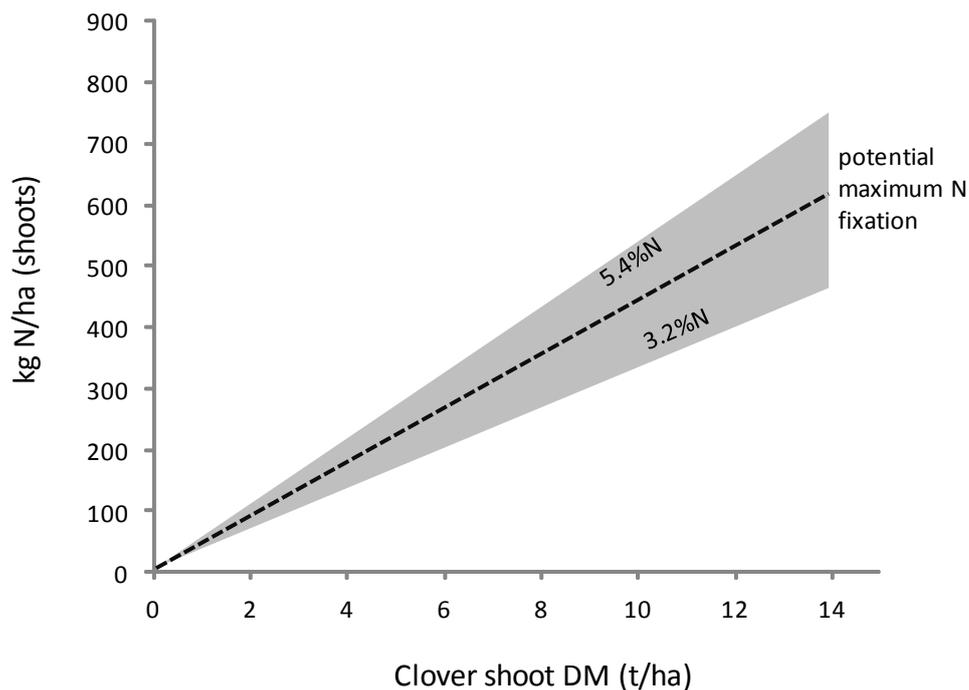


Figure 6 Potential N₂ fixation by clover herbage is set by clover total N in herbage, a function of herbage dry matter and N concentration. The indicated upper and lower limits around the central line result from the range in N content (%) observed for white clover across 71 dairy pastures in south-west Victoria (Riffkin et al 1999a). The slope of the dotted line is 44.8 kg/tonne (based on a mean N concentration of 4.48%).

3.2 Problems of measurement

Methods for field measurement of N₂ fixation have been detailed in Unkovich *et al.* (2008) and summarised by Peoples *et al.* (2009). These reports highlight that there are substantial obstacles to the reliable quantification of N₂ fixation in the field and no available methodology is optimal. Those methods which use the stable isotope ¹⁵N are considered the more reliable and also give time-integrated values. The natural ¹⁵N abundance ($\delta^{15}\text{N}$) methodology is currently the most widely deployed approach to field

measurement of N₂ fixation in temperate legumes. Under controlled conditions the relative activity of the nitrogen fixing enzyme, nitrogenase, can be compared in different treatments using the acetylene reduction assay (e.g. Mundy *et al.* 1988), and while the assay can be applied to field samples it cannot provide reliable quantitative estimates of symbiotic N₂ fixation at the field (kg ha⁻¹ yr⁻¹) scale (Unkovich *et al.* 2008). Other non-isotopic techniques (N difference, N balance, regression equations) do not measure N₂ fixation directly but rely on a suite of assumptions that are very often invalid and this reduces their usefulness in many situations. Regression equations relating clover growth to the amount of N₂ fixed are becoming popular (e.g. Carlsson and Huss-Danell 2003; Eckard *et al.* 2001a; Gourley *et al.* 2010; Ledgard *et al.* 1999) but these may not be as widely applicable as one might hope. This approach is considered in more detail on page 23, but results of their application in Australia are not considered to constitute measurements of N₂ fixation in the present review. Studies reporting quantitative field estimates of N₂ fixation in Australian dairy systems are outlined in Table 3.

3.2.1 Accounting for whole plant N

From the point of view of dairy production the N contained in legume roots that might have been input from N₂ fixation may not be as important as it is in cropping systems (see e.g. Khan *et al.* 2003). However, it represents a N input to the system and as such can provide for fertility build up and N supply to companion grasses when roots senesce and the N becomes more readily available for microorganisms. This may be particularly important when studying N balances or when modelling mineral N availability in dairy pasture soils. None of the reports in Table 3 include measurement of the total N in legume roots, a task which remains an ongoing challenge (McNeill *et al.* 1997). In the absence of such measurement the pragmatic approach has been to apply fixed ratios of shoot:root N and multiply the ratio by the amount of shoot N fixed to get total N₂ fixation (Unkovich *et al.* 2010). However, in the absence of the aforementioned root N measurements (see also Wichern *et al.* 2008) it is difficult to have confidence in the ratios proposed. For white clover a multiplication factor of 1.7 times herbage N was proposed for estimating total clover N (herbage + stolons + roots, Jorgensen and Ledgard 1997) and this has been applied in several studies (e.g. Peoples *et al.* 2001; Eckard *et al.* 2007). However, most of the Jorgensen and Ledgard data came from pot studies where the plants were ungrazed/uncut and only grown for a few weeks. How such leaf/stolon + root N ratios might relate to field ratios for grazed perennial clover is unclear. They had one contrasting data point for a grazed field experiment but this was not compared to the glasshouse experiments although they were plotted on the same graph. In a field study of subterranean clover using mowing, McNeill *et al.* (1997) estimated below-ground plant N and came up with a similar 1.75 ratio for estimating total plant N. Unkovich *et al.* (2010) give a value of 2.0 for lucerne, based on a pot study. It is not clear how such multiplication factors might apply across grazing/cutting regimes, soils, water availabilities, soil fertilities or species and thus some caution must be exercised in their use.

Nevertheless application of these approximate ratios might result in a more accurate estimate of total N₂ fixed than if they were not applied at all and root N were ignored.

Table 3 Studies quantifying legume N₂ fixation in Australian dairy, high-rainfall or irrigated perennial pastures.

Reference	Location	Notes
White clover		
Riffkin <i>et al.</i> 1999a (see also Riffkin <i>et al.</i> 1997)	sw Victoria	survey of 71 pastures, qualitative (%Ndfa) rather than quantitative (kg N ha ⁻¹), methodology: δ ¹⁵ N
Riffkin <i>et al.</i> 1999b (see also Riffkin <i>et al.</i> 1997)	sw Victoria	three sites, quantitative seasonal and annual estimates, methodology: δ ¹⁵ N
Pakrou and Dillon 2000	se SA	compared perennial and annual grazed pastures, quantitative annual estimates, methodology: δ ¹⁵ N
I. Fillery (CSIRO)	sw WA	six farms, quantitative annual estimates, methodology: δ ¹⁵ N
McKenzie <i>et al.</i> 1998	sw Victoria	one site, N fertiliser rates, quantitative for 3 months after N applications, only 9% clover, methodology: δ ¹⁵ N
Mundy <i>et al.</i> 1988	nth Victoria	one site, varied soil water content and N fertiliser rate, semi-quantitative, measurement period of hours extrapolated to days, methodology: acetylene reduction
Mundy 1987	nth Victoria	fertiliser N rates, 70 days, methodology: ¹⁵ N isotope dilution
Smith <i>et al.</i> 1993	nth Victoria	irrigation rates with saline water, quantitative seasonal (6 months), methodology: ¹⁵ N isotope dilution
Peoples <i>et al.</i> 1995	NSW	irrigation frequency, legume content comparisons, 109 days, methodology: δ ¹⁵ N
Lucerne		
Yang <i>et al.</i> 2011	se SA	surveyed 20 irrigated lucerne hay fields, quantitative (seasonal) estimates, methodology: δ ¹⁵ N
Gault <i>et al.</i> 1995	ACT	irrigated lucerne, fertiliser and inoculation treatments, quantitative annual estimates for 3 years, methodology: δ ¹⁵ N
Brockwell <i>et al.</i> 1995	ACT	irrigated lucerne, fertiliser and inoculation treatments, quantitative seasonal estimate, methodology: δ ¹⁵ N

3.3 Grazed white clover pastures

I have only been able to find 12 reports of field measurement of N₂ fixation in Australian high rainfall/irrigated perennial pastures (Table 3), although there are a number of other reports on rain fed,

annual or lower rainfall, perennial pastures (see Peoples and Baldock 2001). Reference is given in Peoples and Baldock (2001) to white clover in the study of Rochester *et al.* (1998) but I was unable to find specific reports on perennial clovers in that data source.

3.3.1 Annual inputs

For white clover, only three of the datasets in Table 3 (Riffkin *et al.* 1999b, Peoples *et al.* 1995, Pakrou and Dillon 2000. I Fillery (CSIRO) pers. comm .2012) include annual N₂ fixation estimates, the remainder of the datasets are for shorter periods of time. The work of Riffkin *et al.* (1999b) at three rain-fed sites in south-west Victoria demonstrated that N₂ fixation was primarily limited by the low legume (white clover) content, averaging only 8% across the three sites. Thus annual N₂ fixation input in herbage was only 19–22 kg N ha⁻¹, with the total amount (including roots) being perhaps *ca* 1.7 times this (Jorgensen and Ledgard 1997) at 32–37 kg N ha⁻¹ yr⁻¹. These values may be slightly below the average for the region, with an average clover content double these (19%) across the 71 dairy pastures examined across the region (Riffkin *et al.* 1999a).

In a recent study in Western Australian dairy pastures (Table 4), similar low legume contents constrained N₂ fixation to 2–87 kg ha⁻¹ yr⁻¹ across two years and six farmlets (I. Fillery pers. comm. 2012). The higher value in Farmlet 6 was for a perennial pasture whereas the other pastures contained annual legumes.

Table 4 N fixation by clover in farmlets. Values in parentheses are shoot N fixation x 1.75 to account for N fixation above- and below-ground, based on work of McNeill et al. (1997).

** Farmlet 6 is a perennial legume pasture, while 1 to 5 contain annual legumes. (Data from Dairy Australia Greener Pastures project, per Ian Fillery, CSIRO)*

Year	N fixation in clover (kg N ha ⁻¹) allocated to each farmlet					
	1	2	3	4	5	6*
2006	8 (14)	4 (7)	7 (12)	3 (5)	2 (4)	87 (152)
2007	18 (32)	10 (18)	9 (16)	5 (9)	6 (11)	50 (88)

The most comprehensive study of the nitrogen stocks and flows in an Australian dairy pasture comes from the work of Pakrou and Dillon (2000). This study is invaluable because it used isotopic measurement of N₂ fixation rather than estimation as has been used in several other N balance studies (e.g. Eckard *et al.* 2001a; Eckard *et al.* 2007; Gourley *et al.* 2007). The South Australian study by Pakrou and Dillon (2000) compared a perennial, irrigated white clover/ryegrass pasture (**Error! Reference source not found.**) with a rain fed, annual subterranean clover based pasture (**Error! Reference source**

not found.) In contrast to the abovementioned studies, this involved the sowing of a white clover/ryegrass pasture and comparing this irrigated pasture with an adjacent, rain-fed, unrenovated annual *Trifolium* pasture. In the irrigated white clover pasture legume content was just above 50%, and in the rain-fed annual pasture about 25%. Both pastures were grazed by cows, with utilisation rates around 70%.

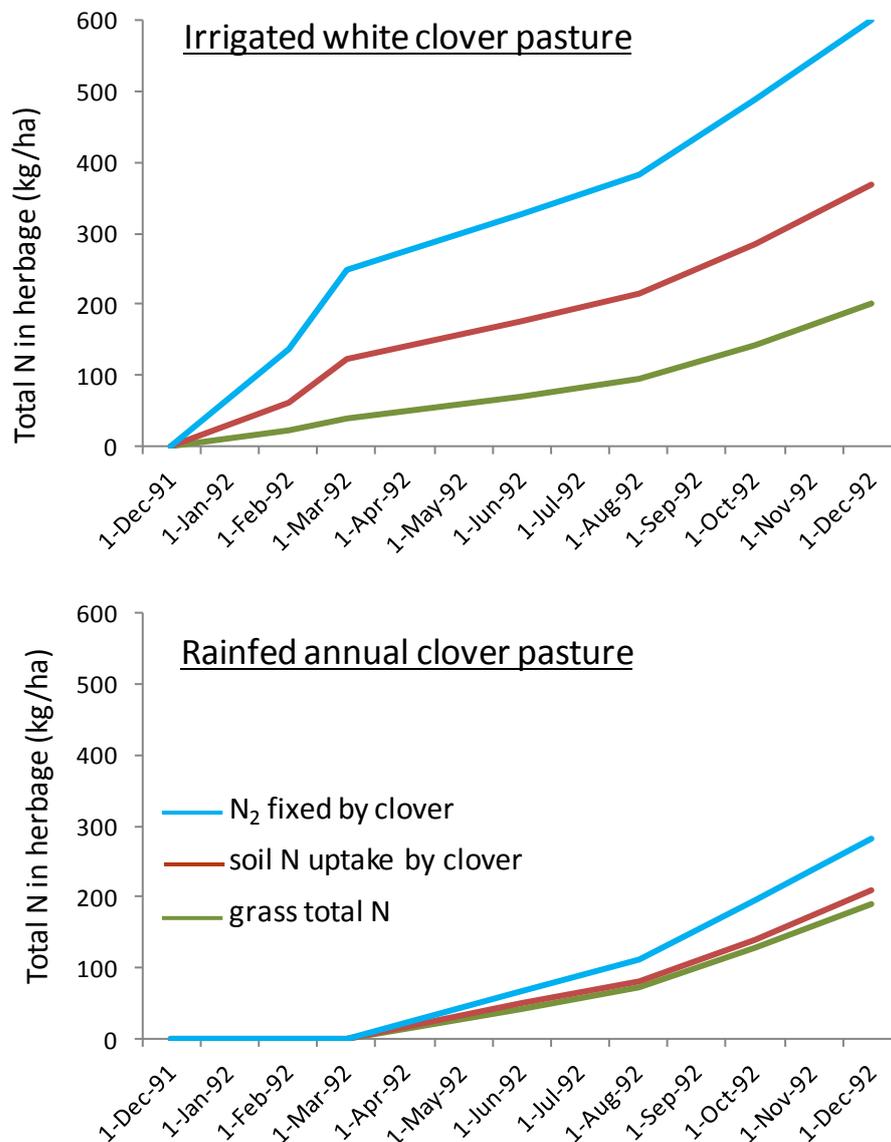


Figure 7 Cumulative plant nitrogen acquisition in an irrigated white clover and rain fed annual clover pasture in the south east of South Australia. (Plotted from the data of Pakrou and Dillon 2000).

Over the 12 month study period the irrigated white clover pasture fixed 231 kg N ha⁻¹ in the harvested herbage whereas the annual subterranean clover based pasture only fixed 75 kg N ha⁻¹ (Figure 7). The difference between the two pastures was clearly due to the increased productivity of the white clover pasture with irrigation, to the longer growing season afforded by this, and to the high clover content when compared to the annual pasture. In the annual pasture, grass N uptake dominated the accumulation of

herbage N whereas in the perennial pasture, clover accounted for 66% of total herbage N. In the annual pasture, soil mineral N uptake by herbage totalled 208 kg ha⁻¹ for the growing season while N₂ fixation contributed only 75 kg ha⁻¹, clearly soil mineral N supply provided for the bulk of plant N requirements, thus limiting N₂ fixation. The key element of these results is the substantial fixation of N₂ when pasture productivity (17.2 t ha⁻¹) and clover content (>50%) are high. Interestingly, although productivity of the annual pasture (12.2 t ha⁻¹) was 70% of the irrigated perennial pasture, herbage N accumulation totalled only 47% of that of the perennial pasture. Why the N concentration in herbage was lower in the annual pasture is not clear, but might relate to differential grazing management (see Unkovich *et al.* 1998).

The final quantitative estimate of N₂ fixation in a white clover pasture is that of Peoples *et al.* (1995), comparing a clover dominant (85%) with a grass dominant (60%) pasture over 109 days, with low or high irrigation frequency. Few details of the experiment are given in the Peoples *et al.* review paper. Results are as one might anticipate, with greater total N accumulation in both pastures under lower soil water deficits, and greater N₂ fixation with higher pasture clover content and clover N yield (Table 5).

Table 5 N₂ fixation by white clover over 109 days in clover dominant (85%) or grass dominant (60%) pastures irrigated after 60mm evaporation (high frequency) or 120mm evaporation (low frequency). (From Peoples et al. 1995).

Pasture type	Irrigation frequency	Clover N yield (kg ha ⁻¹)	N fixed (%)	N fixed (kg ha ⁻¹)
Clover dominant	low	108	61	66
	high	145	62	90
Grass dominant	low	66	67	44
	high	93	71	66

Based on the exhaustive survey of Riffkin *et al.* (1999a) white clover dependence on N₂ fixation in Australian dairy pastures is typically ca 65%, indicating reasonable N₂ fixing capacity. However, the actual amounts of N₂ fixed are very much limited by low clover dry matter production as a consequence of low clover content in most pastures. Much higher rates of N₂ fixation are achievable, with up to 294 kg N ha⁻¹ yr⁻¹ being recorded for a recently sown, irrigated white clover pasture (Pakrou and Dillon 2000). In a review of perennial forage legumes in temperate/boreal environments, Carlsson and Huss-Danell 2003 report N₂ fixation by white clover to be up to 545 kg N ha⁻¹ yr⁻¹. However, they did not include data on white clover from Australia. Mason *et al.* (1987) measured irrigated pure white clover pasture annual dry matter production of almost 23 t ha⁻¹ in northern Victoria, which, according to Figure 6 would provide for potential annual N₂ fixation of >1000 kg N ha⁻¹. This is higher than any value in the literature for any N₂ fixing system, but nevertheless shows that the potential with this species is very high. In current dairy systems this potential is not being realised due to low pasture legume contents.

3.3.2 *The Achilles heel: low white clover content of pastures*

Similar low white clover contents of pastures were previously reported in an exhaustive survey of Australian temperate pastures (Hill and Donald 1998; Pearson *et al.* 1997), and also earlier in Victoria (Ward and Quigley 1992). It would thus appear that pasture clover contents, and potential N₂ fixation in Australian perennial pastures has probably not improved in almost 20 years, regardless of the increased application of fertiliser N. Farmers appear reluctant to re-sow legumes (Ward and Quigley 1992). It may well be that, for well managed, N fertilised, intensively grazed perennial ryegrass/white clover pastures, equilibrium clover contents are around 20% resulting in the fixation of no more than ca 100 kg N ha⁻¹yr⁻¹, similar to that observed in the UK (Andrews *et al.* 2007; Parsons *et al.* 1991) and NZ (Woodfield and Clark 2009), although Jarvis (1993) suggested that in the UK, dairy pastures were typically much lower in both clover content (<10%) and the amount of N₂ fixed (10 kg ha⁻¹ yr⁻¹). These low clover contents are likely to be suboptimal in terms of dairy production (Woodfield and Clark 2009) as well as N₂ fixation and thus efforts to increase N₂ fixation should be rewarded with increased milk production efficiency.

In the absence of cattle grazing and the associated deposition of high rates of urine and dung, which increase soil mineral N and most likely depress N₂ fixation (Haynes and Williams 1993; Ledgard *et al.* 1999), dependence on N₂ fixation may be higher. For example, in the irrigated pure lucerne systems of south-eastern Australia (Yang *et al.* 2011) lucerne dependence on N₂ fixation averaged 65% and annual N₂ fixation in herbage was estimated to be >200 kg N ha⁻¹.

3.4 Lucerne hay systems

While grazed lucerne pastures are used in Australian dairy systems they are of relatively minor importance compared to white clover/ryegrass pastures. However, they are important for hay production that feeds directly into the dairy system. Table 3 indicates just three studies quantifying N₂ fixation of irrigated lucerne in Australia, with the only two of those (Brockwell *et al.* 1995; Gault *et al.* 1995) providing annual N₂ fixation estimates being experimental sites in the ACT.

The Gault *et al.* (1995) study measured N₂ fixation using δ¹⁵N natural abundance, in newly established, irrigated lucerne stands cut for hay, over a three year period. Experimental treatments were (1) no rhizobial inoculation and superphosphate only in the year of sowing (9 kg P ha⁻¹), (2) rhizobial inoculation plus annual applications of superphosphate, and (3) no rhizobial inoculation, annual application of superphosphate and nitrogen fertiliser (33 kg N ha⁻¹). Dry matter production and N₂ fixation increased dramatically after the first year (Figure 8), reaching 284 kg N ha⁻¹ yr⁻¹ for the inoculated and P fertilised treatment in the third year, although this was only marginally more than for the second year for all treatments (269–275 kg N ha⁻¹). In the third year, the uninoculated treatment which had not received annual applications of P fertiliser fixed much less than the other treatments. The authors

considered that total N₂ fixation (including root N) over the three year period exceeded 1400 kg N ha⁻¹ in the annual P fertilised treatments.

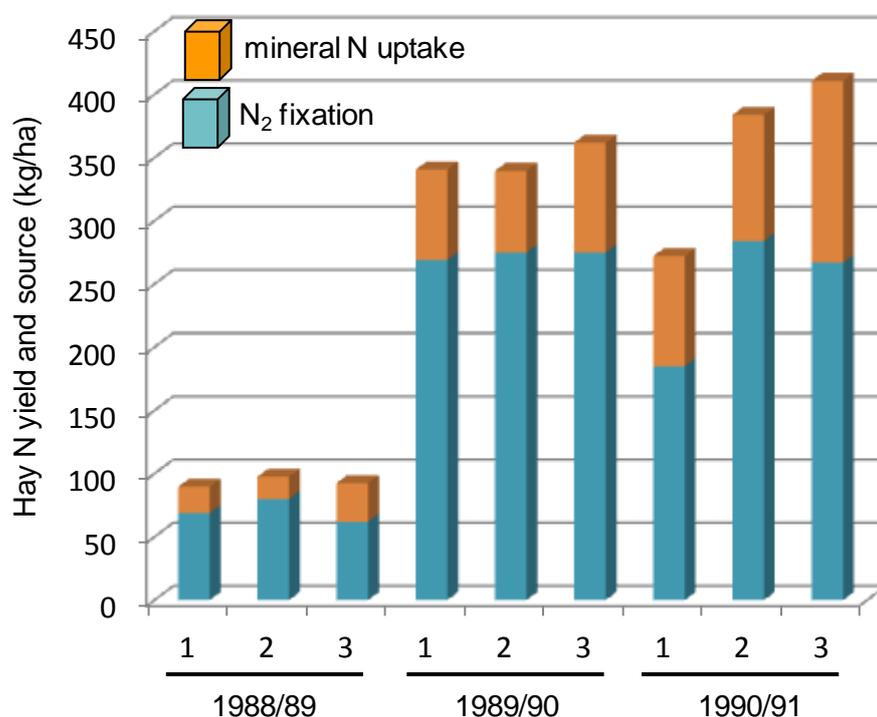


Figure 8 Sources of nitrogen for irrigated lucerne hay in the first three years after establishment, (1) uninoculated and superphosphate only in the year of sowing (9 kg P ha⁻¹), (2) inoculated plus annual applications of superphosphate, and (3) uninoculated, annual application of superphosphate and nitrogen fertiliser (33 kg N ha⁻¹). (Plotted from the data of Gault *et al.* 1995).

This study shows that the potential for N₂ fixation in irrigated lucerne is very high, provided that attention is paid to crop nutrition. The removal of 10–12 t ha⁻¹ yr⁻¹ of hay exports significant quantities of nutrients, aside from N, and these would need to be replaced if growth and N₂ fixation is to continue uninhibited.

The above treatments were also applied to a four year old lucerne stand at the same site (Brockwell *et al.* 1995) and N₂ fixation ranged from 83–97 kg N ha⁻¹ over the six month period of study, giving a nominal annual rate similar to that of Gault *et al.* (1995) at the same site. From the data of Figure 9, it would appear that N₂ fixation continues unabated at a constant rate over the warmer months where irrigation water is applied.

The final example of field measures of N₂ fixation in lucerne systems comes from Yang *et al.* (2011) who surveyed N₂ fixation in 18 irrigated lucerne hay fields in the south east of South Australia. The estimates of N₂ fixation were for standing dry matter at the time of sampling, in a system which typically has three hay cuts per year. Mean N₂ fixation in standing biomass (Table 6) was 73 kg N ha⁻¹, or 65% of lucerne herbage N. What time period these values might represent was not able to be established, but the authors

considered that, on average, annual values were likely to be three times those observed, giving a value very similar to the annual N₂ fixation indications from the studies of Brockwell *et al.* (1995) and Gault *et al.* (1995). The South Australian study also indicated that these lucerne stands continued to fix N₂ many years (>25) after they were established.

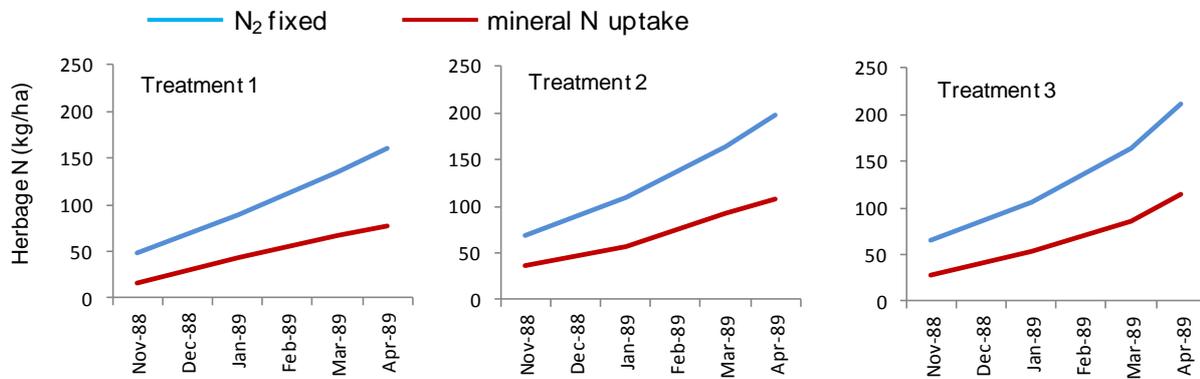


Figure 9 Cumulative seasonal N₂ fixation and mineral N uptake in a four year old irrigated lucerne stand grown for hay. Treatments same as for Figure 8. (Plotted from the data of Brockwell *et al.* 1995).

Table 6 Summary of N₂ fixation data from a survey of 18 irrigated lucerne stands cut for hay in the south east of South Australia (from Yang *et al.* 2011).

	%Ndfa	N fixed	mineral N uptake
mean	65	73	44
min	33	33	9
max	90	122	90

Together these data indicate that irrigated lucerne hay crop systems continue to fix considerable amounts of N over time. In contrast to grazed white clover systems, these hay systems export substantial quantities of N in herbage. Furthermore, they are often only grazed lightly such that the build up of soil mineral N does not occur to the extent that is seen in intensively grazed white clover pastures. In this case it is not the legume species which are driving the massive differences between lucerne and white clover in N₂ fixation input, but rather the presence of the animals, and the differential management of the systems in which the legumes are utilised.

3.5 Grazing and N₂ fixation

A detailed review of the impacts of grazing animals on legume N₂ fixation are given in Menneer *et al.* (2004). The key element of grazed dairy systems is the excretion by cattle of at least 75% of the N they ingest as herbage as urine and dung (Whitehead 1995). Maximal N₂ fixation is likely to come from well managed hay systems rather than grazed systems, because optimal clover content can be more easily managed and the urinary and dung N returns do not suppress N₂ fixation. However, this does not mean that ungrazed systems will have greater N₂ fixation than grazed systems. Ungrazed mixtures of clover and grass are likely to become grass dominant with shading reducing clover growth and N₂ fixation (Sanford *et al.* 1995). In a study of an annual subclover pasture grazed by sheep in Western Australia (Unkovich *et al.* 1998) a more heavily grazed pasture had lower grass growth and greater N₂ fixation than a lightly grazed pasture. While increased grazing pressure can favour clover growth over grasses, in practice the magnitude of this generally appears quite small as the effect occurs at the lighter end of grazing intensities (Doyle *et al.* 2000). Increased grazing pressure usually increases the N (protein) content of clover (Unkovich *et al.* 1998), and indeed other pasture species (Kelly *et al.* 2006). The work of Pakrou and Dillon (2000) highlights the significance of the mineral N flux under grazing. Under irrigated, grazed white clover pasture, the flux of N through the soil mineral N pool was estimated to be 687 kg N ha⁻¹, more than half of which was derived from animal returns (**Error! Reference source not found.**). The figure also highlights the significant role that N₂ fixation can play when there is a high clover content, even in the presence of intensive grazing. Under the annual pasture, mineralisation of soil organic N was driving the available N pool (**Error! Reference source not found.**), being no higher when the animals were on the pasture than when they were absent (see Pakrou and Dillon 2000). Excretory N returns from grazing animals are the key influence on sward N dynamics and N₂ fixation in dairy systems.

In terms of N₂ fixation the key elements to note in the perennial pasture of Pakrou and Dillon (2000) :

- the legume (white clover) content was high (57%) because the pasture had been sown only two years before, this is atypical for Australian dairy pastures where legumes contents are commonly <20%
- because the legume content and legume dry matter production (9.8 t/ha) was high, N₂ fixation was also high (236 kg N/ha), excluding an additional 59 kg/ha (25%) estimated for clover roots
- after mineralisation (687 kg N/ha), cattle intake (419 kg N/ha) and grass mineral N uptake (389 kg N/ha), N fixation was the fourth highest N flux in the system
- N₂ fixation was greater than the combined N losses estimated from leaching, NH₃ volatilisation and denitrification (209 kg N/ha) and thus the system appeared to be in an approximate N balance, despite there being no N fertiliser inputs

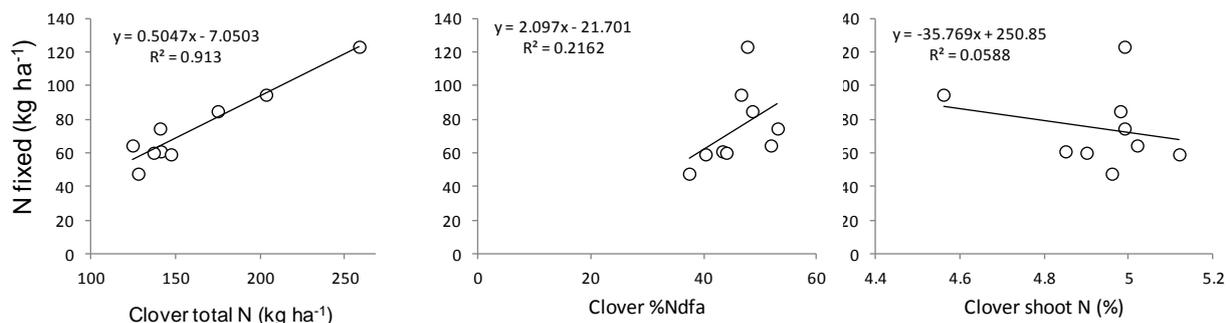
The key elements to note for the annual, rain fed pasture were:

- N_2 fixation was much lower than for the white clover based pasture because (a) subterranean clover is an annual and only grows for part of the year (b) the clover content (25%) was less than half that of the perennial pasture, and (c) the annual pasture was rain fed, not irrigated.
- most of the clover N was fixed (80%)
- the system had a marginally negative N balance overall
- the fixation rate of $100 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ in this pasture is higher than for Australian dairy pastures generally because there were no fertiliser N inputs.

3.6 Differences in N_2 fixation capacity between species and cultivars

Differences in cultivars are unlikely to be of quantitative importance for N_2 fixation input in Australian dairy systems. However, where differences in clover productivity are able to be expressed, then those cultivars with greater shoot biomass would fix more nitrogen. This has not been examined specifically for Australian cultivars and N_2 fixation has not been considered in the Australian white clover breeding program (pers. comm. Carol Harris, NSW DPI, Jan 2012). However, data on nine white clover cultivars from New Zealand (Ledgard *et al.* 1996) indicated that differences between cultivars in the amount of N_2 fixed are mostly related to dry matter production driven differences in clover total N accumulation, rather than to inherent differences in the N_2 fixation efficiency or shoot N concentration (Figure 10). While all three of these are used to calculate the amount of N fixed, it is clearly legume dry matter production which is the driving force in this dataset, and indeed in most others (Unkovich *et al.* 2010).

Figure 10 Correlation between clover shoot total N, clover dependence on N_2 fixation (%Ndfa) or



clover shoot N concentration (%), and the amount of N_2 fixed (kg ha^{-1}) for nine white clover cultivars in New Zealand. (Plotted from the data of Ledgard *et al.* 1996).

In an earlier study of differences in N_2 fixation between white clover cultivars in New Zealand (Ledgard *et al.* 1990), it was concluded that as there were no inherent differences in the capacity of different cultivars to fix nitrogen, N_2 fixation was not a basis for substituting one for another. Generally speaking, in breeding for maximum dry matter or total N accumulation, clover breeding programs might indirectly select for maximal N_2 fixation. However, this does not mean that N_2 fixation is optimal or has been

selected for, because it may well be that even with the best available plant material, N₂ fixation could still be limiting growth, due, for example to poorly effective rhizobia. With respect to cultivar performance in N₂ fixation, in a number of pasture legume species it has been shown that there is a strong interaction between legume cultivar and rhizobium strain, such that optimal N₂ fixation potential is achieved with specific combinations of pasture legume cultivar and rhizobial strain (see e.g. Ballard *et al.* 2003).

Differences in N₂ fixation between species of legume will be driven as much by differential management of species/systems and environment, as by inherent differences between legume species.

4 Modelling N₂ fixation in dairy systems

As field measurement of biological N₂ fixation is complex and expensive (Unkovich *et al.* 2008) modelling approaches to estimate N₂ fixation hold significant attraction. The basis for model design can be either empirical (e.g. Hogh-Jensen *et al.* 2004; Unkovich *et al.* 2010) or dynamic mechanistic (e.g. Boote *et al.* 2008). Empirical approaches tend to correlate measured N₂ fixation rates with other, more easily measured pasture properties, fit regression equations to the resulting dataset, and then apply those regressions elsewhere in time or space. Dynamic simulation models attempt to mimic the primary biological and physical processes driving plant growth (Sinclair and Seligman 1996), including N₂ fixation, and, therefore, they attempt to be universally applicable upon local parameterisation. Such so called mechanistic or dynamic simulation models are usually only semi-mechanistic as they typically include some empirical approaches. Liu *et al.* (2010) reviewed a large number of approaches to modelling N₂ fixation and the reader is referred to this thorough exposé of N₂ fixation modelling, the detail of which is outside the scope of the present review.

4.1 Empirical relationships

An example of a typical empirical model for estimating N₂ fixation is given in Figure 11 which relates legume shoot dry matter production to the amount of N₂ fixed. This figure is for herbage N fixed, an additional fraction can be added for fixed N possibly contained in roots.

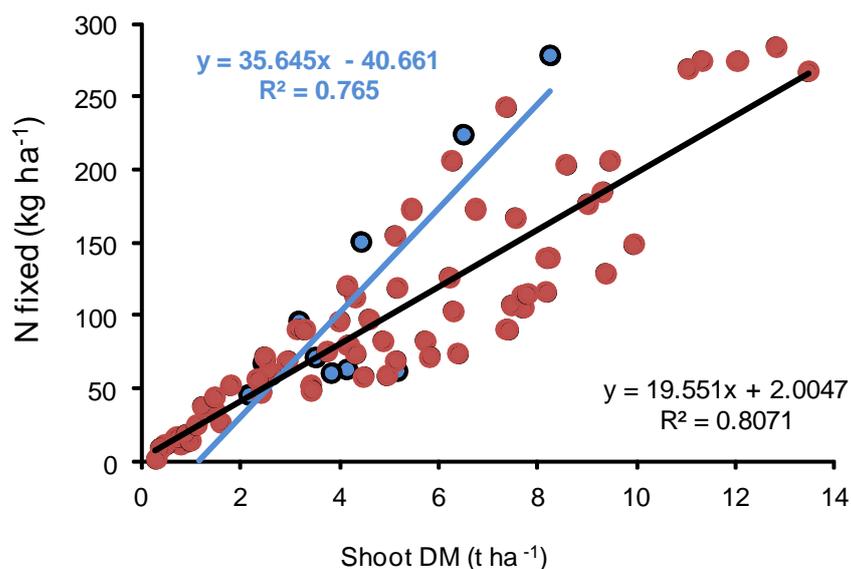


Figure 11 Correlation between clover shoot dry matter and the amount of N_2 fixed, and fitted regression equations for white clover and lucerne grown in Australia. Data from Unkovich *et al.* (2010).

The pros and cons of such approaches are detailed in Unkovich *et al.* (2010) and Liu *et al.* (2010). The primary limitation of such approaches is that, aside from the influence of dry matter production, they are naive to other possible drivers of N_2 fixation, such as soil fertility, temperature, water availability, grazing intensity, non-legume pasture content, and microsymbiont performance. The net effect of such factors is of course inherent in the observed data and so has been captured for the data points presented. The problem is that once the regression is applied in another situation (time or place), these inherent effects may not apply at the application place/time.

Carlsson and Huss-Danell (2003) found significantly different regressions for grazed and mown white clover pastures, and thus the regressions are not transferable between such management regimes. Thus applying relationships in Australia which have been developed elsewhere (e.g. Eckard *et al.* 2001a; Eckard *et al.* 2007) is fraught with danger, particularly if applied too specifically. Such regressions have no experience beyond their derivation dataset and thus other regressions might have equal validity. For example Carlsson and Huss-Danell (2003) gave linear regressions between white clover dry matter and N fixed accounting for 91% (clover/grass) to 55% (legume monocultures) of the measured amount of N_2 fixed., without accounting for N fertiliser application.

Examples such as those in Figure 11 may approximate behaviour across regions but are unlikely to be correct at any given point and should only be applied at the scale at which the regression is derived. That is, if the data are derived from a range of treatments within a single field or farm, they could not be

reliably extrapolated outside of that field or farm. Conversely, a regression across a range of fields or regions might usefully be applied across such a scale, but is not likely to apply at sub field or region scale. The regressions cannot be reliably used in situations where they have no previous experience. In this way they are different to dynamic simulation models which often respond to local environmental and management influences.

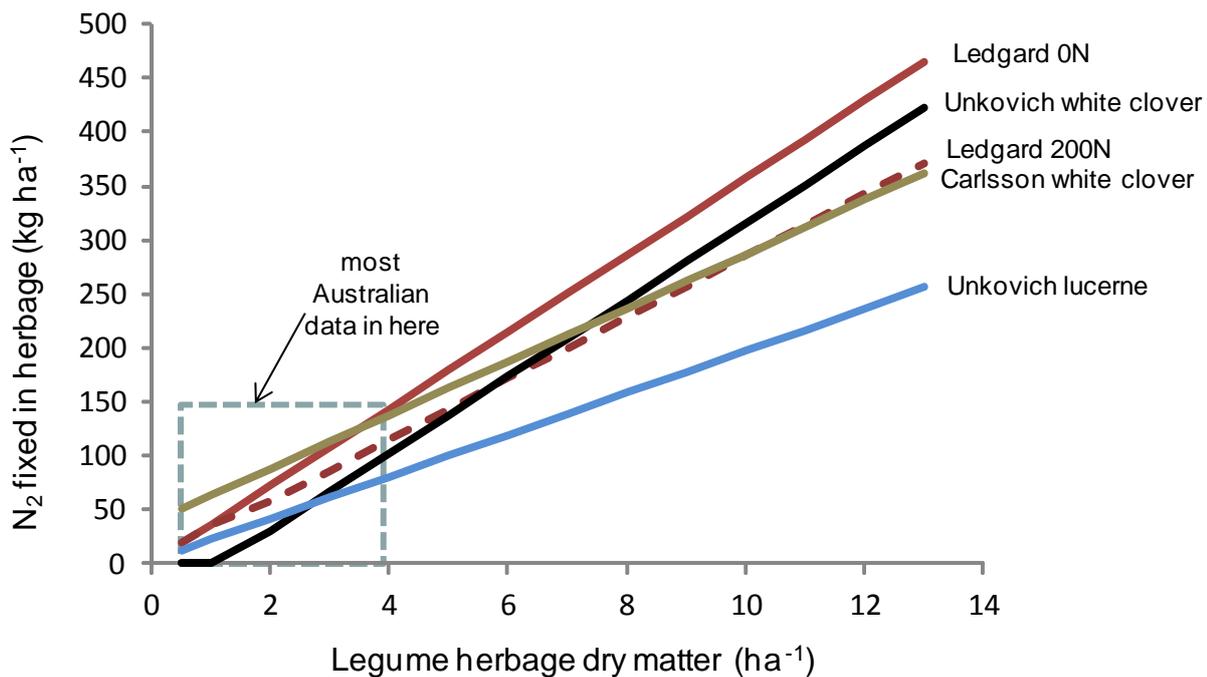


Figure 12 Comparison of different regression equations used to estimate N_2 fixation in white clover or lucerne from clover shoot dry matter. Details of the regressions are given in Table 7.

In the study of Ledgard *et al.* (2001) the white clover N concentration did not drop below 4.5%, whereas this was close to the average for 71 pastures investigated in Victoria (Riffkin *et al.* 1999a) and in the analysis of broader Australian data by Unkovich *et al.* (2010) the mean shoot [N] for white clover was given as 3.2%, which could account for a significant difference in the slope of the regression lines. Indeed Figure 12 looks much like Figure 6. Furthermore, the clover N_2 fixation in the Ledgard study did not exceed 94 kg N ha^{-1} whereas in the Unkovich dataset the maximum was 278 kg N ha^{-1} and in the Carlsson dataset it exceeded $400 \text{ kg N ha}^{-1} \text{ yr}^{-1}$. As much of the evidence indicates that clover content and clover dry matter production are low in Australia ($\leq 4 \text{ t ha}^{-1}$) the relevant part of Figure 12 is near the origin. At 2 t ha^{-1} clover dry matter N_2 fixation could range from $30\text{--}87 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ depending on which regression equation is used. Further complications arise because in some instances significant N_2 fixation would be indicated with no clover dry matter (Figure 12, Carlsson regression). This can occur with regressions when they are extended beyond their experience, or where the responses may indeed not be linear, as is likely to be the case at the lower end of the range when soil mineral N will become increasingly important.

Table 7 Regression equations relating clover herbage dry matter (kg ha^{-1}) to N_2 fixation in shoots for perennial legumes.

Reference	Legume	Regression
Ledgard 2001	white clover	$=\text{DM}*(0.0358-3.59*10^{-5}*\text{N fertiliser rate})$
Carlsson and Huss-Danell 2003	white clover (generic)	$=\text{DM}*0.025+37.2$
	white clover (monoculture)	$=\text{DM}*0.00.016+57.9$
	white clover (mixtures)	$=\text{DM}*0.031+23.9$
	red clover (generic)	$=\text{DM}*0.023+8.4$
	red clover (monoculture)	$=\text{DM}*0.016+16.5$
	red clover (mixtures)	$=\text{DM}*0.026+7.4$
	lucerne (generic)	$=\text{DM}*0.012+38.8$
	lucerne (monoculture)	$=\text{DM}*0.0.013+12.3$
Unkovich <i>et al.</i> 2010	white clover	$=\text{DM}*0.036 -40.661$
	lucerne	$=\text{DM}*0.0196 +2.007$

Given that a value of ca 4.5% N for herbage seems typical for grazed white clover (Figure 6 and Ledgard *et al.* 2001), a shoot:root N ratio of 1.7 (Jorgensen and Ledgard 1997) and the average dependence of white clover on N_2 fixation in western Victoria of 65% (Riffkin *et al.* 1999b), this implies a total N fixation for current systems averaging 50 kg t^{-1} clover shoot dry matter, or in shoots only 29 kg t^{-1} herbage. While this might provide a useful rule of thumb for pastures of low (<25%) legume content from which the data have been derived, for higher legume content dairy pastures other factors may play a part in changing %Ndfa or herbage N concentration and thus alter the relationship between dry matter and N_2 fixed.

4.2 Dynamic simulation models

In the review of Liu *et al.* (2010), nine mechanistic/process based models of N_2 fixation were identified. Commonalities were the scaling of a maximum daily N_2 fixation rate as a function of some combination of temperature, soil water, soil mineral N, plant carbon availability, and plant development stage. The implementation of these various factors in a range of models is shown in Table 8. Eight of the models have been used for perennial legume pasture species (white clover or lucerne).

Table 8 Factors used to scale maximum daily N₂ fixation rate in various “mechanistic” N₂ fixation models. Adapted from Liu *et al.* (2010) with SGS/DairyMod added and an indication of whether the model has been used for white clover ✓ (* or lucerne)

Model	temp.	water	mineral N	plant C	growth stage	white clover	reference
Sinclair		✓		✓			Sinclair 1986
EPIC		✓					Cabelguenne <i>et al.</i> 1999
Hurley	✓	✓	✓	✓		✓	Thornley 2001
Schwinning			✓	✓		✓	Schwinning and Parsons 1996
CropGro	✓	✓		✓	✓		Boote <i>et al.</i> 2008
SOILN	✓	✓	✓			✓	Wu and McGechan 1999
APSIM		✓	✓		✓	*	Robertson <i>et al.</i> 2002
Soussana			✓			✓	Soussana <i>et al.</i> 2002
STICS	✓	✓	✓		✓	✓	Brisson <i>et al.</i> 2009
GrassGro		✓	✓		✓	*	Moore <i>et al.</i> 1997
SGS/DairyMod			✓			✓	Johnson <i>et al.</i> 2008

When reviewing models the first consideration is the purpose/objective of the modelling required. There are many models, either specifically for N₂ fixation, or which have N₂ fixation as a component, but each has been built with a different specific purpose in mind. For the present purposes it is assumed that the modelling objective is to quantify changes in legume N₂ fixation in response to management and climate, rather than legume physiological responses to climate and management. Relevant pasture simulation models which have been used in Australia are given in Table 9, along with their N₂ fixation simulation capacity.

Table 9 Legume N₂ fixation simulation capacity of dynamic pasture models used in Australia

Model	N ₂ fixation functions	Reference
GrassGro	a fraction of the net remaining demand for N, affected by nodule mass, developmental stage, soil moisture availability and NO ₃ ⁻ -N	Moore <i>et al.</i> 1997 Moore pers. comm.
DairyMod	a minimum of 20% of legume N is from fixation, where mineral N cannot meet legume N demand then N ₂ fixation tops up herbage N to the optimal shoot [N], but constrained by plant C availability	Johnson 2005; Johnson <i>et al.</i> 2008 I Johnson pers comm.
SGS Pasture Model	as above	Johnson 2005; Johnson <i>et al.</i> 2003
APSIM	a function of daily growth rate, up to a maximum daily N ₂ fixation rate, with a legume specific factor for relative suppression of N fixation by soil mineral N	Robertson <i>et al.</i> 2002
GRASP	does not incorporate N ₂ fixation	McKeon <i>et al.</i> 1982

In GrassGro the potential N₂ fixation rate is calculated as the total plant N demand less N translocated from belowground reserves and N recycled from shaded leaves, multiplied by a factor for the development of nodules in early growth. This potential rate is then scaled back by low water content and high mineral N, weighted according to a nodule depth distribution (Andrew Moore, CSIRO, pers. comm.).

In the DairyMod tool, N₂ fixation is linked directly to photosynthesis and a value of 6 mg C respiration / mg N fixed used as a carbon cost, thus reducing growth of N₂ fixing clover compared to non-fixing clover. Earlier versions of the model constrained N₂ dependent clover to 0.6 of the growth of mineral N dependent clover, although this has recently been removed. A minimum of 20% of legume N comes from N₂ fixation under all conditions. Legumes are not limited for N, with N₂ fixation topping herbage N up to the optimal value (Johnson 2005). Graham (2008) provides a review of the DairyMod tool although does not discuss legume N₂ fixation.

In APSIM (Robertson *et al.* 2002) N₂ fixation occurs when there is insufficient mineral N to meet plant N demand, but with the sensitivity of with which N₂ fixation is switched on in the presence of mineral N being a cultivar specific parameter. While the model does not currently have an interaction between soil mineral N and nodulation, the N₂ fixation routines are currently being revised and nodule mass will become an integral part of the N₂ fixation simulation routines.

While DairyMod, APSIM and GrassGro have the capacity to model N₂ fixation I can find no published model output showing N₂ fixation by pasture or crop legumes, or a comparison of model output with measured N₂ fixation data. While the models often show good correlation of model simulated and measured dry matter production or total N, the validity of these models nevertheless remains essentially untested in terms of N₂ fixation. The N₂ fixation routines in both APSIM and DairyMod are currently being revised (pers. comm. M. Robertson (CSIRO) and I. Johnson (IMJ)).

None of the available models include any consideration of the population dynamics, effectiveness or environmental responses of the microsymbiont and thus will be unable to simulate responses of the symbiosis to management and environment in the field. Those models which ignore the microsymbiont dynamics will inevitably have limited capacity over time. There is no physiological process-based model tested for specific study of N₂ fixation in Australian dairy systems. Until the N₂ fixation routines in the available models have been tested against measured data they offer no more in terms of predictive N₂ fixation capacity than a suitably calibrated empirical model.

5 Environmental costs and benefits of N₂ fixation

A fair assessment of the environmental costs and benefits of legume N₂ fixation in dairy systems can only be achieved with consideration of a gamut of factors impinging on the environmental balance sheet for a dairy farm. While this is beyond the scope of the present review we can briefly consider some of the issues feeding into and out of legume nitrogen in dairy farming systems. More thorough environmental analyses of dairy farming systems can be found in a recent volume (de Klein *et al.* 2008; Kleinman and Soder 2008; Nash and Barlow 2008) and a range of other relevant articles (Andrews *et al.* 2007; Ledgard *et al.* 2009; Ridley *et al.* 2004; Woodfield and Clark 2009).

Because urinary N returns from dairy cattle concentrate soluble N at rates equivalent to $\geq 1000 \text{ kg ha}^{-1}$ (Haynes and Williams 1993) this provides the primary point of soluble N excess, and thus the greatest opportunity for environmental impact. Generally to minimise losses of N *via* denitrification, leaching or ammonia volatilisation a “tight” N cycle is required, necessitating the maintenance of some N limited grass to “mop up” available N (Parsons *et al.* 1991). However, a system with slightly N deficient grass may limit feed quantity and quality and is generally not considered optimised in terms of animal production (Eckard 2001). This is thus not usually recommended from a milk production perspective but could provide significant environmental benefits.

In a study in the UK, Andrews *et al.* (2007) considered the relative merits of (1) an unfertilised perennial ryegrass/white clover pasture (2) a perennial ryegrass pasture receiving $200 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, and (3) a perennial ryegrass only pasture supplied with $350\text{--}400 \text{ kg N ha}^{-1} \text{ yr}^{-1}$. From a N cycling and NO₃⁻ leaching

perspective, pastures (1) and (2) were considered equal as the unfertilised pasture had similar N input from N₂ fixation, and with a similar grazing regime the amount of N cycling through the animals was about the same. The pasture with the higher fertiliser N addition rate (3) was considered to have a greater N footprint due to increased leaching and nitrous oxide (N₂O) emissions. Generally it was considered that with similar N inputs, pasture productivity and grazing intensity the environmental N footprint would be about the same, that is there may be no inherent advantage in N₂ fixation per se in terms of N cycling impacts. The analysis of Andrews *et al.* (2007) did not include the magnitude of N₂ fixed in clover roots and thus may have underestimated the difference between N inputs in treatments.

Generally then, if contrasting systems (grass v grass/clover) are equally as productive and have the same stocking rates or animal products output, they are likely to have very similar environmental costs/benefits. This is because most of the environmental footprint from dairy systems comes from the livestock N returns not the N input per se. While substitution of fertiliser N with clover fixed N might improve the environmental balance sheet on farm, the benefit is likely to be marginal where best practise fertiliser management is used.

Excretal N is the primary source of nitrous oxide emissions from dairy systems (de Klein *et al.* 2008; Ledgard *et al.* 2009). Although legume N₂ fixation was previously thought to contribute directly to N₂O emissions, this has been shown not to be the case (Rochette and Janzen 2005) and so the direct N₂O footprint of legume fixed N₂ is minimal. If one were also to include energy costs of urea fertiliser manufacture (0.73 – 2.14 kg CO₂-e kg⁻¹ Ledgard *et al.* 2011), then substituting fixed N₂ for fertiliser N should have some greenhouse gas (GHG) mitigation potential (Ledgard *et al.* 2009), but not if pasture clover contents are low. Andrews *et al.* (2007) considered that savings in CO₂-e by substituting 200 kg N for fixed N would be negligible on a global scale but very significant on a ha⁻¹ basis. Nitrogen fertiliser manufacture accounts for about 1% of total global CO₂-e emissions. In the future if legumes with condensed tannins become available (Woodfield and Clark 2009) additional GHG benefits in terms of reduced CH₄ emissions should accrue.

High land use intensity in the dairy industry is the primary cause of environmental problems resulting from excess nitrogen (de Klein *et al.* 2008). While similar, well managed clover/grass and grass only pastures are likely to have the same local environmental impact, whole system or life cycle analysis (LCA) suggests that overall, pastures which contain N₂ fixing legumes would have a lower net environmental impact than nitrogen fertilised pastures (Ledgard *et al.* 2009). While ungrazed legume dominant hay systems would appear to have a much lower environmental impact than intensively grazed pastures as the primary animal driven mineral N fluxes would be avoided, this ignores the fact that the hay will still be fed to animals and the excretal N returned elsewhere. Although in this case it could be more effectively managed.

6 Managing N₂ fixation in Australian dairy pastures – where to from here?

Australian dairy systems have made the inevitable drift from the exploitation of legume N in extended grazing systems to short rotational grazing of N fertilised pastures that has characterised the development of intensive, modern dairy systems elsewhere in the world. This is due to a perceived increase in system efficiency by increasing the stocking rate to utilise more of the pasture, and then supplementing the otherwise underfed cows (Lemerle *et al.* 1992). Such a system increases the return of urinary and dung N to pastures, further reducing legume content. It is this high intensity grazing rate that is exerting significant influences on N₂ fixation by clover, through defoliation, treading and returns of urinary N which cause direct reductions in N₂ fixing (nitrogenase) activity and in clover persistence. However, as legumes have a number of special benefits to dairy cows and to farming systems, they are likely to have a continuing, perhaps increasing role in dairy systems in the future, provided that investment is made in the appropriate areas.

The clover contents of typical dairy pastures are clearly below the optimum required for effective N₂ fixation input, and perhaps below what might be optimal in terms of animal nutrition and milk production (Harris *et al.* 1997) and efforts to increase N₂ fixation should be rewarded with both improved animal production efficiency, and environmental benefits. Generally lower rates of N application and moderate intensity grazing favour white clover persistence and abundance in mixed pastures (Kelly *et al.* 2006). Legume herbage has distinct advantages over grasses in terms of animal production and warrants inclusion in dairy pasture systems. The preference by grazing animals for white clover herbage over companion grasses is likely due to its higher digestibility and lower NO₃⁻ concentration (Horadagoda *et al.* 2009). The fact that clover is able to obtain its own N requirements from the atmosphere provides an opportunity to reduce input costs and the environmental impact of dairy agriculture.

In high rainfall and irrigated pastures, clover contents should be able to be increased, with multiple benefits, including N₂ fixation. However, under rain fed conditions where summer droughts occur, perennial legume persistence and N₂ fixation are likely to be more difficult to maintain, and occasional resowing will be required. Housed animal systems with cut-and-carry forage are likely to be more reliant on legumes and N₂ fixation, whereas intensively grazed pastures will inevitably have lower clover contents, higher returns of urinary and dung N (intensified through the addition of supplementary feeding when pasture supply is limited), increasing the downward pressure on legumes and N₂ fixation.

Eckard *et al.* (2001b) point out that reduced N fertiliser use and increased dependence on legumes have now occurred in Europe, a trend which might follow here. Whether this alone will be sufficient to boost pasture legume content and N₂ fixation to the required level is not clear. It is likely to also require lower stocking rates which is somewhat anachronistic to the current management paradigm in Australian dairy

systems which focus on pasture utilisation efficiency rather than N use efficiency. In any event if pasture legume contents are increased there will be a requirement for monitoring of legume growth and N₂ fixation to ascertain whether the other factors highlighted here begin to constrain N₂ fixation (rhizobia effectiveness, nematodes, grazing intensity and excretal N returns). One alternative option worth exploring might be the spatial separation of clover and grass as suggested by Woodfield and Clark (2009), with potential increases in N₂ fixation input and scope for spatial management of fertiliser, and improved milk production. Differences in the N₂ fixing potential (growth) of white clover cultivars are likely, as are differences in responses to available N (Doyle *et al.* 2000) but these have not been explored for Australian clover varieties.

Complex dynamic simulation models are probably not required to predict the likely outcome of changes in pasture legume content in terms of N₂ fixation. This should be able to be modelled relatively simply, or with simple regression models such as that shown in Figure 13. The DairyMod, APSIM and GrassGro models all have some capacity for N₂ fixation simulation, but this is yet to be exploited. A comparison of model outputs in terms of N₂ fixation against measured data are required to ascertain if the current models have anything to offer.

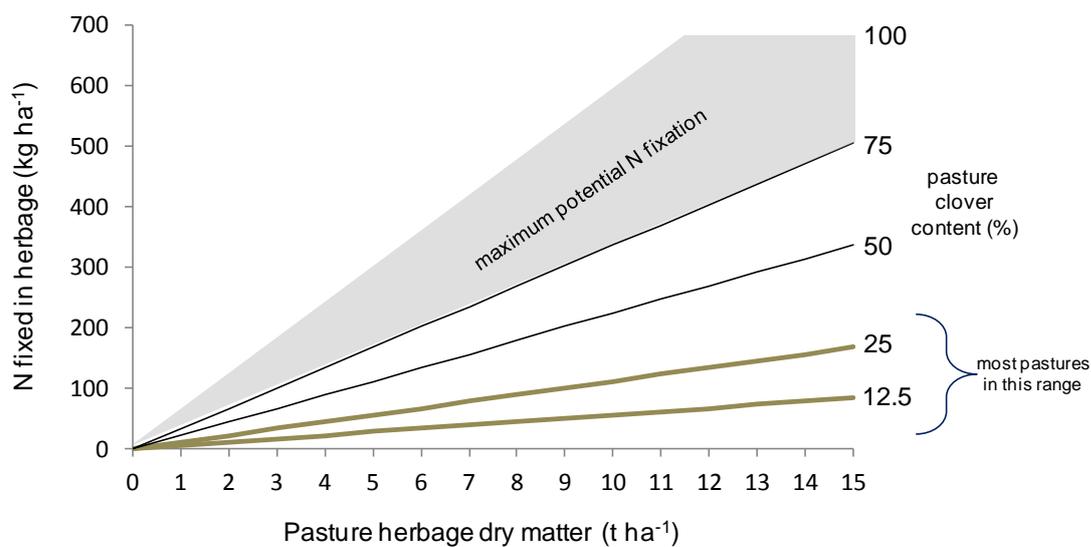


Figure 13 Potential nitrogen fixation by clover, assuming herbage N content of 4.48% and 100% dependence on N_2 fixation for a range of pasture clover contents. Maximum potential N_2 fixation is ca $700 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ depending on clover N content. Most Australian dairy pastures have a clover content below 25% and a %Ndffa of ca 65%, so actual N_2 fixation in clover herbage must typically be much less than $80 \text{ kg N ha}^{-1} \text{ yr}^{-1}$.

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